Molecular and Cytogenetic Approaches to the Analysis of Chromosomes in Human Preimplantation Embryos.

by

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To my father, mother and brother for believing in me.
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Abstract

The original focus of the research for this thesis was concentrated on establishing strategies to detect chromosome imbalance in embryos derived from chromosomal rearrangement carrier parents. Optimising conditions and investigating the possibility of different probe combinations was an important part of this study. The research carried out involved the gathering of data regarding segregation patterns during meiosis in gametogenesis, as well as acquiring information as to the mechanisms involved in the formation of mosaic and chaotically dividing embryos. Dual and triple probe combinations were developed, and evaluated in 69 surplus IVF embryos, using FISH. In the normally developing group 77% were uniformly normal, while in the abnormally developing group, 24% were normal. PGD strategies were designed for forty-four referrals, 20% of which reached the embryo biopsy stage of PGD. Over 13 PGD cycles, a total of 166 oocytes were retrieved and 113 of the resulting embryos were biopsied. FISH analysis showed that 29% of the embryos were normal for the chromosomes tested, whilst 71% were chromosomally unbalanced. The three live births and one biochemical pregnancy achieved, despite the poor history in almost all cases, is proof that a policy of biopsying two cells from embryos consisting of 6 or more cells and a single cell from 4 or 5 cell embryos is compatible with a positive outcome and reduces the risk of misdiagnosis in cases of balanced/aneuploid mosaics.

The use of Comparative Genomic Hybridisation (CGH) was investigated as an alternative, global strategy for PGD. The final part of the research for this thesis involved assessing the efficiency of CGH, improving the protocol for optimised use on single cells, and its application to human embryonic material. Results suggested that CGH is a laborious and technically demanding technique not standard enough for routine clinical application. It therefore proved not to be the best approach to PGD for sub fertile patients carrying translocations since deletions and small duplications were not readily detected in single cells. Delineating the involvement of the WHS for a referred patient by examining the status of selected polymorphic markers, and performing molecular cytogenetic analysis of embryonic DNA samples from fetal tissues obtained from social terminations, were projects performed as part of the training required for CGH analysis on single cells.
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List of Abbreviations

LIST OF ABBREVIATIONS.

Abbreviations commonly found in this thesis;

ADO  Allele dropout.
Alu-PCR  Alu polymerase chain reaction.
AMA  Advanced maternal age.
BAC  Bacterial Artificial Chromosome
bp  Base pair.
BSA  Bovine serum albumen.
cAMP  Cyclic adenine monophosphate.
cDNA  Complementary deoxyribonucleic acid.
CBAVD  Congenital bilateral absence of the vas deferens
CCD  Charged coupled device.
CEPH  Centre d'Etude Polymorphism Humain.
CF  Cystic fibrosis.
CGH  Comparative genomic hybridisation.
CISS  Chromosomal in situ suppression.
Contig  Contiguous.
CPM  Confined placental mosaicism.
CVS  Chorionic villus sampling.
dATP  Deoxyadenosine triphosphate.
dCTP  Deoxycytidine triphosphate.
dGTP  Deoxyguanosine triphosphate.
dNTP  Deoxynucleoside triphosphate.
dTTP  Deoxycytidine triphosphate.
dUTP  Deoxyuridine triphosphate.
DAPI  4',6-diamidino-2-phenylindole.
der  Derivative chromosome.
DNA  Deoxyribonucleic acid.
DNase  Deoxyribonuclease.
DOP-PCR  Degenerate oligonucleotide primed – polymerase chain reaction.
DTT  Dithiothreitol.
EDTA  Ethylene diamine tetraacetic acid.
ESHRE  European Society for Human Reproduction and Embryology.
ET  Embryo transfer.
FISH  Fluorescent in situ hybridisation.
FITC  Fluorescein isothiocyanate.
FSH  Follicle stimulating hormone.
G-banding  Giemsa banding.
GnRH  Gonadotrophin releasing hormone.
List of Abbreviations

GPS  Glutamine/Penicillin/Streptomycin.
hCG  Human chorionic gonadotrophin.
HCl  Hydrochloric acid.
HFEA  Human Fertilisation and Embryology Authority.
HGMP  Human genome mapping project.
ICM  Inner cell mass.
ICSI  Intracytoplasmic sperm injection.
ISCN  International System for Human Cytogenetic Nomenclature.
Ig  Immunoglobulin.
IVF  In vitro fertilisation.
kb  Kilobase pairs.
LH  Lutenising hormone.
μg  Microgram.
μl  Microlitre.
MI  First meiotic division.
MII  Second meiotic division.
Mb  Megabase pairs.
M-FISH  Multi-target/Multiplex - fluorescent in situ hybridisation.
mg  Milligram.
ml  Millilitre.
MPF  Maturation/M-phase promoting factor.
mRNA  Messenger ribonucleic acid.
ng  Nanogram.
nl  Nanolitre.
OR  Oocyte retrieval.
PBI  First polar body.
PBII  Second polar body.
PBS  Phosphate-buffered saline.
PCR  Polymerase chain reaction.
PGD  Preimplantation genetic diagnosis.
PHA  Phytohaemagglutinin.
POC  Product of conception.
rec  Recombinant chromosome.
RIF  Recurrent implantation failure.
RM  Recurrent miscarriages.
RNA  Ribonucleic acid.
RNase  Ribonuclease.
SO  Spectrum Orange.
SG  Spectrum Green.
SR  Spectrum Red.
SA  Spectrum Aqua.
Sg  Spectrum Gold.
SDS  Sodium dodecyl-sulphate.
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<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>SKY</td>
<td>Spectral karyotyping.</td>
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<tr>
<td>SSC</td>
<td>Standard saline citrate.</td>
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<tr>
<td>TE</td>
<td>Trophectoderm.</td>
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<tr>
<td>TOP</td>
<td>Termination of pregnancy.</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol.</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate.</td>
</tr>
<tr>
<td>UPD</td>
<td>Uniparental disomy.</td>
</tr>
<tr>
<td>WHS</td>
<td>Wolf Hirschhorn Syndrome</td>
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<tr>
<td>WHSCR</td>
<td>Wolf Hirschhorn Syndrome Critical Region</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume for volume.</td>
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<tr>
<td>w/v</td>
<td>Weight for volume.</td>
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<td>YAC</td>
<td>Yeast artificial chromosome.</td>
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CHAPTER 1

INTRODUCTION
1.1 Early Human Development.

Humans are characterised by an extraordinarily low fecundity in comparison to other mammalian species used as animal models to study the mechanisms of embryogenesis (Stevens et al., 1997). Hormonal studies reveal that only 25% of menstrual cycles result in implantation in fertile women trying to conceive with an estimated 20%-25% of these conceptions failing to progress to clinical pregnancies (Wilcox et al., 1988; Ellish et al., 1996). The first trimester has been proven to be the most critical for pregnancy survival, while 10%-15% of clinical recognised pregnancies spontaneously abort by forty weeks (Edmonds et al., 1982; Wilcox et al., 1988).

This may be a reflection of the level of aneuploidy present within human embryos. Studies show that over half of all spontaneous abortions have abnormal chromosome constitutions. At conception, the incidence is far higher, as shown by work on human gametes and preimplantation embryos over the last decade (Hassold et al., 1986; Eiben et al., 1990; Sherman et al., 1991; Angell et al., 1995; Abruzzo and Hassold, 1995; Hunt and Hassold, 2002). The incidence of aneuploidy and its effects on fertility, pregnancy wastage, malformation syndromes and on the parental source and meiotic stage of origin of aneuploid gametes, have been an active subject in terms of research since the 1960's, as aneuploidy accounts for a very high percentage of chromosomal abnormalities in general. However, the primary mechanisms leading to human aneuploidy are only now becoming clearer.

The major genetic elements that can determine embryo viability in the human embryo involve errors occurring during one of the three stages: gametogenesis, fertilisation or early embryogenesis. Due to the ethical issues surrounding research on human embryos, many of the principles have been extrapolated from research in other species.
1.1.1. Gametogenesis and Meiosis.

In all multicellular organisms that are diploid the complex process that apportions the new chromosomes equally between daughter cells is called mitosis and is a basic requirement for growth and differentiation throughout development. An important type of cell division departing from the plan of mitosis is meiosis, the form of cell division in which haploid germ cells (23 chromosomes) are produced from diploid cells (46 chromosomes) giving rise to gametes. Apart from the obvious halving of the chromosomes in daughter cells, a major difference between meiosis and mitosis is the fact that during female meiosis the gamete is blocked at precise phases of the cell cycle until a specific stimulus removes the block.

Meiosis involves a single round of DNA replication followed by two successive chromosome segregations; meiosis I and meiosis II. Meiosis I involves chromosome pairing and recombination between non-sister chromatids, and yields two haploid daughter cells. In meiosis II the duplicated chromosomes divide, yielding four haploid daughter cells. The chromosome number is restored to its diploid state again with the fusion of the male and female gametes at fertilisation. The recombination of homologous chromosomes during meiosis leads to exchange of material between maternally and paternally derived chromosomes. This process called crossing over is the source of new combinations of genes in the next generation.

Given the disparity in the numbers of the gametes required for fertilisation between males and females, it could be expected that the more precious commodity, eggs, would be subject to more stringent quality-control mechanisms. However, the limited data suggests just the opposite, supporting the higher incidence of maternally-derived aneuploidy. Cytogenetic techniques are difficult to apply to gametes, with MII oocyte chromosomes of poor morphology and sperm nuclei requiring labour-intensive heterospecific fertilisation techniques to yield metaphase chromosomes (Rudak et al., 1978). Several studies have been performed to show the frequency of the incidence of aneuploidy. Average sperm karyotypes show 1-4% aneuploidy compared to 15-20% seen in oocytes (Martin et al., 1991; Pellestor et al., 1991; Jacobs, 1992; Benkhalifa et al., 1996). In part this reflects the higher sensitivity of mammalian spermatogenesis to meiotic disturbances compared to oogenesis where lack of a cell cycle checkpoint can lead to aneuploidy (LeMaire-Adkins et al., 1997). Good evidence of more stringent control mechanism operating during male meiosis
has been shown in the mouse and provides a reason for the difference in the error rate between oogenesis and spermatogenesis (Odorisio et al., 1998). In a more recent study on meiotic genes using a mouse model, the authors suggest that faced with adversity, male meiosis grinds to a halt, whereas female meiosis soldiers on (Hunt and Hassold, 2002). The result of this female “robustness” comes with a cost, however, because aneuploidy appears to be increased in the resultant oocytes.

1.1.1.1 Spermatogenesis.

Spermatogenesis begins at puberty and continues throughout adult life. The process of spermatogenesis can be divided into three phases; proliferation, meiosis and differentiation. These are associated with specific germ cell types—spermatogonia, spermatocytes and spermatids. At puberty the testes secrete the steroid hormone testosterone, triggering the growth of the testes, maturation of the seminiferous tubules, and commencement of spermatogenesis. Testosterone causes the primordial germ cells to undergo mitosis and differentiate into spermatogonia. The cells that will undergo spermatogenesis arise by mitosis from the spermatogonia.

Spermatogenesis takes place continuously from puberty to death. In the human male, each spermatogenesis cycle takes approximately 64 days. Spermatogonial mitosis occupies 16 days. The first meiotic division lasts about 8 days and the second 16 days. Spermiogenesis requires about 24 days (Metz and Monroy, 1985; Eddy and O’Brien, 1993).

For 2% of the male population however, the process is defective resulting in abnormally low sperm counts (<20x10^6 /ml, oligozoospermia) or total absence of sperm (azoospermia). The discovery that some of these men had a deletion for a region of Yq termed AZF (azoospermia factor) (Tiepolo and Zuffardi, 1976) lead to characterisation of gene families involved in spermatogenesis, with those mapped including DAZ (Reijo et al., 1995) and RBM (Ma et al., 1993). A number of different aetiologies can disrupt the pattern of spermiogenesis and the epithelium of the tubules is very sensitive to toxins or to ischaemia.

Of the 200-600 million sperm deposited near the cervical canal during sexual intercourse only a few hundred will complete the journey to the uterine tubes taking 5-45 minutes, with the majority of the remainder becoming non-viable after 24 hours (Settlage et al., 1973). Prior to this human sperm undergo a terminal step of functional maturation called capacitation in the female genital tract, a prerequisite for...
Introduction

fertilisation whereby changes in the acrosome occur to prepare it to release the enzymes required to penetrate the zona pellucida. More particularly, glycoproteins are lost from the acrosomal region and motility increases (de Lamirande et al., 1997).

1.1.1.2 Oogenesis.

Oogenesis is discontinuous and begins during fetal life. Oogenesis begins during the sixth week of development when oogonia in the ovary start to proliferate mitotically and form primordial follicles, consisting of primary oocytes surrounded by a layer of somatic epithelial cells (Siracusa et al., 1985). These diploid oocytes then enter MI before arresting at diplotene of prophase I. At birth, as with most mammalian species, all oocytes are arrested in the germinal vesicle stage and remain in this quiescent state until puberty (Baker, 1963). An estimate of 200 000 germ cells are available for the reproductive life span at puberty when recruitment of some of these primordial follicles begins. However, over 99% of follicles undergo atresia rather than ovulation, a degenerative process leading to cell death.

The hormones of the female cycle control folliculogenesis, ovulation, and the uterine condition. At female puberty the secretion of hypothalamic, pituitary, and ovarian hormones control a menstrual cycle with the purpose of producing a single female gamete and a uterus in a condition to receive a fertilised embryo. The hypothalamus produces gonadotrophin releasing hormones (GnRH) stimulating the pituitary gland to increase its secretion of the two gonadotropic hormones (gonadotropins): follicle stimulating hormone (FSH) and in later stages luteinizing hormone (LH). These hormones promote folliculogenesis involving growth and differentiation of 5-12 primary (preantral) follicles. This time of maturation is characterised by proliferation of the surrounding follicular cells as well as secretion of oestrogen. The oocyte is covered by a glycoprotein called the zona pellucida (Heikinheimo & Gibbons, 1998) and in the progression from primary (preantral) to secondary (antral) follicle, a single follicle becomes dominant and the remainder degenerate as a fluid filled space known as the antrum forms whilst the oocyte surrounded by cumulus cells becomes positioned eccentrically within the follicle (Wassarman and Albertini, 1993). During this follicular maturation, the oocyte is transcriptionally and translationally very active accumulating high levels of mRNA and the cytoplasmic volume increases some 300-fold (Driancourt and Thuel, 1998). The oocyte accumulates specific RNA macromolecules required at a later stage for
the control of embryogenesis. The endogenous LH surge induces the final nuclear maturation of the oocyte within the next 24-36 hours (Heikinheimo & Gibbons, 1998). In the final maturation time the nuclear membrane of the germinal vesicle breaks down (GVBD), the primary oocyte completes MI with extrusion of the first polar body, before arresting again at the metaphase stage of MII. It is now known as a secondary or MII oocyte (Wassarman and Albertini, 1993). Progression from the first to the second meiotic arrest is called oocyte maturation and ovulation should now commence. At ovulation the mature follicle ruptures to release the MII oocyte surrounded by a layer of cumulus cells. Under the influence of LH the remaining follicle forms the corpus luteum, which secretes progesterone to prepare the endometrial lining of the uterus for implantation. If fertilisation does not occur, oestrogen and progesterone levels fall and twelve to fourteen days later, menstruation begins (Moore, 1988).

1.1.2 Fertilisation.

Fertilisation is a complex interaction between sperm and oocyte that takes place in the ampulla of the uterine tube. This multi-step process begins with the specific recognition of complementary receptors on the surfaces of the two gametes and terminates with syngamy, the union of maternal and paternal chromosomes. Binding of the sperm head to the oocyte is mediated by at least three major glycoproteins, ZP1, ZP2 and ZP3 consisting the zona extracellular matrix (Longo, 1997). This process has been particularly well studied in the mouse homologues arranged as ZP2/ZP3 heterodimers cross-linked with ZP1 dimers, showing that sperm/ZP3 binding initiates a cascade of signalling events culminating in exocytosis of the acrosome (Bleil and Wassarman, 1983; Wassarman et al., 1999). Following the binding on the ZP it undergoes the acrosome reaction, resulting to the release of certain digestive enzymes allowing the sperm to penetrate the zona pellucida, and the subsequent exposure of the inner acrosomal membrane. The fusion also causes the oocyte to resume meiosis completing the second meiotic metaphase and rapidly proceeding through anaphase producing another polar body. The chromosomes of the oocytes and the sperm are respectively enclosed within the female and the male pronuclei, which fuse with each other to produce the single, diploid nucleus of the
fertilised zygote. This moment is the zero time point of embryonic development (Larsen, 1997).

1.1.3. In Vivo Preimplantation Embryo Development.

Initial work in this field was performed on embryos recovered from hysterectomies, and analysis involved thorough morphological descriptions (Hertig et al., 1954; 1959). However, Steptoe and Edwards were the first to be successful with In Vitro Fertilisation (IVF) in 1978, which paved the way for detailed studies to be performed on early embryos.

Pronuclei formation commences about 8-14 hours post insemination and the first cleavage division about 32 hours post insemination. The embryo should have divided into 4 cells by 48 hours and 8 cells by 72 hours post insemination. By day 4 embryos should reach the morula stage and by day 5-6 embryos form the blastocyst. During the first days of development, the zygote travels down the oviduct and undergoes cleavage that subdivides the zygote without increasing its size. In fact, with each division the resulting blastomeres are half the size of the parent ones, and they become increasingly tightly connected as compaction occurs to form the 16-32 cell embryo with an appearance of a small mulberry therefore called a morula (90-120h post-insemination) (Trounson et al., 1982). This is a time characterised by intense DNA synthesis and replication.

Activation of the zygote genome is essential for the embryo’s further development, which requires reprogramming the pattern of gene expression. Studies involving the use of cDNA libraries constructed from human preimplantation embryos have shown the presence of sequences corresponding to known housekeeping genes and tissue-specific genes as well as completely novel ones as yet uncharacterised, all of which vital to the complex process of embryogenesis (Buraczynska et al., 1995; Adjaye et al., 1997; Daniels and Monk, 1997; Morozov et al., 1998). At the 4- to 8-cell stage in humans the expression of the embryonic genome increases while maternal mRNA rapidly disappears. Although it is likely that transcripts from the embryonic genome influence development from very early stages (Daniels and Monk, 1997), embryos treated with transcription inhibitors progress to the 4- to 8-cell stage presumably reliant solely on maternal mRNA/proteins and it is at that stage that the main switch from maternal to embryonic control is thought to occur.
Introduction

(Tesarik et al., 1986; Braude et al., 1988). This is in contrast to the earlier 2-cell stage embryonic genome initiation in the mouse (Flach et al., 1982) and later 16-cell stage in bovine embryos (Telford et al., 1990). With regards to the metabolic requirements of the embryo during the preimplantation period, activation of the embryonic genome is of great importance. Prior to genome activation pyruvate and lactate are the primary energy sources. However, following the activation of the embryonic genome, metabolism shifts to glucose. The precise amino acid requirements of the preimplantation embryo are unknown but they certainly vary before and after genome activation (Gardner, 1998).

To facilitate and coordinate the multipart process of growth and differentiation two types of intracellular junctions have been described as communicative devices between cells, appearing from the 8-cell stage onwards. Firstly there are the structural tight junctions and desmosomes forming in the outer most cells, anchoring the cells together and forming a permeability seal isolating the interior of the embryo from the external environment. Certain integral and peripheral proteins such as occludin and cingulin (ZO-1) compose the tight junctions. Desmosomal proteins are synthesised between 8-16 cells. They are points of intercellular contact that ‘bolt’ the cells together. Secondly, there are the low resistance junctions known as gap junctions allowing intracellular connection between the cytoplasm of two cells so that small proteins and ions can be exchanged. Direct transfer through the gap junctions includes metabolites and second messangers (cAMP) (Bennet et al., 1997). In human embryos gap junctions are not apparently well developed until the early blastocyst stage when intracellular communication is clearly seen between inner cell mass (ICM) cells (Dale et al., 1991).

Experimental splitting of 2- to 8-cell embryos from a number of species demonstrates the totipotency of single blastomeres in these early cleavage stages to produce viable offspring (Tarkowski and Wroblewska, 1967; Willadsen, 1980; Johnson et al., 1995). The exact stages at which such totipotency becomes progressively restricted to pluripotency before final terminal differentiation, is the subject of debate (Gardner et al., 1996; Edwards and Beard, 1997; 1999). There is some evidence of polarity from the uneven distribution of some cytokine and transcription factor molecules following cleavage divisions, although the significance of this is unclear (Antczak and Van Blerkom, 1997; 1999). Studies involving the random injection of fluorescent tracer dye into human embryos cultured until the
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blastocyst stage showed that blastomeres can give rise equally to either ICM or TE at least up until to the 8-cell stage (Mottla et al., 1995).

The next stage of embryo development is compaction where blastomeres flatten against each other and form junctions between them. Boundaries are no longer distinguishable and the embryo becomes polarised. The apical surface faces the outside of the embryo and the basal surface faces inwards. Intercellular adhesion is mediated by the redistribution of uvomorulin, a calcium dependent adhesion molecule. Although factors triggering the onset of compaction are unknown, it has been suggested that is regulated by post-translational modification of specific proteins such as E-cathedrin (uvomorulin) which accumulates in the regions of intracellular contact and is uniformly distributed on the blastomeres’s surface. Following compaction the embryo, now described as a morula, shows an increase and change of pattern in RNA, protein and phospholipid synthesis, resulting in differentiation of the cells to inner cell mass (ICM) which will form the embryo proper, and the outer cells forming the epithelial layer of trophectodrem (TE) which will form the placenta and extra embryonic tissue (Fleming et al., 2001). The side of the blastocysts containing the ICM is called the embryonic pole and the opposite side is called the abembryonic pole.

There is no size change observed in the early blastocyst (day4/5) however, active fluid accumulation in the blastocoel cavity causes it to expand as. Fluid filled cavities develop between the blastomeres by action of a Na+K+ATPase (cavitation). While the embryo transforms from morula to blastocyst as it enters the uterus, proteolytic enzymes are produced by the TE causing the embryo to “hatch” free of the zona and implant. Although the molecular basis of hatching and implantation is unknown, it is suggested to be regulated by gene expression of a variety of growth factors and their receptors (Sharkey et al., 1995).

1.1.4. Infertility, In Vitro Fertilisation (IVF) and Embryo Culture.

Infertility is defined as an involuntary failure to conceive within 12 months of unprotected intercourse. Approximately 90% of couples with normal fertility will conceive in the 1st year, whilst 95% will have conceived within 2 years (Greenhall and Vessey, 1990). The epidemiological distribution of the main causes of infertility
show unexplained infertility accounting for 30%, ovulatory disorders (27%), tubal
damage (14%), endometriosis (5%), cervical mucus problems or uterine abnormalities
(5%) and male factor (19%) (Data from effective Health Care: The Management of
Infertility, 1999). The first successful birth following IVF treatment was reported in
1978 by Steptoe and Edwards (Steptoe and Edwards, 1978). This medical
breakthrough heralded the beginning of real hope for thousands of infertile couples.
Over the last 25 years, superovulation regimens have been fine-tuned, methods to
prepare the sperm have been improved, and our understanding of the needs of the
early embryo has expanded, contributing to the increase in the number of patients that
can be treated successfully. The pregnancy rate varies between centres according to
the patient group treated but overall the actual livebirth per cycle or ‘take home baby’
rate is about 25%, which declines rapidly as maternal age increases over 36 years
(HFEA Annual Report, 2000). Of all pregnancies following IVF 29% are multiple
with a significant proportion of triplets (5%). Obstetric and perinatal complications
associated with IVF are largely attributable to the high incidence of multiple
pregnancies at risk of pre-term delivery and low birthweight. There is no evidence
that fetal abnormalities are more common in children conceived after assisted
conception (about 2-3%), compared to their peers conceived normally. In longitudinal
studies, child development appears normal (SART, 1992; FIVNAT, 1995). Similarly
although a single embryo cryopreservation study has suggested an associated
abnormal increase in body weight in adult mice (Dulioust et al., 1995), human births
from cryopreserved/thawed embryos do not differ significantly from those for fresh
IVF embryos or natural conceptions (Wennerholm et al., 1997).

1.1.4.1. Intracytoplasmic Sperm Injection (ICSI).

Micro-assisted fertilisation techniques (MAF) based on IVF technology have
been developed since the late 1980s specifically to address the problem of male factor
infertility. These include partial zonal dissection (PZD) making a breach in the zona
by mechanical dissection to facilitate sperm entry (Malter and Cohen, 1989), and sub-
zonal insemination (SUZI) involving placement of two to five single sperm into the
perivitelline space (Laws-King et al., 1987). These techniques have now been
superseded by the technique of choice, intracytoplasmic sperm injection (ICSI)
involving direct microinjection of sperm into the cytoplasm of a mature metaphase II
egg that has been denuded of all surrounding cumulus and corona cells. All types of
sperm may be injected and sperm does not need to be acrosome reacted or capacitated for fertilisation to occur. ICSI is an invasive but very successful method of treatment that bypasses all natural barriers to conception facilitating the sperm-oocyte interaction (Palermo et al., 1992). ICSI has been developed specifically to address the problem of male factor infertility, allowing even azoospermic men to father children.

These infertile men are a diverse group exhibiting various testicular histology ranging from Sertoli-only types to varying degrees of meiotic and maturation arrest. Obstructive cases include congenital bilateral absence of the vas deferens (CBAVD) associated with cystic fibrosis mutations (Chillon et al., 1995), whilst non-obstructive cases show a well-documented two to ten fold increase in the incidence of karyotypic abnormalities, half of which involve the sex chromosomes, compared to the general population (Chandley et al., 1975; Retief et al., 1984; Scholtes et al., 1998) as well as an array of Y chromosome deletions (Tiepolo and Zuffardi, 1976; Reijo et al., 1995). Therefore, significant worries remain about ICSI as it by-passes the natural barriers to fertilisation potentially resulting in fetal abnormalities (Cummins and Jequier, 1994). These fears appeared to be confirmed with reports of an increase in sex chromosome aberrations in ICSI babies (Bonduelle et al., 1995; 1998; In’t Veld et al., 1995; Tournaye et al., 1995) and the inheritance of paternal chromosome rearrangements (Testart et al., 1996; In’t Veld et al., 1997; Meschede et al., 1997) and Y deletions (Kent-First et al., 1996), however with the exception of hypospadias no overall increase in major congenital abnormalities has been noted (Wennerholm et al., 2000). As a precaution routine karyotyping is now recommended prior to ICSI (Martin, 1996; Persson et al., 1996; Rosenbusch et al., 1996).

1.1.5 Ethical and Legal Considerations of Human Embryo Research.

Rapid developments in reproductive medicine and science regularly present new social and ethical issues requiring the authority to formulate ethical policy and review its existing guidance. Following embryo transfer the surplus embryos resulting from superovulation could be cryopreserved, discarded, donated to another couple undergoing fertility treatment following informed patient consent, or made available for research. The research performed on such embryos targets improvement of IVF culture conditions, further understanding of human preimplantation embryo development, and testing therapeutic approaches such as preimplantation genetic
diagnosis (PGD) (Edwards, 1982). The surplus IVF generated embryos donated to research are referred to as pre-embryos separating them from later post-implantation embryonic and fetal stages resulting from induced abortion.

What poses the main ethical constraint for scientific research on human embryos is the matter of; when does human life and personhood begin? (Bennet and Harris, 1997). As there is a variety of cultural, religious, and moral beliefs in today’s society it is understandable that the response to the above posed question varies greatly. Islam views the first feeling that life exists in the womb as significant, Judaism attaches importance to the actual potential of the embryo for implantation but the fetus is only considered a separate being at birth whilst Roman Catholicism recognises life as beginning at the moment of conception and as such all embryo research is unacceptable (Schenker, 1998). At present, the most widely accepted position is an intermediate one regarding the embryo as “potential human life” and therefore granting it appropriate ethical consideration and respect (Fasouliotis and Schenker, 1998).

The wide media coverage regarding advances in Assisted Reproduction Technology (ART) is the source of most of the ethical concerns raised today concerning what could become possible in the future. Cloning, prenatal diagnosis, and gene therapy (Fletcher, 1978; Fiddler and Pergament, 1995; Fiddler et al., 1999) are research fields mainly linked with fear of excessive embryo and fetal experimentation. Consequently most countries where this technology exists now have in place, or are in the process of defining, ethical guidelines or legislation to regulate human embryo research.

In the UK the authority was established by the Human Fertilisation and Embryology Act 1990, following a long period of debate about the social, ethical and legal implications of developments in ART. Two of the main issues underlying the legislation were concerns about the creation and use of human embryos outside the body, and about the storage and use of genetic material (gametes or embryos) for the treatment of others. The five purposes for which embryo research is currently permitted are: (1) promoting advances in the treatment of infertility, (2) Increasing knowledge about the causes of congenital disease, (3) Increasing knowledge about the causes of miscarriage, (4) Developing more effective techniques of contraception and (5) Developing methods for detecting the presence of gene or chromosome abnormalities in embryos before implantation. The HFEA is required under section 25
of the HFE Act to: “Maintain a code of practice giving guidance about the proper conduct of activities carried on in pursuance of a licence under this Act and the proper discharge of the functions of the persons responsible and other persons to whom the Act applies”. The main elements of the Code of Practice are: staff, facilities, assessing patients and donors, information, consent, counselling, use of gametes and embryos, storage and handling of gametes and embryos, research, records, and complaints. Research is only permitted up to fourteen days post-conception at the stage immediately proceeding formation of the primitive streak, a seemingly arbitrary choice but one which signifies the last stage at which twinning may occur. Prohibited are reproductive cloning, inter-species fertilisation or transfer of human embryos into other species and gene therapy in the pre-embryo.

Other countries with legislation regulating research on human embryos include: Australia, Spain, USA, France, etc. Legislation in France places much stricter controls on all types of this work but has recently allowed PGD under specific circumstances linking it to prenatal diagnosis rather than embryo research (Viville and Nisand, 1997). In the USA, the legality of such research is more complicated since both federal and state laws apply as well as recommendations of other bodies such as The American Fertility Society Ethics Committee. Federal law prohibits funding for any research on human embryos and as such all work is carried out by private institutes (Viville and Pergament, 1998). In Germany the Embryo Protection Act prohibits the creation of embryos for any purpose other than achieving a pregnancy.

“Designer babies” and PGD raise real ethical dilemmas in certain unusual cases (Braude et al., 1998; Braude et al., 2001). For example a couple suffering from achondroplasia might request PGD to avoid embryos that would be lethal in utero, but wish to select only heterozygous embryos that would give rise to children with achondroplasia rather than unaffected embryos, to suit their lifestyle. A similar dilemma might occur with inherited deafness. In a recent case a non-hearing child was deliberately conceived to be deaf like its lesbian parents (Savulescu et al., 2002). Sex selection referred to as “family balancing” remains controversial (Gleincher and Karande, 2002) and many consider it not to be a legitimate use of PGD (Robertson, 2002). Finally, the attempt to save the life of a sibling by having another child provide a suitable tissue match can be rationalised and commended however, this process has met with great controversy (Boyle and Savulescu 2002). Issues of consent and protection of children’s autonomy become paramount in these cases and should form
the focus for giving an approval in cases or countries where more general methods for regulation of PGD are in force (Braude et al., 2002).

1.2 Molecular and Cytogenetic Techniques.

The growing needs of diagnostics and research in the field of chromosome abnormalities have lead to constant improvement and expansion of the range of laboratory techniques available to detect them. Karyotyping, being the first technique allowing analysis and identification of all 23 pairs of chromosomes with respect to number and morphological structure, is still considered the most widely applicable technique in clinical and research cytogenetics.

A valuable and innovative technique, FISH has combined DNA hybridisation techniques with fluorescent microscopy, allowing direct visualisation of a specific DNA sequence onto metaphase chromosomes, interphase nuclei or DNA strands and has been described as the most important achievement in the field of cytogenetics in the last 20 years (Bui et al., 2002). Although the approach using radio-isotope labelled probes was developed in 1969 (John et al., 1969; Pardue and Gall, 1969), it was the introduction of simplified fluorescent detection system in the 1980s (Manuelidis et al., 1982; Cremer et al., 1986; Pinkel et al., 1986; Lichter et al., 1988) which allowed this technique to find a wider use outside specialist research laboratories and lead to the diversification in related techniques seen today (Lichter and Cremer, 1992). The combination of FISH and karyotyping has lead to the development of innovative techniques such as spectral karyotyping (SKY-FISH) (Schrock et al., 1996) and multi-fluorochrome karyotyping (M-FISH) (Speicher et al., 1996).

The Polymerase Chain Reaction (PCR) using oligonucleotide primers and a thermostable DNA polymerase (Saiki et al., 1985), is another key technique designed to enrich a DNA sample for a specific fragment, amplifying it to a level at which it can be visualised and subjected to further genetic analysis. The marriage of molecular and cytogenetic approaches is termed molecular cytogenetics and has dramatically expanded both the range and the precision of routine chromosome analysis, leading to the development of revolutionary techniques such as: microarrays (DNA chips) and Comparative Genomic Hybridisation (CGH), (Jackson, 2002). CGH is a combination of cytogenetic and molecular based
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techniques, which in a single hybridisation allows the copy number of all 23 pairs of chromosomes to be assessed in situations that do not allow standard methods of chromosomal analysis to be used (Kallionemi et al., 1992). Microarrays, or the spotting of numerous specific DNA clones or targets to an analysable matrix unites cytogeneticists and molecular geneticists with a relatively common approach to resolving questions about the genome, especially following the event of mapping a total of 8,484 human bacterial artificial chromosomes (BACs) to single chromosomal bands, tagged with a piece of known unique sequence (Cheung et al., 2000; Antonarakis, 2001).

1.2.1 Karyotyping

The number, sizes and shapes of the metaphase chromosomes constitute the karyotype. Following certain modifications, the methods Tjio and Levan used in 1956 to demonstrate that the normal human somatic cell contains only 46 chromosomes, are now universally employed in all cytogenetic laboratories to analyse the chromosome constitution of an individual, which is known as a karyotype. Giemsa or G-banding gives each chromosome a characteristic and reproducible pattern of light and dark bands. Quinacrine (Q), Reverse (R), and Constitutive Heterochromatin (C) banding, are variations in banding techniques allowing chromosome classification. The banding pattern of each chromosome is specific and can be shown in the form of a stylised ideal karyotype known as ideogram (Therman and Suuman, 1993).

Karyotyping can be used on any tissue with living nucleated cells, which undergo division. Most commonly lymphocytes from peripheral blood, amniotic fluid and tissue fibroblasts are cultured and using either colcemid or colchicine may be arrested at metaphase. Karyotyping has long been the technique of choice for prenatal diagnosis since the first amniocentesis and chorionic villus sampling (CVS) procedures were performed. Moreover, karyotyping has been applied to oocytes, polar-bodies, spermatozoa and embryos (Plachot et al., 1988, Clouston et al., 2002). However, it has always been a very time-consuming and labour-intensive technique. Even though development of in-situ technologies such as fluorescent in situ hybridisation (FISH) have decreased culture reporting time significantly (Van Opstal et al., 1993) and computer systems have been developed to automate the karyotype procedure, the results still take a great deal of time to obtain (Evans et al., 1999). Following the advent of FISH, recent advances have lead to the development of
spectral karyotyping (SKY) (Schrock et al., 1996), and multi-fluorochrome karyotyping (Speincher et al., 1996) which will be reviewed in chapter 1.2.2.3.

1.2.2 Fluorescent In Situ Hybridisation (FISH)

1.2.2.1 Principles of FISH.

FISH is a molecular cytogenetic technique for enumerating chromosomes combining DNA hybridisation techniques with fluorescent microscopy and relies on the property of single strands of DNA to hybridise to complementary target strands and form a stable DNA hybrid duplex when perfectly base-paired as seen in Fig. 1.1. During FISH a specific DNA fragment is tagged with a compound that fluoresces. This combination is termed a probe. FISH probes are quite sensitive DNA molecules being able to detect regions even of the size of 0.5 kb on metaphase chromosomes (McNeil and Ried, 2000). Cloned DNA fragments are usually the constitution of FISH chromosomal probes, which are characterised by the ability to anneal only to their complementary DNA sequences (Wells and Levy, 2003). Vectors commonly used to obtain DNA probes in increasing order of insert size (200b-1.5Mb) include plasmids, cosmids, P1s, P1 derived artificial chromosomes (PACs), bacterial artificial chromosomes (BACs) and yeast artificial chromosomes (YACs).

Pretreatment with proteolytic enzymes and/or RNase digestion increases the accessibility of the DNA probe to the sample on the slide with metaphase or interphase cells. Denaturation of the slide and probe follows causing the double DNA strands to separate which are then left to reanneal under optimal conditions, the length of time depending on probe type. The stringency with which the probe binds is controlled during post-hybridisation treatment by varying the temperature, ionic strength and concentration of formamide washes to destabilise and remove imperfectly base-paired probe/target duplexes. In this way only probe bound stably to its complementary target remains. After hybridization the slide is observed under ultraviolet light of the proper wavelength, and any region where the labeled DNA fragment has bound fluoresces (Jackson, 2002).

Incorporating a labeled nucleotide into the probe DNA can be performed by a variety of techniques such as: nick translation, end-labeling, and PCR (Rigby et al., 1977). Initially, FISH experiments relevant to human embryos employed indirectly labelled probes (Griffin et al., 1991, 1992, 1993, 1994).
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Fig. 1.1: FISH principles

The double stranded DNA of the chromosome and the probe, give rise to single strands of DNA following denaturation. Chromosomes and probe are allowed to reanneal, as single stranded DNA hybridise to complementary target strands and form a stable DNA hybrid duplex when perfectly base-paired.

These probes were labelled with a hapten such as biotin and digoxygenin which then required the application of fluorophore-linked immunoglobin reagents (Langer et al., 1981). This immunocytochemical detection of ‘indirectly-labelled’ probes allowed the flexibility to amplify a weak FISH signal, where necessary by sequential applications and was especially useful for visualising smaller probes. However, indirect labelling is a time-consuming process with non-specific background fluorescence potentially making interpretation difficult. Directly labelled probes, where the fluorochrome is attached to the probe itself, improve the time-frame of the FISH experiments as they can be visualised immediately post-hybridisation (Wiegant et al., 1991, 1993). Moreover, the specificity of the fluorescent detection is clearly enhanced as the non-specific binding of immunoglobin reagents, which can result in high background fluorescence, is significantly reduced (Harper et al., 1994a). Fluorophores commonly used are those based on fluorescein (green) or rhodamine (red), although this selection has widened considerably in the last few years with the introduction of a new generation of commercial fluorophores such as the Cyanine, Blue, and Gold dyes (Yurov et al., 1996).

Probes are defined according to the nature of the DNA sequence and can be divided into four broad groups as seen in Fig. 1.2.
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Repeat sequence probes anneal to repetitive specific sequences located at the centromeric, pericentric, or telomeric regions of target chromosomes. These include the widely used α-satellite/centromeric probes (b), which hybridise specifically to the centromeres of individual chromosomes (Willard, 1985). Such specific centromeric probes are available for the majority of chromosomes, although some also co-hybridise to another chromosome that shares a homologous sequence (chromosome pairs 5 and 19, 13 and 21, 14 and 22). Heterochromatic probes include those binding to the heterochromatin usually situated below the centromere, seen in chromosomes 1, 9 and Y. All repeat sequence probes hybridise in a short time, in some cases less than an hour, and are particularly suitable for direct labeling. The signals obtained are very bright and the whole FISH procedure can be performed within two hours (Harper et al., 1994c).

Locus specific or unique sequence probes (c) uniquely identify specific nucleic acid sequences on a target chromosome, such as those encoding genes. Depending on the type of clone, these probes may range from small cDNA fragments in plasmids, to much larger blocks of genomic DNA in YACs. Alu PCR has been employed to achieve probe DNA amplification following the YAC culture. The presence of the human Alu sequence hybridizing to all chromosomes resulting in a R-band pattern and loss of the specific probe signal poses an important problem in the use of such probes. This problem is overcome by chromosomal in situ suppression (CISS) (Lichter et al., 1988) in which Cot-1 DNA is added to the probe DNA binding and blocking the repeat sequences in the probe DNA allowing the probe to bind to the unique sequence that it targets. In the case of commercially available locus specific probes the probes are premixed with blocker DNA as well to exclude hybridisation to other loci. Since these probes have to find a unique sequence they require longer...
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hybridisation (between 2-12 hours) to allow the less abundant copy number sequence to reanneal.

Telomeric probes specifically recognize the repetitive sequence TTAGGG, and can be used for the simultaneous identification of all telomeres, while chromosome-specific “sub-telomeric” probes (d) bind and specifically recognise subtelomeric, chromosome-specific repetitive sequences (McNeil and Ried, 2000). Each probe is estimated to be within 300kb from the end of the each chromosome arm (National Institutes of Health and Institute of Molecular Medicine Collaboration, 1996).

Chromosome paints (a) contain a cocktail of DNA sequences hybridising to an entire chromosome or chromosome arm and are derived either from a pool of clones picked from a chromosome specific library (Fuscoe et al., 1989) or from flow-sorted chromosomes amplified by PCR (Vooijs et al., 1993). As with locus-specific probes, these paints are co-hybridised with competitor DNA and require an extended hybridisation time to allow low copy number sequence to reanneal. Chromosome paints provide excellent fluorescent intensity, improved chromosome coverage, highly specific signal and a low background noise, but in general are only suitable for metaphase work.

1.2.2.2 Applications of FISH.

The development of FISH techniques to examine the presence or absence of a particular DNA sequence or to evaluate the number or organisation of a chromosome or chromosomal region has revolutionized both research and clinical cytogenetics. Definition of chromosome anomalies in prenatal, postnatal, and preimplantation genetics is critical for predicting pregnancy outcomes and future quality in life and FISH is a powerful means for determining many types of chromosome anomalies.

The growing availability of commercially produced probe cocktails enabled FISH to become an outstanding routine diagnostic tool in the cytogenetic laboratory. For example, microdeletion probes consist of a probe specific for the locus or region of deletion associated with the microdeletion syndrome, as well as a control probe for accurate identification of the chromosome of interest. Their use has proved very useful for the detection of a range of microdeletion syndromes, providing rapid same-day results (Ligon et al., 1997). Other recently developed locus-specific probes have successfully confirmed complex or cryptic chromosomal rearrangements (Speleman
et al., 1992; Stankiewicz et al. 1997; de Die-Smulder et al. 1999) as well as showing the origin of marker chromosomes (Blennow et al., 1995). Genetic anomalies and complicated karyotypes associated with cancer continue to be discovered through the use of FISH employing commercially available DNA oncology and pathology probes (Thein et al., 1995; Guan et al., 1998; Tamura et al., 1998). The wide selection of existing centromeric probes allows determination of chromosome-specific ploidy on solid tumours, fresh or archived biopsy specimens, and on cultured and uncultured preimplantation, prenatal, postnatal and hematologic samples. Chromosome-specific ploidy analysis is increasingly becoming a critical technique for clinical researchers in areas like breast and myeloid cancers while common aneuploidies in uncultured amniocytes have been detected for rapid prenatal diagnosis (Klinger et al., 1992; Zheng et al., 1992; Ward et al., 1993; Bryndorf et al., 1997). Telomeric regions lost due to deletions, and unbalanced translocations have been associated with idiopathic mental retardation, as well as cryptic rearrangements (Flint et al., 1995; Warburton et al., 2001). Research has shown that subtelomeric DNA contains a high density of genes (Flint et al., 1997). DNA probes for the terminal regions of chromosome arms are proving to be useful research tools for a number of disorders involving the telomeric regions (NIH/IMM Collaboration 1996). FISH is also an ideal means to analyse samples where good quality metaphase preparations are difficult to obtain such as oocytes (Dailey et al., 1996; Mahmoud et al., 2000), embryonic nuclei (Munné et al., 1993b; Harper et al., 1994a) and sperm nuclei (Spriggs et al., 1996; Van Hummelen et al., 1997).

Microdissection (Meltzer et al., 1992) and flow-sorting (Carter et al., 1992) can be employed for detection of unknown markers, by isolating the region of interest and subsequently subjecting it to amplification by PCR to produce a DNA probe for FISH. This reverse painting has been frequently used for prenatal and postnatal samples (Müller-Navia et al., 1995; Senger et al., 1997), markers associated with tumours (Zhang et al., 1993; Jonveaux et al., 1996) and characterisation of somatic cell hybrids (Jones et al., 1992).

Although, FISH has been characterised as one of the most robust and efficient techniques, it has certain limitations. Performing FISH on interphase cells is not as efficient as metaphase FISH (Ruangvutilert et al., 2000b), while the phenomena of hybridisation failure (Harper and Wells, 1999) and of cross-hybridisation (Verlinsky et al., 1995) pose major concerns. Overlapping loci, signal splitting (due to DNA
replication) or abnormal fluorescence may lead to ambiguous results and misdiagnosis (Voullaire et al., 2000; Munné et al., 1996b; Munné et al., 1998d). It has been shown that in the case of multicolour-FISH where more than one probe is hybridized, as well as in "sequential hybridization" where the FISH procedure is carried out in two or three sequential rounds, the hybridization efficiency is compromised decreasing significantly by each extra round or extra probe added (Liu et al., 1998). The accuracy of the FISH procedure per probe per interphase cell has been established to be around 91-96% for α-satellite sequences (Ruangvutilert et al., 2000b). However, as many as six different chromosomes have been simultaneously analysed by Munne et al. (1998) and Gianaroli and colleagues (1999), achieving a total of nine chromosome studied per cell with a second sequential hybridisation, but for the purposes of aneuploidy screening rather than specific diagnosis.

1.2.2.3 Multiplex-FISH (M-FISH) and Spectral Karyotyping (SKY).

The important diagnostic role that FISH plays in the characterization of chromosomal abnormalities remains limited by the number of spectrally distinguishable fluorochromes or fluorochrome combinations. Since 1990 many groups have tried to maximise the number of probes that can be used employing combinatorial or Boolean labelling ie: combining labelled probes in different proportions. First demonstrated by Nederlof et al., (1990) with three fluorophores to detect four chromosomes, combinatorial labelling remains a strategy that although in theory many combinations are possible, in practice the number of combinations are dependent on the sensitivity of the imaging system. Since then, several groups have developed combinatorial labelling, to the detection of six loci on a single chromosome (Wiegant et al., 1993), to seven chromosomes using the same number of labels (Ried et al., 1992), while other groups have applied combinations of chromosome paints and YAC probes to produce chromosomal bar codes for up to seven chromosomes (Ried et al., 1992; Lengauer et al., 1993).

It was in 1996 that Speicher et al., (1996) attempted combinatorial labelling of twenty-four chromosome paints using five fluorophores, reporting the genesis of Multiplex-FISH or Multi-fluorochrome Karyotyping. Following hybridisation a monochrome CCD camera with multiple optical filters was used to capture each channel sequentially before merger to form a final image. Analysis for M-FISH is based on the presence or absence of probe signal for each fluorophore at each pixel.
that is then compared to the labelling strategy to identify the chromosome (Speicher et al. 1996). In the same year the group of Schröck et al., employed a dedicated custom-designed imaging spectrometer system, in a method known as spectral karyotyping or SKY-FISH. This finely samples each pixel for all fluorescence across the spectrum simultaneously producing a set of interferograms, before data processing to form a spectral image. Analysis is based on comparison of the interference pattern for each pixel with stored data on chromosome interference spectra, the pixel is then allocated to a matching chromosome (Schröck et al. 1996). Both techniques permit the simultaneous visualisation of all human chromosomes using 24 chromosomes-specific probe paints labelled with different combinations of fluorochromes. SKY-FISH has been employed to successfully identify particular de novo supernumerary marker chromosomes as well as, de novo unbalanced structural rearrangements, proving to be of beneficial role for diagnostic and counselling purposes, due to its reliability and speed (Haddad et al., 1998). SKY-FISH has already been used to examine chromosomes from oocytes and polar bodies (Marquez et al., 1998). Finally, it has been used on blastomeres and polar bodies where the nucleus has been converted to a metaphase (Evsikov & Verlinsky, 1999; Willasden et al., 1999). Henegariu and colleagues (1999) developed an alternative multicolour karyotyping technique, called colour-changing karyotyping (CCK) using only 3 fluorescent dyes to discriminate up to 41 different DNA probes when each probe is labelled with no more than 3 fluorescent or hapten-modified nucleotides. CCK discriminates the chromosomes not by ratio-labeling or pseudo-banding, but through the difference in signal strength between direct-fluorescent and antibody-detected chromosomes, and it uses only one hybridisation and two imaging steps.

1.2.3 PCR

1.2.3.1 Applications and Limitations

The polymerise chain reaction (PCR) is a technique to amplify specific stretches of DNA and consists of several cycles of three steps: template denaturation, primer annealing and primer extension (Bredbacka, 2001). PCR is a powerful molecular technique for quickly amplifying a particular DNA fragment to a stage that can be further analysed by other methods (Saiki et al., 1985). The success of PCR in achieving this objective has enabled it to become one of the most important methods.
in genetic testing having numerous applications in basic research and medicine. A variety of modified techniques have been developed for different purposes, including forensic analysis, prenatal diagnosis (PND) and PGD of single gene disorders. More sophisticated and modern techniques have been devised to better suit the needs of its applications. The introduction of single cell PCR enabling PGD on human embryos allowed many single gene disorders to be diagnosed using DNA from single cells (Handyside et al., 1989; Sermon et al., 2002). Since then great emphasis has been given to overcome significant challenges by optimising reaction conditions developing a rapid but robust diagnostic assay effective at the level of the single cell. The most significant problems that are taken into consideration in the most updated PCR protocols include contamination, amplification failure (AF) and allele dropout (ADO) (Wells and Sherlock, 1998; Sermon, 2002). These will be reviewed in section 1.5.4. PGD for Single Gene Defects.

1.2.3.2 DOP-PCR.

Whole Genome Amplification (WGA) methods lead to non-specific amplification of the entire genome that have been employed in order to provide sufficient DNA quantities from a single cell so as to perform many independent PCR amplifications (Zhang et al., 1992; Wells et al., 1998). With Primer Extension Preamplification (PEP) at least 70%, and 90% of the genome is amplified more than 30 times according to Zhang et al., 1992, and Wells et al., 1998 respectively. Another efficient and reliable WGA method is degenerate oligonucleotide primed PCR (DOP-PCR) (Telenius et al., 1992). DOP-PCR amplifies a similar proportion of the genome to PEP, but to a much more significant level. DOP-PCR followed by comparative genomic hybridization (CGH) analysis of tumour DNA has been described by several investigators (Speicher et al., 1993; James and Varley, 1996; Kuukasjarvi et al., 1997; Wells et al., 1999). Applying DOP-PCR to single fibroblasts, buccal cells, amniocytes and human blastomeres, Wells et al. (1998) showed that it could provide DNA sufficient for performing 100 subsequent PCR amplifications as well as CGH analysis (Wells et al., 1998). Particularly, Wells and colleagues (1998) successfully determined the copy number of every chromosome and conducted numerous molecular tests on the same cell using a combination of DOP-PCR-CGH and quantitative Fluorescent PCR (QF-PCR).
A significant drawback of DOP-PCR and of other WGA techniques is that amplification of repetitive DNA sequences, such as short tandem repeats is error-prone when performed on WGA products (Wells et al., 1998). In some studies over 50% of fragments amplified are found to differ from their expected size, presumably due to slippage of the DNA chain during product generation (Wells and Sherlock, 1998). The low annealing temperatures that characterize all WGA protocols may underlie this problem (Wells et al., 1998). Despite hopes that WGA strategies might reduce the incidence of allele drop out, Wells and Sherlock (1998) found ADO rates after PEP and DOP-PCR to be comparable to those obtained by direct amplification of single cell loci.

1.2.3.3 Fluorescent-PCR (F-PCR).

The traditional methods for visualizing PCR products following electrophoresis include ethidium bromide or silver staining. Both techniques need nested PCR to ensure sufficient amplified fragments for visualization. In addition, the use of radioactively labeled primers can be employed for visualization and is a more sensitive, but time consuming technique than the first two. The advent of fluorescent PCR technology (Hattori et al., 1992) has enabled more far reaching diagnostic applications to be considered. Fluorescent-PCR (F-PCR) is a modification of PCR technology using fluorescent primers and an automated DNA sequencer and has improved both PCR accuracy and sensitivity (Ziegle et al., 1992). The application of oligonucleotide primers attached to fluorescent molecules gives rise to amplified products labeled with fluorescent dye. When these F-PCR products migrate under electrophoresis to the position where the laser bisects the gel, the fluorescent molecules are activated by the laser and give a signal of a specific wavelength, subsequently detected by a CCD detector and analyzed by computer software. The size analysis is as precise as a single base pair difference. It is of major importance that fluorescent PCR is compatible with heteroduplex analysis (Rommens et al., 1990), SSCP (Ellison et al., 1993), chemical mismatch cleavage (CMC) (Rowley et al., 1995), as well as ARMS (Sherlock et al., 1998).

Sensitivity and speed are both strong points of the technique reducing the need for nested PCR and increasing the speed of diagnosis (Wells and Sherlock 1998). One of the main advantages of F-PCR is that the allele drop out (ADO) rate is significantly lower than in conventional PCR since, due to its high sensitivity it may still
demonstrate the presence of both alleles with one greatly over-amplified in comparison to the other (Findlay et al., 1995a; Sherlock et al., 1998; Sermon et al., 1998a). F-PCR has successfully been employed for PGD of single gene defects such as cystic fibrosis and myotonic dystrophy (Sermon et al., 1998; Piyamongkol et al., 2001).

Several investigators have documented the diagnostic value of using short tandem repeat markers (STR) and quantitative fluorescent PCR (QF-PCR) assays for the rapid prenatal detection of chromosome aneuploidies (Mansfield, 1993; Pertl et al., 1994, 1996, 1997; Adinolfi et al., 1997). When fluorescent dyes are incorporated in the products of PCR amplification, the STR markers can be rapidly analyzed and quantified using an appropriate DNA sequencer (Adinolfi et al., 1997). This technique allows the detection of the major chromosomal abnormalities within 24 h after amniocentesis, CVS or fetal blood sampling (FBS) (Pertl et al., 1999). However, despite the successful diagnosis of aneuploidies from prenatal samples (Pertl et al., 1994, 1996, 1997; Toth et al., 1998; Verma et al., 1998) and small clumps of cells (Tutschek et al., 1995; Sherlock et al., 1997) application of this procedure to a single cell has been shown to be unreliable in approximately 25% of cases due to the phenomenon of preferential amplification (Sherlock et al., 1998).

1.2.4 CGH

CGH is a FISH related technique, which in a single hybridisation allows the copy number of all 23 pairs of chromosomes to be assessed, providing a copy number karyotype based on cells from any give DNA sample. As a result quantitative fluorescent analysis using a digital imaging system is able to detect duplications or deletions in the sample DNA mapped to a target metaphase; ploidy abnormalities and balanced rearrangements however remain undetected. CGH was developed in 1992 (Kallionemi et al., 1992) to detect sequence copy number changes in solid tumours. Further progress of CGH provided molecular cytogenetics with a versatile technique that answers many of the deficiencies of FISH and conventional cytogenetic analysis, allowing the entire genome to be scanned in a single step (Wells and Levy, 2003).

1.2.4.1 Principles and Applications of CGH.

CGH originally developed for cytogenetic analysis of solid tumours involves the use of equal quantities of sample genomic DNA and control DNA which are
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differentially-labelled and hybridised to a normal metaphase spread (Kallioniemi et al., 1992). DNA from the sample to be tested is labelled with a green fluorescent tag while DNA from a sample previously determined to have a normal karyotype is labelled with a red fluorescent tag. The two DNAs are then simultaneously hybridised to normal metaphase chromosomes on a microscope slide. If there is no chromosomal imbalance in the sample (i.e. the green test DNA has the same karyotype as the red control DNA) then DNA fragments of both colours compete equally for hybridisation sites along the chromosomes. Equal hybridisation of red and green DNA produces a yellow colour. However, if the test sample contains an excess of chromosomal material, trisomy 21 for example, then green DNA fragments for this chromosome will be more abundant than their red equivalents and will out compete the red DNA fragments for hybridisation sites. This effect is only seen on the over-represented chromosome and results in a greenish coloration. Conversely if the test sample has a deficiency of chromosomal material, such as a monosomy, then a predominantly red coloration is seen on the chromosome in question. Generally, excess chromosomal DNA is detected as an intensification of the colour of test DNA, whereas deletion appears as an intensification of the colour of the normal DNA (Handyside and Delhanty, 1997). The principles of CGH are depicted in Fig. 1.3.
Fig. 1.4 CGH analysis and interpretation of normal male 46,XY DNA.

Fig. 1.4: (a): A CGH experiment in which DNA from a normal male (test DNA labelled with green fluorescent dye) and DNA from another normal male (reference DNA labelled with red fluorescent dye), have been hybridised to a normal set of male chromosomes. (b): Interpretation of 10 metaphases from the same experiment captured and karyotyped. (c) and (d): Karyotype analysis of chromosomes of a single captured metaphase in (c): black and white showing a banding pattern similar to giemsa staining used to karyotype the chromosomes (d) showing the chromosomes following hybridisation.
Fig. 1.3 Principles of Comparative Genomic Hybridisation.

Fig. 1.3: Comparative genomic hybridisation. Sample DNA (labelled green) and 46,XY reference DNA (labelled red) are simultaneously hybridised to normal chromosome spreads. The ratio of red:green fluorescence along the axis of each chromosome is calculated by computer. An excess of red fluorescence indicates a deficiency in the sample for the chromosome under analysis, while an excess of green reveals regions of the genome that are over-represented.

Fig. 1.4 shows a captured image and subsequent analysis and interpretation of a control CGH experiment, where two DNAs both of normal karyotype are hybridised to normal metaphase chromosomes. CGH reveals any sequence copy number changes (gains, amplifications, or losses) in a particular specimen and maps these changes on normal chromosomes (Kallionemi et al., 1992; du Manoir et al., 1993). A composite karyotype is produced using a digital analysis system. Equal quantities of any loci (disomy) are assigned a profile ratio of 1.0. Changes in the copy number will deviate from this by a factor of 0.5 X n (where n is the number of copies of any locus). Therefore, a partial chromosome gain (trisomy) will produce a ratio of 1.5 and a loss (monosomy), a ratio of 0.5 (Chang and Mark, 1997).

The standard resolution of this technique is in the range of 10-40Mb but varies according to the regions analysed and degree of amplification (Kallioniemi et al., 1992; 1994), although much higher resolutions of 1-5Mb have been recently reported (Kirchhoff et al., 2001). Since the smallest autosome is in excess of 50Mb, CGH analysis provides a very powerful and sensitive method for detecting duplications and deletions of significant size (Wells and Levy, 2003).

One of the few limitations of CGH is that it only detects relative alterations on chromosome copy number and cannot detect changes that involve the entire set of chromosomes, therefore is unable to detect abnormalities of ploidy (Wells and Delhanty, 2000). Although providing information on imbalances on all chromosomes, unlike conventional karyotyping, CGH fails to provide information on chromosomal architecture.
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The limited resolution of CGH implies that small imbalances such as those resulting from translocations with very distal breakpoints could be missed, as unreliable ratio changes may appear at the telomeric regions (Kallionemi et al., 1994). Moreover, other regions of the karyotype including centromeres and heterochromatic segments also show variation in the profile and these regions are normally excluded from CGH (Kallionemi et al., 1994). Certain chromosomes (1p, 17, 19, 22) are prone to show frequent enhancement of the test signal and are also excluded from the analysis (Moore et al., 1997). The most significant limitation in utilizing CGH in a clinical setting and especially in IVF, relates to the technical complexities of the technique, as the method has been described as time consuming, labour intensive, and requiring expertise with several cytogenetic and molecular genetic techniques (Wells and Levy, 2003). The disadvantages of CGH are thoroughly discussed in section 4.2.

Originally developed as a cancer research tool, the initial application of CGH involved direct analysis of genomic DNA from tumour specimens. Rising above the usual problems encountered with the use of conventional cytogenetics CGH did not require time-consuming and laborious techniques of cell culture, or obtaining poor quality metaphase spreads consisting of short chromosomes of low mitotic index unsuitable for G-banding analysis. Therefore, the major advantage of CGH to highlight chromosomal regions of amplification or deletion is that it can be applied to any sample from which DNA can be extracted in a rapid single step, without reliance on the cytogenetic preparations needed for standard FISH or karyotyping.

The use of CGH for the analysis of tumours revealed a number of new recurring chromosomal gains, amplifications, losses and deletion sites. There have been over 1400 articles published that have employed CGH to delineate cytogenetic changes in cancer specimens (Wells and Levy, 2003). A number of groups have employed CGH to show previously unrecorded areas of presumed tumour suppressor gene deletion and oncogene amplification in cell lines and solid tumours (Kallioniemi et al., 1994; Kokkola et al., 1997; Van Roy et al., 1997). Some of the tumours studied included; Uveal melanomas (Becher et al., 1997), small-cell lung carcinomas (Ried et al., 1994; Levin et al., 1995), gliomas (Schrock et al., 1994), sarcomas (Forus et al., 1995), and breast cancer (Kallionemi et al., 1994). CGH has also shown its prognostic value by detecting chromosomal alterations in neoplasms including node-negative breast cancer (Isola et al., 1995), renal cell carcinomas (Moch et al., 1996), Uveal and
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cutaneous melanomas (Prescher et al., 1996; Wiltshire et al., 1995) and bladder
cancer (Kallionemi, et al., 1995). CGH has also been applied to neoplastic samples
relevant to reproductive medicine, such as: prostate cancer (Joos et al.,
1995;Visakorpi et al., 1995), testicular germ cell tumours (Korn et al., 1996), ovarian
tumours (Kiechle et al., 2001), and endometrial cancer (Suehiro et al., 2000).

Similarly in clinical cytogenetics CGH has been applied to prenatal and
paediatric samples as well as mitotically inactive cells derived from products of
conception (Wells and Levy, 2003) to characterise marker chromosomes, cryptic
deletions and complex rearrangements, and the origin of intrachromosomal
duplications (Bryndorf et al., 1995; Ghaffari et al., 1998; Daniely et al., 1999;
Kirchhoff et al., 2001;Griffin et al., 1998). CGH has also been applied to material
from spontaneous abortions (Daniely et al., 1998), in one study combined with flow
cytometry for ploidy analysis (Lomax et al., 2000). Tabet et al. (2001) used CGH to
analyse trophoblast cells from spontaneous abortions, intrauterine fetal death, and
malformed fetuses, circumventing the need for culture. In a study performed by Fritz
and colleagues (2001), cytogenetic analysis of culture failures by CGH was performed
to re-evaluate chromosome aberration rates in early spontaneous abortions. The
investigations suggested that the contribution of chromosome aberrations to first
trimester pregnancy loss is nearly 70%.

1.2.4.2 Analysis of Single cells Using CGH.

Wells et al., (1999) reported successful application of CGH for diagnosing
chromosome imbalance in single cells. To provide enough DNA for the CGH
procedure a variety of whole genome amplification (WGA) techniques were
employed and subsequently assessed for their respective efficiencies (Wells et al.,
1999). These included; degenerate oligonucleotide primed PCR (DOP-PCR), tagged
PCR (T-PCR), primer extension preamplification (PEP), and Alu-PCR. Most genomic
DNA CGH protocols utilise between 100 ng and 1 μg of test DNA, equivalent to
more than 10,000 cells. However, the success of subjecting a single cell to DOP-PCR,
for both molecular and cytogenetic analysis, provided adequate amounts of DNA for
CGH as well as over 90 separate PCR amplifications (Wells et al., 1999). This was
the first reliable application of CGH in a research context to single cells from
fibroblasts, buccal cells, amniocytes, and blastomeres from human preimplantation
embryos. Single cell CGH has also been successfully employed to assess clonal
evolution of genetic variants in complex populations, by subjecting single micrometastatic cells isolated from bone marrow of cancer patients to CGH (Klein et al., 1999). Since the first report of successful application of single cell CGH, continuous investigations aim to improve the technique’s fidelity. Huang and colleagues (2000) conducted a comparative study aiming to define the optimal protocol for single cell CGH. The study employing differently labelled probes and hybridisation combinations concluded that DOP-PCR-CGH homo-hybridisation (amplified test DNA vs. amplified reference DNA), especially when combined with labelling by nick translation is reliable and reproducible. However, the most important product of the first successful single cell CGH application was the potential use of CGH as a diagnostic tool for detecting chromosomal abnormalities in human preimplantation embryos. DOP-PCR has been successfully employed as a means to amplify single human blastomere DNA allowing comparative genomic hybridisation to be undertaken by many groups worldwide (Wells and Delhanty, 2000; Voullaire et al., 2000; Malmgren et al., 2002).

1.3 Chromosomal Abnormalities

It was not until 1956 that the human chromosome number was identified by Tjio and Levan, and three years later Lejeune et al., (1959) connected genetic disease with deviations from the right number of chromosomes. Chromosomal abnormalities comprise cytogenetically detectable alterations in the normal karyotype, and may be either structural in nature involving physical rearrangements of chromosomes, or numerical involving the loss or gain of individual chromosomes (aneuploidy) or whole chromosome sets (polyploidy). In cases where more than one genetically distinct cell line is present, there may be either a common or separate zygotic origin (mosaicism and chimaerism respectively). Gardner and Sutherland (1996) reported a frequency of 8.3% of chromosomal abnormalities in newborns being responsible for an estimated 25% of mental deficiencies and 10% of congenital malformations (Baird et al., 1988; Hook, 1992). Approximately 60% of spontaneous abortions in the first trimester and 5% of stillbirths are caused by chromosomal abnormalities (Hassold et al., 1986; Eiben et al., 1990). In the world of Assisted Reproduction, following karyotyping 11-40% of embryos present with chromosomal abnormalities (Angell et al., 1986; Plachot et al., 1987; Papadopoulos et al., 1989; Zeneses et al., 1992a; Jamieson et al., 1994; Clouston et al., 1997), with much higher rates of up to 80%
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reported for abnormally fertilised or morphologically poor embryos (Wimmers and Van der Merwe, 1988; Bongso et al., 1991; Pellestor et al., 1994). These percentages indicate that pregnancy loss is frequently a result of natural selection against chromosomal aberrations.

1.3.1 Structural Chromosomal Abnormalities.

Structurally abnormal chromosomes are formed from the rearrangement, deletion or duplication of chromosomal segments leaving the karyotype either genetically balanced or unbalanced. Structural abnormalities include translocations (reciprocal or Robertsonian), deletions, inversions, insertions, ring chromosomes and isochromosomes. Non-homologous fusions together account for the rest. Fig. 1.5 shows eight types of balanced rearrangements.
Fig. 1.5: Structural Chromosomal Rearrangements.

a Reciprocal Translocation

(i) A derA B
(ii) A derB B

b Robertsonian Translocation

(i) A derA:B B

(c) Chromosomal Insertions

(i) A insA

(d) Chromosomal Inversions

(i) A invA

(e) Ring Chromosome

(Fig 1.5: Eight types of balanced rearrangements are illustrated. Chromosomal translocations a Reciprocal, b Robertsonian, c Insertions (i) interchromosomal, (ii) intrachromosomal between-arm and (iii) intrachromosomal within-arm. d Chromosomal inversions (i) pericentric and (ii) paracentric. e Ring chromosome.

A, B - Normal homologue. der, ins, inv, r - Rearranged chromosome. → Breakpoints.

(Therman et al., 1989).
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Structural chromosomal aberrations may be familial or de novo in nature with an estimated mutation rate of $1 \times 10^{-3}$ (Jacobs, 1981) and are seen in 5% of spontaneous abortions (Hassold et al., 1986; Eiben et al., 1990) and between 0.2-0.6% of livebirths, the majority balanced (Hook and Hamerton, 1977; Nielsen and Wohlet, 1991; Jacobs et al., 1992). Balanced forms are not associated with any adverse phenotypic effects unless, extremely rarely, a gene disruption, cryptic deletion or functional imbalance (uniparental disomy, UPD) is indicated (Maraia et al., 1991; Bonthron et al., 1993; James et al., 1994). However, some reports have indicated a link between de novo apparently cytogenetically balanced rearrangements and increased risk of congenital defects (Jacobs et al., 1974; Warburton, 1991). As a result most structural anomalies are only identified if a couple presents with a history of chromosomally abnormal conceptions. These reproductive problems arise due to abnormal meiotic segregation and/or production of recombinant chromosomes at gametogenesis, the degree of which varies according to the chromosomes involved, the size and location of the rearranged segments, whether complete synapsis is achieved, the position of crossovers and the individual (reviewed in Gardner and Sutherland, 1996). Information on meiotic analysis can be obtained via FISH or karyotyping sperm investigations. Such studies proved that in contrast to the origin of numerical abnormalities, chromosomal rearrangements arise more frequently during paternal meiosis (Chandley, 1991). Sperm karyotyping data resulting from heterospecific fertilization studies show that this frequency may be as high as 13%, although the possibility that this may be an artefact of this particular technique has been suggested (Martin et al., 1991; Jacobs, 1992). Balanced karyotypic rearrangements are often linked with impaired spermatogenesis, especially in cases of severe oligozoospermia ($<10 \times 10^6$/ml), with at least 4-7% of ICSI referrals in this category (Retief et al., 1984; Bourrouillou et al., 1985). This may result from disturbances caused by the meiotic pairing configurations adopted by rearranged chromosomes, particularly to the X-Y bivalent (Forejt, 1979; Johannisson et al., 1993). In other cases spermatogenesis proceeds normally and the reasons for this discrepancy are unclear, indeed reports show both fertile and infertile men in the same family with the same translocation (Palmer et al., 1973; Rosenmann et al., 1985).

The faithful repair of DNA damage such as chromosomal double stranded breaks (DSBs) is crucial for genomic integrity. Aberrant repair of these lesions can result in chromosomal rearrangements. Examining the role of DNA damage on the
formation of chromosomal aberrations shows that the presence of two DSBs in a single cell can alter the spectrum of repair products that are recovered (Richardson and Jasin, 2000). The possibility that a chromosomal rearrangement might disturb the meiotic behaviour of chromosomes not involved in the rearrangement and favour non-disjunction is a controversial issue. It has been suggested that any general interchromosomal effect for structural rearrangements in both male and female carriers and any unrelated aneuploidies in their children are likely to be entirely random events (Martin et al., 1991; Schinzel et al., 1992; Estop et al., 2000). However, Pellestor et al., (2001) asserts that in infertile carriers, in contrast to fertile ones the risk for interchromosomal effect appears to be real and should be taken into consideration in the genetic counselling of infertile couples with a male partner carrying a chromosomal rearrangement. In addition, it is generally known that infertile men have a higher incidence of de novo chromosomal abnormalities in sperm than fertile men do. Having postulated that the causes of IVF implantation failure and recurrent pregnancy loss are the same (Stern et al., 1998), balanced parental rearrangements may be implicated in the pathogenesis of IVF implantation failure and genetic evaluation should be considered as part of the investigation of these patients (Stern et al., 1999). The fact that certain couples carrying a chromosomal rearrangement suffer only a slight reduction in fertility and others a detrimental one is a consequence of the multifactorial nature governing the production of viable or non-viable gametes as the production of unbalanced gametes of a particular type is influenced by the size of the imbalance (Cohen et al., 1994).

**Reciprocal translocations (a)** arise when a two-way exchange of material takes place between two non-homologous chromosomes. A break occurs in one arm of each chromosome and the portions of chromosome material distal to the breakpoints switch positions. The portions exchanged are the translocated fragments and the rest of the chromosome including the centromere is the centric fragment. The breakpoints leading to reciprocal translocations are generally observed in the Q-dark regions of chromosomes (Therman et al. 1993). It has been suggested that chromosome 1 is the most susceptible chromosome participating in reciprocal translocations (Tharapel et al. 1989). Breakpoints can occur theoretically anywhere on a chromosome and therefore each translocation event is unique, with the exception of the most encountered reciprocal translocation t(11;22)(q23.3;11.2) (Zackai et al.
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1980; Estop et al. 1999; Van Assche et al. 1999; Armstrong et al. 2000), which has been described in approximately 110 families from different ethnic and racial groups (Iselius et al. 1983). These findings led Therman et al. (1993) to suggest that there could be a homologous region in the 11 and 22 chromosome segments, which caused high rates of pairing and crossing over. On chromosome 11 the translocation occurs within a short palindromic AT-rich region (ATRR). Likewise the breakpoint on chromosome 22 has been localised within an in full first ATRR that is part of another palindrome. Recent studies suggest that it is likely that the palindromic ATRRs produce unstable DNA structures in 22q11 and 11q23 that are responsible for the recurrent t(11;22) translocation (Kurahashi et al., 2000).

About 1 person in 625 of the general population is a reciprocal translocation heterozygote (Van Dyke et al., 1983), but it is markedly higher in selected groups, such as 1% in infertile men (Guichaoua et al., 1990) and up to 3% in men characterised by oligospermia and in couples referred for ICSI as impaired spermatogenesis is also frequently seen in male balanced translocation carriers (Testart et al., 1996). The translocation may have arisen de novo or it could be widespread with many carriers throughout a family.

It is generally known that carriers of balanced reciprocal translocations show an increased risk of having chromosomally abnormal offspring in addition to infertility and spontaneous abortions. A few translocations are associated with a high risk (20%) of producing malformed and mentally retarded offspring. Others are of intermediate level of risk (around 5-10%), but some carriers have a risk of only 1% or even less. In most cases however, there is an increased miscarriage rate.

There are five human acrocentric chromosomes 13, 14, 15, 21, and 22 that are involved in Robertsonian translocations (b). The majority of these occur between chromosomes 13 and 14 (74%) or chromosomes 14 and 21 (8%) whilst the remaining eight other possible non-homologous fusions account for the rest (Therman et al., 1989). Those involving the fusion of the homologue (homologous translocation) are very rare. Non-homologous translocations can be transmitted through many generations, while the homologous ones are always seen as a de novo event. There are three ways for the formation of a Robertsonian translocation: fusion at the centomere (centric fusion), giving a monocentric chromosome, a whole arm reciprocal translocation, with breakage in one short arm and one long arm giving a mononcentric...
chromosome, and finally, union following breakages in both short arms giving a dicentric chromosome (or after the suppression of one centromere, a monocentric.) Most t(13;14) and t(14;21) cases show consistent breakpoints however, highly variable breakpoints have been found in the less common translocations (Page et al., 1996; Page and Shaffer, 1997), possibly reflecting the presence of homologous repetitive sequence on chromosomes 13 and 21 which is inverted on chromosome 14 and may cause preferential pairing (Therman et al., 1989). The homologous Robertsonian translocation is most likely to have formed not during meiosis, but after conception and probably in one of the very first cell divisions.

The importance of balanced reciprocal translocations lies in their behavior during meiosis I, where the gametes involved in the translocation can segregate in manners resulting in gross chromosomal imbalances (Mueller and Young, 2001). During meiotic prophase I balanced translocations may adopt characteristic conformations to facilitate synapsis such as trivalent arrangements for Robertsonian translocations as seen in Fig. 1.6 and pachytene quadrivalents for some reciprocal translocations as seen in Fig. 1.7. The segregation pattern followed depends on the chromosomes involved and the breakpoints. However, there are several segregation patterns according to which the two rearrangements can segregate at anaphase, with eight possibilities for a quadrivalent and four for a trivalent, but only alternate segregation of derivative chromosomes and normal chromosomes to opposite poles will result in the production of normal or balanced gametes. Jalbert and colleagues have suggested a few ground rules according to which the segregation outcomes of an individual translocation carrier can be predicted, based on the lengths of the centric and the translocated segments (Jalbert et al., 1980;1988). Fig.1.8 summarises the meiotic behaviour of reciprocal translocations. The most common mode of segregation reported in human embryos (Iwarsson et al. 2000; Simopoulou et al., 2003) has been found to be the alternate segregation mode. This finding is in line with studies of meiotic segregation in spermatozoa on male translocations carriers (Estop et al. 1995; Honda et al. 1999), while the adjacent-2 segregation pattern is rarely found (Estop et al. 1995; Gardner and Sutherland, 1996; Iwarsson et al. 2000).
Fig. 1.6 Robertsonian translocations-Meiotic pairing and segregation patterns.

**Gametes of alternate segregation**

**Gametes of adjacent segregation with disomy and nullisomy.**

**Gametes of adjacent segregation with disomy and nullisomy.**

**3:0 Double aneuploidy**
Fig. 1.7 The pachytene quadrivalent at meiosis I of a balanced reciprocal translocation.

The figure illustrates how a balanced reciprocal translocation involving two chromosomes leads to the formation of a quadrivalent pachytene in meiosis I. The quadrivalent is formed to preserve homologous pairing (reproduced from Mueller and Young, 2001).

Fig. 1.8 Segregation patterns of chromosomes involved in a balanced reciprocal translocation during meiosis I.

The figure depicts the alternate (2:2), adjacent-1 (2:2), adjacent-2 (2:2), and 3:1 segregation patterns that can be generated by the segregation of chromosomes involved in a reciprocal translocation, during meiosis I. The dashed lines indicate the different axes of segregation (reproduced from Braude et al., 2002).

In adjacent-1 non-homologous centromeres segregate together. In adjacent-2 homologous centromeres segregate together. Both adjacent-1 and adjacent-2 lead to a combination of partial monosomy and trisomy in the zygote.

3:1 segregation can lead to tertiary trisomy (with 2 normal chromosomes and one derivative chromosome in the gamete), and tertiary monosomy (with one derivative chromosome in the gamete). 3:1 can also lead to interchange trisomy (with 2 derivative chromosomes and one normal chromosome in the gamete), and interchange monosomy (with one normal chromosome in the gamete).

In 4:0 also known as double aneuploidy both normal and both derivative chromosomes segregate together in the gamete leading to disomy and nullisomy for both chromosomes involved in the translocation.
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**Ring chromosomes** (e) are very uncommon. The classic mode of formation is breakage in both arms of a chromosome, with fusion of the points of the fracture and loss of the distal fragments. Therefore a partial monosomy for the distal short arm and the distal long arm results. Although cases of completely normal individuals with autosomal rings have been described (McGinniss *et al*., 1992; Wintle *et al*., 1995; Paoloni-Giacobino *et al*., 1998), most are associated with severe phenotypic abnormalities with only X chromosome rings showing viability. The stability of the rings varies from chromosome to chromosome and 99% of rings arise sporadically (Kosztolanyi *et al*., 1991). Their inability to undergo complete mitotic division explains their presence in only a proportion of cells resulting in mosaicism for monosomic cell lines. In the fraction of cases where reproduction is an option, meiotic and mitotic disturbances presumably lead to high levels of gametic chromosomal imbalance and impaired fertility, although reports of stable familial rings show that this is not inevitable (Kosztolanyi *et al*., 1991; McGinniss *et al*., 1992). Extra marker chromosomes including ring chromosomes of unidentifiable origin occur with a frequency of approximately 1 in 2500 in human population (Warburton, 1991). It is assumed that phenotypes of patients with marker chromosomes are mainly due to the chromosomal origin and euchromatic content of the marker and its tissue specific distribution (Gardner and Sutherland 1996). The risk of non-satellited marker chromosomes prenatally is 14.7%, 70% of which occur as non-mosaic (Warburton, 1991). Most patients with additional ring chromosomes are mosaic either because of post zygotic ring formation or instability of the ring during cell division (Chen *et al*., 1995).

**Insertions** (c) or insertional translocations (IT) involve three breaks, the first two release an interstitial segment, which is then inserted into the gap created by the third break. If a single chromosome is involved, this can be described as a shift. In the interchromosomal insertions a segment of one chromosome is inserted interstitially into another chromosome. The insertion could be direct when the inserted segment is inserted with the same orientation with respect to the centromere (Schninzel, 1984), or it could be inverted when the orientation towards the centromere is reversed (Hamden *et al*., 1985). Kajii *et al.* (1987) described the difficulties of determining whether the insertion is direct or indirect. Of these the interchromosomal type predominates with very few intrachromosomal cases reported although this may be an ascertainment bias with some within-arm insertions confused with inversions (Madan and Menko, 1992; Van Hemel and Eussen, 2000).

Interchromosomal insertions are rare chromosomal rearrangements with an incidence of 1:80000, of which nearly 80% are referred because of congenital abnormalities and mental retardation Van Hemel and Eussen, 2000). In the study performed by Van Hemel and Eussen, (2000) a maternal origin was seen in 59.5%, a paternal origin in 26.6% and 13.9% were de novo. During meiosis for the interchromosomal insertion the insertional segment could be disregarded.
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according to its size and the homologues would synapse with segments matching for
as much of their length as they are able (Goldman et al., 1992). However, the
insertional segment could be thrown into a loop to accommodate this and homologues
would pair along their full lengths, which would bring some non-matching segments
alongside each other (heterosynapsis) (Gardner and Sutherland, 1996). The possible
gametes give rise to four possible segregant types normal, balanced, duplication and
deletion of the inserted fragment, which would lead to pure partial trisomy, and
monosomy. The viability of the conceptuses depends on the degree of the aneuploid
states. The risk of an abnormal offspring in the case of an interchromosomal insertion
is in the range of 10-50% (Gardner and Sutherland, 1996)

In the case of intrachromosomal insertion either within or between arm,
crossing over can occur in one or the other insertion loop following complete
synapsis. According to whether it is direct or inverted or where the crossing-over
takes place recombinant chromosomes are formed giving rise to certain imbalances
involving duplications and deletions of the inserted fragment, dicentric or acentric
chromosomes, as illustrated in Fig. 3.6 The risk of having a child with an unbalanced
karyotype when carrying an intrachromosomal insertion has been estimated to be 15%
by Madan and Menko (1992). However, the review by Gardner and Sutherland (1996)
presumes a range from near 50% to zero in the individual case with a higher risk if
one of the segments is small and the other one long.

Insertions are amongst rearrangements implying the highest reproductive risk.
This risk is greater for the small segment insertion than the large segment insertion,
however, in the case of the meiotic recombination product carrying a duplication there
appears to be no clear relationship between the phenotype and the length of the
duplicated segment (Wilson et al., 1985). The risk of a chromosomally liveborn
offspring will depend on the probability of crossing over and the specific chromosome
segments involved (Farrell et al., 1992). The recombinant chromosome can have a
duplication or a deletion of different segments depending on whether there is meiotic
crossing over in the inserted or the interstitial non-inserted segment. Several of the
insertions have been difficult to interpret and some could be mistaken for paracentric
inversions. Caution is therefore indicated in interpreting parental karyotypes of a child
with a deletion or duplication particularly if it is interstitial (Madan and Menko,
Inversions (d) involve a two-break rearrangement in a chromosome and the segment formed by the breaks is reversed. If the inverted segment includes the centromere then the inversion is pericentric, if however, the centromere is not involved the rearrangement is known as paracentric. Excluding the common inversions of heterochromatin 1qh, 9qh, and 16qh generally considered normal variants, inversions occur in about 1 in 2-5000 births (Gardner and Sutherland, 1996).

In the case of pericentric inversions, during synapsis at meiosis in order for the homologous chromosomes to be paired, classically there is a loop formed, but with very large or small inversions other configurations are possible. Following unequal numbers of crossovers within the inverted segment there is formation of two unbalanced recombinant chromosomes one with duplication of the distal non-inverted fragment and deletion of the other end of the chromosome and the other having the opposite arrangement. The chromosome imbalance is directly proportional to the size of the duplications and deletions respectively. Therefore, the smaller the inverted fragment the greater the chromosomal imbalance and the likelihood of miscarriage of the conception. Conversely, with a large inverted segment, the unbalanced regions will be small and offspring carrying the recombinant chromosomes may be viable (Daniel, 1981). It has been reported that pericentric inversion of chromosome 2 causes repeated abortions, congenital abnormalities, and mental retardation (Fryns et al., 1987). Carriers of inversions involving chromosomes 4 and 5 are also at risk of having unbalanced offspring with Wolf-Hirschhorn and Cri-Du Chat microdeletion syndromes (Beemer et al., 1984; Hirch and Baldinger, 1993; Villa et al., 1995; Ogle et al., 1996) It has been estimated that a carrier of an inversion has a risk of 5-10% to have a child with a viable imbalance if that inversion has already resulted in the birth of an abnormal child (Gardner and Sutherland, 1996).

In the case of paracentric inversions the crossing-over during synapsis at meiosis will lead to the formation of recombinant chromosomes that are either acentric, and incompatible with survival or dicentric and unstable during cell division and therefore are not compatible with life either (Worsham, 1989). Therefore, the risk of the birth of an abnormal child resulting from a carrier parent of a balanced paracentric inversion is almost non-existent. An estimated 4% of the offspring from paracentric inversion carriers are unbalanced possibly as a result of U-loop formation; this incidence could be higher in the early stages of development (Pettenati et al., 1995).
1.3.1.1 Wolf-Hirschhorn Syndrome

Chromosomal syndromes can be classified as follows: duplication syndromes with an additional segment of chromosome material; deletion syndromes, in which a segment is lacking; and breakpoint disruption syndromes, in which only one or a few genes may be mutated. There are more recorded deletion than duplication syndromes and with the development of finer cytogenetic methods, smaller deletions ("microdeletions") associated with milder phenotypes are being discovered (Singh et al., 2002). Wolf-Hirschhorn syndrome (WHS) is a multiple malformation syndrome, characterized by mental and developmental defects caused by partial deletion of the short arm of human autosome 4p16.3 (Hirschhorn et al., 1965; Wolf et al., 1965). The milder Pitt-Rogers-Danks syndrome (PRDS) (Pitt et al., 1984), was recently shown to be caused by deletions within the same 4p16.3 region (Wright et al., 1998). Thus PRDS is a mild form of WHS (Battaglia and Carey, 1998). The molecular analysis of patients with WHS and patients with PRDS employing a series of cosmids across a 4.5.Mb region of 4p16.3 lead to the conclusion that the two conditions arise as a result of the deletion of similar genetic segments and suggested that the differences in respect to their clinical image are due to allelic variation in the remaining homologue (Wright et al., 1998).

Genotype

The WHS is a contiguous gene syndrome involving more than one gene. The critical region is the region of overlap of deletions producing typical WHS and is 165 Kb long and very gene dense. The WHSCR is defined by the loci D4S166 proximally and D4S3327 distally (Wright et al., 1997). It was inferred that a few genes, or just one residing in this region are responsible for the WHS phenotype, possibly by acting as transcriptional regulators of other genes. Three candidate genes have been independently reported. The results of sequencing of the WHSCR revealed the existence of 9 transcripts for one of which homology has been found with a developmental gene in Drosophilla and the same is considered to be the most strong candidate for causing the WHS phenotype, at least in hemizygous form (Stec et al., 1999). This gene (WHSC1) for two-thirds of its length overlaps with the distal half of the WHSCR (Stec et al., 1998). The critical zone for development of the WHS was described by Wright et al. (1997) to be located distal to the FGFR3 gene as well as the Huntington's disease linked G8 (D4S10) marker. Further work by Wright and colleagues (1999) resulted in defining the second candidate gene (WHSC2) falling
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entirely within the WHSCR. The third candidate gene for WHS, LETM1 (leucine zipper/EF-hand-containing trans-membrane) is likely to be pathologically involved in seizures (endele et al., 1999). It lies distal to the WHSCR and is an excellent candidate gene for neuromuscular impairment in WHS. Recent studies identified certain chromosomal regions-including the WHSCR- where aberrations had an evident association with seizures, possible due to disruptions of epileptic genes (Singh et al., 2002). The WHS critical region (WHSCR) of approximately 165kb has been defined on the basis of two atypical interstitial deletions (Wright et al., 1997; Fang et al., 1997). As most WHS associated deletions, either terminal or interstitial are much larger than the WHSCR, usually including all the candidate genes, in a very recent study Zollino and colleagues (2003), proposed a new critical region for WHS, referred to as WHSCR-2. It falls within a 300-600-kb interval in 4p16.3, between the loci D4S3327 and D4S98-D4S168.

**Phenotype**

WHS patients exhibit a constellation of symptoms including severe growth deficiency, and severe to profound mental retardation with onset of convulsions by the second year of life. Most cases die in early childhood, with survival to adulthood being exceptional (Opitz et al., 1995; Smith et al., 1995). The classical craniofacial anomalies are microcephaly, sacral dimples and characteristic facial features including prominent glabella, hypertelorism, (“The Greek Helmet” appearance), micrognathia, highly arched eyebrows, down-turned “carp” mouth and simple lobeless ears Tachdjian et al., 1992). Other anomalies reported may include a variety of midline closure defects (cleft lip or palate, hypospadias, cryptorchidism), flexion/contracture deformities of hands and feet, skeletal defects (scoliosis, kyphosis), heart defects, haemangiomas, hypoplastic nipples and eye defects, (cataracts, iris defects, microphthalmia, stabismus) (Lurie et al., 1980, Smith et al., 1988, Wilson et al., 1981).

Controversy exists about the correlation between size of deletion and clinical severity of the phenotype. Based on cytogenetically visible deletions Wilson et al. (1981) found no phenotype-genotype correlation. Based on molecularly defined deletion size, Estabrooks et al. (1995) provided a detailed preliminary phenotypic map of HAS 4p16. However, discrepancies between this map and recently described patients were seen with respect to severity of mental retardation (Wieczorek et al., 2000; Zollino et al., 2000), hypospadias (Wright et al., 1997), coloboma (Wieczorek et al., 2000),
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cleft/lip palate and hearing loss (Wieczorek et al., 2000; Zollino et al., 2000). These discrepancies maybe due to the non-specificity of findings, common to many different forms of segmental autosomal aneuploidies, as emphasized by Lurie and Opitz (1995). As additional malformations associated with the WHS are constantly reported, with the clinical manifestations depending on the extent of the deletion, it is extremely difficult to establish a genotype-phenotype correlation. However, minimal diagnostic criteria should be standardized. The signs defining the basic WHS phenotype in patients carrying a large or a crypto-deletion involve; congenital hypotonia, mental retardation, growth delay and seizures (Zollino et al., 2003). On the basis of genotype correlation analysis, dividing the WHS phenotype into two distinct clinical entities a “classical” and a “mild” form is recommended for the purpose of proper genetic counseling (Zollino et al., 2003).

Causes

The diverse mechanisms that are responsible for WHS include; terminal 4p deletions, familial translocations, and de novo complex rearrangements. The frequency of translocations in WHS as a result of parental chromosomal translocations is estimated to be 5-13%, whereas the rate of sporadic translocations was suggested to be 1.6% (Lurie et al., 1980). Wieczorek et al. (2000), showed that de novo translocations are a common mechanism causing WHS, and suggested that apart from FISH, and high resolution banding, molecular probes on apparently normal chromosomes should be employed as means to achieve further delineation of this complex syndrome. In seven cases of WHS, Quarrell et al. (1991) found that there was de novo deletion or rearrangement of 4p. In each case the abnormality had arisen on the paternal chromosome. A paternal age effect however was not observed. Anvert et al. (1991) studied two patients and based on his results suggested that the WHSCR was located within 4p16.3. The origin of the deletion was maternal in one case and paternal in the other. Altherr et al. (1991) reported a molecular deletion in 4p due to a subtle inherited translocation involving chromosomes 4 and 19, giving rise to a Wolf-Hirschhorn phenotype. Another case involving a 2 year-old girl was presented with developmental delay and other phenotypic features suggesting the WHS phenotype. A high-resolution chromosome analysis was performed for both the parents and the child indicating that everything was normal. However, molecular analysis revealed that the child had failed to inherit a maternal allele at 4p16. In the next pregnancy of the couple it was proved with
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prenatal diagnosis that the fetus lacked the same maternal allele again and further cytogenetic analysis of the mother using FISH showed that the mother was carrying a submicroscopic translocation involving chromosomes 4 and 10 (Goodship et al., 1992). Estabrook et al. (1992) reported two families with a satellited chromosome 4 short arm. Satellites and stalks normally occur on the short arms of acrocentric chromosomes, presumably resulting through translocation from an acrocentric chromosome. This was the first report of involvement of a satellited 4p. Following FISH and Southern Blotting analysis it was concluded that a region mapping approximately 150 kb from 4pter was deleted. The patient carrying the deletion had a normal phenotype and did not exhibit any WHS characteristics. It was later presumed by Estabrooks and colleagues (1992), that the origin of the rearrangement was explained based on the homology noted between subterminal repeat sequences on 4p and sequences on the acrocentric short arms.

A study aiming to reveal more on the WHS with respect to its natural history was carried out by Battaglia et al. (1999). Fifteen patients were evaluated and four of the cases were followed up spanning 16 years. The methods of detection of the syndrome varied. Cytogenetic techniques were employed for 13 cases while FISH was used for the remaining two. It is known that the WHS patients have similar phenotypic features but a lot of differences too and it is therefore important to study these patterns looking for any associations. A slow but constant progress in development was observed in all cases during the follow-up period. Therefore, the combined cases of the three centers represent considerable experience, providing new information on several aspects of this important deletion syndrome. More combinatorial studies such as the one described should be performed to accomplish better evaluation of the clinical spectrum of WHS.

1.3.2 Numerical Chromosomal Abnormalities.

Aneuploidy is the most common chromosome abnormality, arising as a consequence of fertilisation with an aneuploid gamete involving a gain or loss of a chromosome. 30% of pregnancies result in fetal wastage due to aneuploidy (Wilcox et al., 1988), 0.3% of liveborns are aneuploid, while 4% of stillbirths are reported to be aneuploid (Hassold et al., 1996). The incidence of aneuploidy in humans is an order of magnitude greater than for other studied mammals and most commonly leads to a
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A high rate of mental retardation and pregnancy wastage between 6 and 20 weeks of gestation (Hassold et al., 1980; Bond and Chandley, 1983; Mahmood et al. 2000).

Aneuploidies reported in live borns are autosomal trisomies or sex chromosome aneuploidies. Complete monosomies are incompatible with life except monosomy X. Trisomy is the most common form of aneuploidy, since 1:2000 spontaneous abortions have been reported to be trisomic for any chromosome apart from 1 (Hassold et al., 1996). Trisomies 13, 18, and 21 along with the sex chromosome trisomies are the only ones represented in the live-born population (Hassold and Jacobs, 1984). The incidence of trisomy 21, is approximately 1:800 livebirths and increases markedly with maternal age. The incidence at conception is far higher but only 24% survive to term (Hassold et al., 1996). Trisomy 21, is the autosomal trisomy most compatible with life. Edwards and Patau syndromes (trisomies 18 and 13), are invariably lethal in the perinatal period (Hook, 1982; Goldstein and Nielsen, 1988). Edwards syndrome has an incidence of 1:10,000 livebirths and increases with maternal age. Again the incidence at conception is higher but 95% are spontaneously aborted and only 5% survive to term (Hassold et al., 1996). There are more female cases of Edwards syndrome than male, presumably due to selection against male trisomic conceptions (Griffin et al., 1996; Huether et al., 1996). Patau syndrome has an incident of 1: 20,000 livebirths again increasing with maternal age (Hassold et al., 1996). Sex chromosome trisomies (47XXX, 47XXY [Klinefelter's], 47XYY), having an incidence of 1:1100 (Thompson et al., 1986), are largely compatible with life presumably buffered to a large extent by X inactivation and the heterochromatic content of Yq. On the other hand, the only monosomy reported to show compatibility with life is monosomy XO [Turner's syndrome], with an incidence of 1:5,000-10,000 female births. This represents only a fraction of all conceptions with this condition, as 98-99% are spontaneously aborted and only 0.3% are born alive (Hassold et al., 1996). No record of autosomal monosomies is available from spontaneous abortions, indicating disruption of pregnancy at even earlier stages prior to recognizable pregnancy loss (Hassold and Jacobs 1984). The incidence of aneuploidy at conception has not been established. However, it has been estimated as 5%, considering the high percentage of pregnancies that are being lost (Hassold et al., 1996).

There are essentially three developmental stages when chromosomal defects, and especially aneuploidy, can arise: gametogenesis, fertilisation and embryogenesis.
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(Delhanty et al. 1995). Errors in gametogenesis are usually considered to be meiotic in origin, whereas fertilisation errors are mainly caused by dispermy, which accounts for two thirds of cases of triploidy, occurring in 1% of conceptions in vivo (Jacobs et al. 1978). Most cases (ranging from 60-100%) of trisomies 13, 16, 21 and X, originate from maternal MI errors whilst the majority (60%) of trisomy 18 cases originate from maternal MII errors (Hassold et al., 1987; 1995; Antonarakis et al., 1992; Bugge et al., 1998; Thomas et al., 2001). A paternal origin is only indicated in a few percent of these trisomies but is responsible for about half of XXY cases (Jacobs et al., 1988) as well as the majority of 45,X cases where over 80% show a missing paternal X (Hassold et al., 1988). Mitotic errors in embryogenesis are implicated in 5-20% of cases, but this may be underestimated as errors in pre-mitotic divisions during gametogenesis may also appear as being of meiotic origin (Antonarakis et al., 1993).

The primary mechanisms, as well as the environmental (physico-chemical) causes resulting in human aneuploidy are not fully understood. However, the primary cause of aneuploidy is non-disjunction: abnormal segregation involving either an entire chromosome or a chromatid at meiosis I or II (Fig. 1.9) or in mitosis (premeiotic or postzygotic) (Griffin, 1996). There is a long list of factors known to affect the process of non-disjunction such as: parental age, recombination of chromosomes, chromosome mover components, differential chromosome susceptibility and certain chemicals.

During meiosis I it is vital that the bivalents held together at chiasmata remain associated until all are correctly aligned on the metaphase plate and anaphase can begin. This explains why a large number of trisomies are associated with meiotic nondisjunction of achiasmate or low exchange chromosomes, (Warren et al., 1987; Sherman et al., 1991; 1994; Hassold et al., 1995). It may also explain the preferential involvement of smaller autosomes, since larger chromosomes have more chiasmata (Cupisti et al., 2003). Non-disjunction of bivalents leading to loss or gain of dyads during meiosis I results in a disomic and a nullisomic gamete (Nakaoka et al. 1998). Premature division of the chromosome centromere before anaphase I leads to loss or gain of monads (chromatids) (Angell et al. 1994).
Fig. 1.9 The consequences of meiotic non-disjunction.

Fig. 1.9a: Non-disjunction during meiosis I.

Fig. 1.9b: Non-disjunction during meiosis II.
In addition, studies in oocytes have revealed that during anaphase I univalents can migrate either as a whole chromosome towards the meiotic spindle, or divide prematurely and move as separate chromatids, with subsequent random segregation (Angell et al. 1994). Both types of nondisjunction have been confirmed in subsequent studies (Cozzi et al. 1999).

Lamb et al., (1996) proposes from this a two hit system to explain the predominance of maternal MI errors in human trisomy. The first event establishes a susceptible pairing configuration in fetal meiosis whilst the second event is an age-related impairment of the meiotic process, such as defective spindle apparatus (Battaglia et al., 1996), which gives an increased risk of nondisjunction. This general interpretation is broad enough to encompass other factors that may contribute to spindle disturbances linked to aneuploidy such as hormonal imbalance and reduced intrafollicular vascularity (Gaulden, 1992; Van Blerkom, 1998). Variation in alphoid DNA size has an important role in trisomy formation, with the risk of non-disjunction being related to how small the alphoid array is (Maratou et al., 2000). Finally, translocations with an emphasis on Robertsonians, accounting for 5% of trisomy 21, are another cause of aneuploidy.

Polyploidy (multiple copies of all chromosomes) is the second most common group of chromosomal abnormalities resulting in a spontaneous abortion in the first or second trimester as progression to term is rare (Book and Santesson, 1960; Cassidy et al., 1977). Errors in cell division can result in polyploidy. However, it is mainly due to polyspermic fertilisation (Hassold et al., 1980; Angell et al., 19986, Zaragoza et al. 2000), with dispermy being the most common cause of triploidy. Complete non-disjunction at MI or MII can also lead to triploidy. In digynic cases this meiotic failure can take the form of non-extrusion of a polar body nucleus which then becomes incorporated into the embryo (Penrose and Delhanty, 1961; Zaragoza et al., 2000). Following DNA replication any meiotic or post-zygotic mitotic failure in cytokinesis can cause tetraploidy or higher orders of ploidy.

1.4 Chromosomal Analysis of Embryos and Mosaicism.

Chromosomal analysis of embryos is one of the most important research fields in the area of preimplantation development. Although karyotyping studies on chromosomal abnormalities on human preimplantation embryos had already provided
important results, it was the advent of FISH enabling the examination of every cell within the embryo that revealed the true extent of chromosomal abnormality in human development. The first report to highlight this was in 1993 when dual FISH gave the first indication of chromosome mosaicism in diagnostic cleavage stage embryos (Delhanty et al., 1993). These findings were confirmed by additional studies (Munne et al., 1993b), and further work by Harper and Delhanty (1996) lead to the classification of chromosome patterns in human preimplantation embryos into four groups; uniformly diploid embryos (for the probes examined), uniformly abnormal (where all nuclei present exactly the same anomalies of chromosomal number), mosaic (where two cell lines are present and some of the nuclei show an abnormal chromosomal constitution while the remaining are diploid) and chaotic embryos (characterised by uncontrolled division, where a different chromosomal constitution is seen in all nuclei) (Delhanty et al., 1997b). Chromosomal mosaicism or the phenomenon of the presence of two or more karyotypically different cell lines within a human embryo has been a subject of study since 1990, being reported as the most common finding. In multiple studies much data has been collected at all embryo developmental stages. From the FISH data a higher rate of abnormalities has been observed than previously reported from karyotyping data. However, since mosaic and chaotic embryos are common, if only 1-2 cells are analyzable from an embryo, then karyotyping would underestimate the level of chromosome abnormalities. The recent development and use of complete karyotyping techniques such as CGH offer a more extensive chromosome screen enhancing the benefits of chromosomal analysis on preimplantation embryos (Wilton, 2002).

**Confined placental mosaicism (CPM)** is characterized by a discrepancy between the chromosomal constitution of the embryo/fetus and the placenta (Ford, 1969; Kalousek and Dill, 1983). CPM occurs in approximately 1-2% of pregnancies studied by chorionic villus sampling (CVS) analysis of cytotrophoblast or mesenchymal stroma at 9-12 weeks of gestational age (Ledbetter et al., 1992; Teshima et al., 1992). According to whether the abnormal karyotype was detected in STC (short term culture) villi, LTC (long term culture) villi, or in both, with a normal karyotype in the fetus, CPM is designated as type 1, 2, or 3 respectively (Kalousek and Barret, 1994; Kalousek and Vekemans, 1996; Wolstenholme, 1996). The reason for the mosaicism being sometimes confined to only part of the conceptus is not...
clearly understood, but it might depend upon the timing of the mitotic error. If the error occurs in the cleavage stage embryo prior to the differentiation of the ICM and the TE, then mosaicism maybe distributed into both the placenta and fetal tissues. If the error occurs at a later stage, the abnormal cells may be confined either to the placenta or to the embryos (Crane and Cheung, 1988; Simoni and Sirchia, 1994). Early embryogenesis consists of a complex sequence of events leading to the formation of distinct embryonic and extraembryonic components. Embryonic cell lineages seem to develop last from three to four cells of the ICM at the time of blastocyst formation (Cross et al., 1994). It is this sequential appearance of specific embryonic and extraembryonic cell lineages that is responsible for the patterns of mosaicism that are observed, whether generalized mosaicism affecting the entire conceptus, or confined to either the embryo or the placenta (Ford et al., 1969; Kalousek et al., 1989; James and West 1994).

Pregnancies diagnosed with CPM show an increased incidence of perinatal complications, pregnancy loss, intrauterine fetal growth restriction (IUGR), and premature labour (Kalousek et al., 1991; Johnson et al., 1990; Breed et al., 1991). However, other clinical reports suggest the effect of CPM on fetal uterine development to be non-existent (Schwinger et al., 1989). Henderson and co-workers (1996), produced “placental maps” showing tissue and site-specific mosaicism, and demonstrated the importance of analysis of multiple placental biopsies, as analysis at one site may not be representative of the entire placenta.

Pregnancies with a 100% aneuploid placenta and a normal diploid fetus are likely to represent trisomic zygote rescue, in which one copy of the trisomic chromosome is lost in the embryonic progenitor cells and the majority of the extraembryonic lineages remain trisomic. In some rescued trisomic zygotes, uniparental disomy (UPD) where both homologues of one chromosomal pair originate from one parent may occur (Hall et al., 1990). UPD can be purely heterodisomic, combined hetero/isodisomic or purely isodisomic, depending on the meiotic division in which the non-disjunctional error occurred and the extent of cross-over between the homologues of the chromosome pair involved (Engel, 1980; Engel and DeLozier-Blanchet, 1991). Isodisomy of chromosomes may lead to autosomal recessive disorders (Engel, 1993; Ledbetter and Engel, 1995), while heterodisomy could
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potentially result to autosomal dominant disorders when the parent contributing both chromosomes is affected. In general, UPD causes developmental disturbances when imprinted regions present on some chromosomes are involved (Bennet et al., 1992; Engel, 1993; Ledbetter and Engel, 1995).

It is believed that trisomic zygote rescue resulting in fetal UPD is associated with CPM type 1 and 3 involving non-mosaic trisomies (Ledbetter and Engel, 1995; Wolstenholme, 1996). In a recent study by Wolstenholme and colleagues (2001), it is suggested that formal demonstration of corrected trisomy with associated UPD analysis is actually unlikely to allow accurate differentiation between those pregnancies which will subsequently have adverse outcomes and those which will result in the birth of small, but non-dysmorphic, apparently normal children (Wolstenholme et al., 2001). The exact mechanism of trisomic zygote rescue is not known; anaphase lagging or non-disjunction in an early postzygotic cell division has been proposed (Kalousek et al., 1991). Los and colleagues, (1998), propose an alternative correction mode, chromosome demolition, as a process of deliberate fragmentation and/or removal of one of the set of three chromosomes during metaphase or anaphase resulting in two disomic daughter cells (Los et al., 1998).

1.4.1 Karyotyping of Embryos.

Karyotyping has been employed by several studies for analyzing human chromosomes in embryonic nuclei (Angell et al., 1986; Plachot et al., 1989; Zenzes et al., 1992; Jamieson et al. 1994). The technique involves incubation of the embryo overnight in colchicine in order to arrest the dividing blastomeres at the metaphase stage. Unfortunately, few nuclei arrest in metaphase and those that do, give poor quality chromosomes that are contracted and very hard to categorize, or more elongated but overlapping (Delhanty and Handyside 1995).

Several conclusions have been drawn from the early studies on karyotyping. Angell and coworkers (1983) examined 8-cell stage embryos revealing a high incidence of chromosomal abnormalities thought to be contributing to early embryonic loss and to the high failure rate after embryo transfer. This was the first report on haploid human embryos with an incidence of 20% suggesting parthenogenic activation of the oocyte which was later confirmed by Plachot (1985). Angell and colleagues (1986) carried out another karyotyping study trying to assess the
contribution of lethal chromosome abnormalities to implantation failure and found non-disjunction giving rise to trisomy, monosomy, nullisomy, as well as structural abnormalities, haploidy and triploidy. Non-disjunction was thought to have occurred at the stage of the second zygotic division according to karyotyping studies by Plachot et al. (1987) who also revealed that the most common anomaly of supposed diploid embryos was mosaicism with 16% of normally fertilized embryos being mainly diploid/haploid and diploid/triploid mosaics (Plachot et al., 1986). Several types of mosaicism have been reported such as: aneuploid mosaicism, and ploidy mosaicism, which further breaks down into haploid, triploid or tetraploid on a diploid background. The conclusion drawn from the above mentioned study regarding implantation development was that only half of the abnormal preimplantation embryos are able to implant, probably by selection against major anomalies (Plachot et al., 1987).

Fertilization and zygote formation has been found to be unaffected by oocyte/sperm abnormalities, based on observations of monosomic and trisomic embryo karyotypes (Zenzes and Casper 1991). In one study the proportions of chromosomes involved in trisomic karyotypes were similar to the patterns in spontaneous abortions (Jamieson et al., 1994). The same study implied that embryos carrying sex chromosome trisomies such as XXX, XXY, and XYY are of equal incidence and that the pronuclear status should not be considered a reliable screening for aneuploidy as this study demonstrated that 30% of normally fertilized embryos carried a chromosome anomaly which comprised mainly of aneuploidies (19%), mosaicism (2%), structural abnormalities (1%) and undetected anomalies of ploidy (7%) (Jamieson et al., 1994).

However, the most important conclusions drawn from karyotyping studies involved correlations of chromosome abnormalities with embryo morphology, maternal age effect, and specific environmental factors. Angell and colleagues (1986) showed that chromosomally abnormal embryos could not be distinguished on morphological criteria from embryos of normal chromosomal constitution based on similar cleavage rates, which was also supported by Jamieson et al., (1994). However, the rate of aneuploidy was estimated to be 12.5% in embryos with equally sized blastomeres and it reached 37% in fragmented embryos, indicating an association between embryo morphology and the incidence of chromosomal abnormalities (Plachot et al., 1986). In a later study Plachot and co-workers (1989) demonstrated...
that only very degenerated and fragmented embryos were shown to display a higher rate of chromosomal abnormalities (78%) when compared with morphologically healthy embryos. Finally, the rate of abnormalities is suggested to be significantly higher in dysmorphic embryos (86.6%) than in good quality embryos (36.6%) (Pellestor et al., 1995). This finding is in agreement with the correlation established between embryo grading and pregnancy rates (Veeck et al., 1983; Erenus et al., 1991), confirming the prognostic value of the grading system as a means of eliminating a large proportion of chromosomally abnormal embryos (Pellestor et al., 1995). A study involving embryoscopic and cytogenetic analysis of missed abortions (with most of the specimens retained in utero), using karyotyping, showed that a total of 75% of the cases involving a missed abortion had an abnormal karyotype, 18% had a morphological defect with a normal karyotype, while no embryonic or chromosomal abnormality could be diagnosed for 7% of the cases (Philipp et al., 2003).

Maternal age was found to be directly proportional to aneuploidy frequency (Angell et al., 1986; Plachot et al., 1987; 1989), particularly affecting aneuploidies of the small satellited chromosomes of Group G (Zenzes and Casper 1991).

Based on a karyotyping study carried out by Plachot and colleagues (1989) it was deduced that IVF does not increase the incidence of chromosomal aberrations when compared to natural conceptions, which was in contrast with the earlier findings of Angell et al. (1983) which suggested that specific environmental factors such as oocyte retrieval and culture are involved in the incidence of aneuploidy. Pellestor et al. (1995) also suggested that certain parameters of IVF might be responsible for the incidence of several chromosomal abnormalities. Ovarian hyperstimulation might be involved in the immaturity or overmaturity of the oocytes retrieved having a serious effect on the fertilization process (Testart et al., 1989), however, others report no such correlation (Tejada et al., 1991; Rosenbusch et al., 1992). Delayed IVF might also lead to triploidy, abnormal cleavage, and fragmentation (Plachot et al., 1988). Moreover, delay in gamete fusion could also lead to asynchronisation of both formation and migration of the male and female pronuclei, resulting in cleavage disturbance and chromosome set fragmentation (Ron-El et al., 1991). In addition to these, the constitution of culture media, the pH temperature variation, light exposure or gas phase could theoretically result in embryo fragmentation (Pellestor et al., 1995).
Clouston et al. (1997) used an improved protocol for karyotyping 6-8-day blastocyst embryos and observed polyploidy, diploid/polyploid mosaicism, non-mosaic trisomy as well as variable chromosomal damage with interchanges, chromosome branching and anomalous chromatid pairing. It was concluded that using this reliable technique that produced good quality G-banded chromosome preparations made it possible to study the chromosomal arrangement of blastocyst stage embryos, and that the phenomenon of chaotic/uncontrolled division was reflected by the chromosomal status of hypodiploidy, hyperdiploidy and structural chromosomal damage (Clouston et al., 1997), also predicted by FISH studies (Harper et al., 1995). However, from karyotypic analysis of human blastocysts it was suggested that mosaic tetraploidy might result from production of bi-nucleate blastomeres due to failure of cytokinesis (Hardy et al., 1993). A recent study by Clouston et al. (2002) on blastocysts, concluded that there was a significant loss of haploid and monosomic embryos, as well as loss of some trisomies, prior to the blastocyst stage. However, no reduction was noted in the level of triploidy. It appears that the general range and incidence of most abnormalities observed in the first trimester are in place by the blastocyst stage. The conclusions are in line with published data regarding earlier and later periods of embryonic development. The study supports the combination of both G-banding and FISH analysis in obtaining knowledge on the cytogenetics of this stage of human development.

Spectral karyotyping (SKY) is a technique which employs 24 chromosome specific probes. Each probe is labeled with different proportions of five separate fluorochromes and observed by spectral imaging, providing a different colour for each human chromosome. It has been applied to human oocytes and polar bodies, being able to simultaneously detect specific aneuploidies as well as de novo structural abnormalities, such as acentric fragments, translocations and marker chromosomes. The analysis on first polar bodies provided useful data for polar body genetic diagnosis. Since the time needed for SKY analysis is less than three days, the method can be used for PGD of aneuploidy or translocation in polar bodies. If future work demonstrates the accuracy of the SKY method for detection of chromosomal anomalies in polar bodies, this assay will be a powerful improvement in PGD (Marquez et al., 1998). However, currently the technique is known to be fairly unreliable especially as it requires good quality chromosome spreads. Therefore, it would be more suitably employed in a research rather than in a clinical setting.
The potential of karyotyping human embryonic single cells for (PGD) was tested by Penketh et al. (1989) by disaggregating zona-free cleavage stage embryos and culturing overnight their individual cells. The difficulty of spreading chromosomes led to unreadable and imprecise results indicating that karyotyping should not be considered as a reliable technique (Delhanty and Handyside 1995).

It has been possible to induce metaphase nuclei in sperm by injecting a single sperm cell directly into hamster oocytes or zygotes to induce metaphase formation (Evsikov & Verlinsky, 1999; Willasden et al., 1999). This technique has been termed nuclear conversion, and it has recently been modified to fuse polar bodies or blastomeres into oocytes or zygotes to induce metaphase formation. In one method human oocytes or abnormally fertilized zygotes were enucleated and the blastomere was injected into the perivitelline space. The oocyte/zygote was given an electric pulse, which causes fusion of the cells. Factors in the cytoplasm then induce the injected blastomere to enter metaphase. Okadaic acid treatment is employed to ensure premature chromosome condensation (Evsikov & Verlinsky, 1999). An alternative protocol used fusion with bovine oocytes to induce metaphase and colcemid to arrest the cell cycle at this stage (Willasden et al., 1999). The chromosomes obtained can be subjected to spectral karyotyping or M-FISH (see chapter 1.2.2.3).

1.4.2 Fluorescent In Situ Hybridisation.

Delhanty and co-workers (1993) showed that surprisingly even apparently normally developing IVF embryos were often chromosomally abnormal, in agreement with the original karyotyping data. Whilst few of these 6- to 10-cell embryos studied were uniformly abnormal, the majority of abnormalities involved mosaicism with a minor aneuploid, polyploid or haploid cell line on a normal diploid background (Delhanty et al., 1993; Munné et al., 1993B; 1995; Harper et al., 1994b; 1995a). The proportion of chromosomally abnormal embryos among normally developing, good quality embryos ranges form 30%-65% (Harper et al., 1995b; Benadiva et al., 1996; Delhanty et al., 1997c; Iwarsson et al., 1999) and the frequency of mosaicism in such embryos ranges from 17% to 50% depending upon the patients selected (Harper et al., 1995a; Benadiva et al., 1996; Delhanty et al., 1997c).

Coonen et al. (1994b) used FISH to study abnormally fertilised polyspermic embryos and detected normal, triploid, and mosaic embryos. The origin of
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diploid/triploid mosaicism is not clear but the presence of an extra haploid set could be a result of dispermy or non-extrusion of a polar body (Tuerlings et al., 1993; Muller et al., 1993). Not surprisingly abnormally developing embryos as a group, including those resulting from abnormal fertilisation were found to show 35-70% frequency of chromosomal abnormality in one or more blastomeres (Munne et al., 1994a, b, 1998a; Benadiva et al., 1996; Laverge et al., 1997; Magli et al., 2001). These were more frequently uniformly aneuploid or polyploid, and mosaicism in these embryos commonly involved a chromosomally abnormal background (Munné et al., 1993b; 1994; Coonen et al., 1994b; Laverge et al., 1997). However, embryo morphology alone cannot be used to predict karyotype, as many good quality embryos have been found to be aneuploid (Munne et al., 1994b; Harper et al., 1995a; Delhanty et al., 1997c; Voullaire et al., 2000).

Aneuploid mosaicism is considered to be the most frequent form of mosaicism observed in human embryos (Munne et al. 1994b). The aneuploid cells may be of meiotic or mitotic (post zygotic) origin. Munne et al., (1997) reports 12% of the embryos included in the study being diploid mosaic (including polyploid and haploid mosaics) and 14% classified as chaotic. Delhanty et al. (1997c) observed 19% of human cleavage-stage embryos to be diploid mosaics, and 25% to be chaotics. It was proposed that the main mechanisms of mosaic aneuploidy were mitotic non-disjunction, which causes a reciprocal loss or gain and anaphase lag (Delhanty et al. 1997c). Veiga et al. (1999) revealed a high prevalence of aneuploid mosaicism in both blastocyst and arrested day 5 embryos of 87.5% and 62.5% respectively. Additionally, Ruangvutilert et al. (2000b) showed that 30% of the day 5 arrested embryos and 21% of the blastocysts were aneuploid mosaics.

Chaotic embryos have been reported in both the cleavage and blastocyst stage (Harper et al., 1995; Delhanty et al., 1997; Evsikov and Verlinisky, 1998) but not at later stages of development, as these embryos would probably arrest and fail to implant. It has been proposed that they are a result of uncontrolled “chaotic” division, which is possibly related to centriole or spindle deficiencies and disturbance of pronuclear syngamy (Klingman et al. 1996). Delhanty and Handyside (1995) noted that the nature of abnormalities observed is reminiscent of the mitotic instability shown in some tumour lines. This has lead them to suggest that these high levels of mosaicism may reflect a deficiency or absence of normal cell-cycle check points in early cleavage before embryonic genome activation occurs at the 4- to 8-cell stage.
Work on Drosophila and Xenopus embryos supports this hypothesis showing that inhibition of DNA synthesis or the presence of DNA damage fail to block progression to M-phase. In yeast, RAD 9 deficiency has been shown to result in a 20-fold higher incidence of chromosome loss compared to normal wild-type cells (reviewed in Hartwell and Weinert, 1989; Weinert and Hartwell, 1990; Ikegami et al., 1997). The possibility that cell-cycle checkpoints do not fully operate during cleavage of the human embryo may also explain the relatively high incidence of various nuclear abnormalities which have been observed (Winston et al., 1991; Hardy et al., 1993). For example, binucleate blastomeres are seen in 15% of human embryos, and are frequently associated with chromosomal abnormalities and appear to result from failure of cytokinesis (Winston et al., 1991; Hardy et al., 1993; Kligman et al., 1996; Staessen and Van Steirteghem, 1998). Most recently, work on inbred mouse strains with elevated levels of Y chromosome nondisjunction has shown that malsegregation in this system is largely restricted to the earliest mitotic divisions (Bean et al., 2001). This suggests that mammalian embryos are indeed susceptible to mitotic nondisjunction in early cleavage stages (Bean et al., 2001) and fits the lack of checkpoint control model.

There is evidence that some couples are particularly prone to produce ‘chaotic’ embryos, those with the most extreme form of mosaicism, in repeated cycles (Delhanty et al., 1997). Several groups have since reported that this tendency is frequent in carriers of chromosomal rearrangements that have been referred for PGD because of poor reproductive histories (Conn et al., 1998; Munné et al., 2000; Iwarsson et al., 2000; Malmgren et al., 2002). A very recent FISH study by Wilding et al. (2003), correlated a low mitochondrial membrane potential with chaotic mosaicism in human preimplantation embryos, but it is unclear whether this is cause or effect.

It has been proposed that embryos containing tetraploid cells, which is the most common abnormality seen at blastocyst stages, may reflect a normal part of development of the trophectoderm as a precursor of the multi-nucleated syncytiotrophoblast. Mosaicism including a tetraploid cell line is a very common finding in both human blastocysts (Benkhalifa et al., 1993; Clouston et al., 1997) and those of other animal species (Long and Williams, 1982; Murray et al., 1986) and may well play a role in normal early development particularly when associated with TE lineages (Angell et al., 1987). These cells may arise as a result of
endoreduplication or endomitosis and possibly play a role in embryo implantation (Drury et al. 1998). The fusion of nuclei in binucleate blastomeres or less frequently blastomere fusion may also lead to polyploidy (Balakier et al. 2000). However, other studies have linked mosaic tetraploidy to poor quality arrested embryos (Wells and Delhanty, 2000). The presence of a haploid cell in a mosaic embryo is difficult to explain, however the underlying mechanism maybe associated with binucleate cell production with a meiotic type of segregation (Delhanty et al. 1997) or with an incorporation of a polar body into the embryo (Staessen et al. 1999).

Aneuploidy, was shown to be increased with the respective increase of maternal age in normal cleavage stage embryos in terms of morphology, while ploidy and mosaicism are closely linked to embryonic dysmorphisms (Munne et al., 1995). Janny and Menezo (1996) found that the percentage of embryos reaching blastocyst stage was significantly reduced for patients above the age of 30. These findings are in agreement with the study performed by Ruangvutilert and colleagues (2000) where maternal age appeared to affect the developmental potential of the embryos. Still, more investigation is required in order to answer the following question “Does maternal age have an effect on all chromosomes, or only a few”?

1.4.3 Comparative Genomic Hybridisation.

To date only a small number of embryos have been studied using CGH to analyse every cell. Two studies were carried out simultaneously on a series of good quality cleavage stage embryos. Both studies, aimed to reveal the true extent of chromosomal abnormalities. Combining the results of the two similar studies conducted by Wells and Delhanty (2000) and Voullaire et al. (2000), the most striking finding was that mosaicism was found to be extremely common affecting 66.6% (16/24) of the embryos included in these studies. This finding confirms the FISH and karyotyping studies performed on cleavage embryos.

The remaining eight non-mosaic embryos analysed from the two studies, included two uniformly abnormal embryos one carrying monosomy 4, and the other one showing monosomy X and trisomy 21 (Down and Turner syndromes). For embryos showing consistent aneuploidy, it is suggested that meiotic errors have occurred (Wells and Delhanty, 2000; Voullaire et al., 2000). The remaining 6 embryos were found to be uniformly chromosomally normal. These results suggest that although a common finding, mosaicism is not a universal feature of human
preimplantation development. It is also of great importance that half of the mosaic embryos detected by CGH contained a normal number of chromosomes in at least half of their cells, also the abnormality seen in the abnormal blastomeres from some of these mosaic embryos involved a single chromosome (Voullaire et al., 2000).

Embryos categorised as chaotic were also found in both studies where a number of cells showed multiple abnormalities, in one case including a range of aneuploidies for 14 different chromosomes (Wells and Delhanty, 2000). CGH analysis showed unique abnormalities such as monosomies affecting the largest chromosomes and also nullisomy. Aneuploidies of chromosomes 1, 2, 4, 7, 10, 11, and 19 were observed, supporting findings from previous karyotyping studies (Watt et al., 1987; Clouston et al., 1997).

In both studies five instances of chromosome breakage resulting in imbalance of specific regions, rather than whole chromosomes, were detected, with one embryo showing reciprocal gains and losses of regions of chromosomes 2 and 7 in sibling blastomeres (Wells and Delhanty, 2000). Again such chromosome breakage has also been recorded in karyotyping studies (Papadopoulos et al., 1989; Zenzes and Casper, 1992; Clouston et al., 1997).

In a following study Voullaire and colleagues (2002) used CGH for aneuploidy screening and detected chromosome abnormality in 60% of single blastomeres biopsied prior to implantation from 20 women with repeated implantation failure. The abnormalities included aneuploidy for one or two chromosomes (25%) and complex chromosomal abnormality (29%). Mosaicism involving a complex abnormality (ie: chaotic) is a more frequent occurrence in these patients than in the previously studied cohort of surplus embryos (Voullaire et al., 2000), and is therefore likely to be related to the history of recurrent implantation failure. This study supports the observation that some individuals are more prone to chaotic embryos than others as suggested by previous FISH studies (Delhanty et al., 1997c; Harrison et al., 2000). In addition, the complex abnormality seen in morphologically normal and actively dividing embryos supports the idea that mitotic checkpoints may not function in the early cleavage embryo (Delhanty and Handyside, 1995; Wells and Delhanty, 2000), and it suggests that disturbance of the normal early embryonic cell cycle might be a pathology associated with infertility and implantation failure (Wilton et al., 2003a).
A recent study by Malmgren and colleagues (2002) analysed 94 blastomeres from 28 embryos generated from 13 couples carrying a balanced chromosomal aberration undergoing PGD. The single cell CGH confirmed most of the unbalanced translocations detected by PGD. As the embryos made available for this study were previously diagnosed as unbalanced regarding the chromosomes involved in the translocation or were considered unsuitable for transfer for other reasons, it was expected to see a higher degree of mosaicism than was encountered in the studies by Voullaire et al. (2000) and Wells and Delhanty (2000). Indeed all of the embryos (100%) were classified as mosaic (containing more than one chromosomally uniform cell line) or chaotic. In this study, a tendency for some couples to be more prone to generate chaotic embryos than others was also seen, as previously described by Delhanty et al. (1997).

It has been shown that 5% of good quality embryos and 12% of poor quality embryos are anucleated (Hardy et al., 1993). Highly mosaic and imbalanced embryos will have a high degree of anucleated cells which could also explain the difference of the CGH efficiencies between the two studies by Voullaire et al. (2000) and Wells and Delhanty (2000) analysing normal IVF embryos, with an estimated CGH efficiency of 97% and 98% respectively, and the most recent study by Malmgren et al. (2002) where the CGH analysis success rate was 70%.

Following the application of CGH on single blastomeres, concerns on the reliability of PGD using FISH to identify chromosomally normal from abnormal embryos were raised. In the case of FISH, probes target a defined region on a chromosome so the status of the rest of the chromosomes is simply assumed lacking conclusive proof. This fact strengthens the argument for the adaptation of CGH for clinical screening of embryos (Wells and Levy, 2003). However, hypothetically more than half of the abnormalities found in the CGH embryo studies could have been excluded using a limited FISH probe set (XY, 13, 18, 21). CGH has limitations since small deletions or amplifications of telomeric regions are difficult to interpret by CGH (Malmgren et al., 2002). Therefore, imbalances involving a translocation with a very distal breakpoint could be missed if CGH is employed as a diagnostic tool in PGD. Similarly, translocations involving regions of the karyotype that are subject to artefacts have to be excluded from CGH analysis (Malmgren et al., 2002). Another limitation of CGH is that it can only detect relative alterations in chromosome copy
number and is therefore unable to identify changes that involve the entire set of chromosomes such as haploidy or tetraploidy.

1.4.4 Developmental Selection and Implantation.

Although a high percentage of embryos are mosaic at the blastocyst stage, it has been shown that the proportion of abnormal cells is less than 20% and the majority are tetraploid (Evsikov & Verlinsky, 1999; Ruangvutilert et al., 2000). This finding supports that implantation and further embryo development may be possible for mosaic and generally chromosomally abnormal embryos (Harper and Delhanty 1996). However, chaotics or those with a majority of abnormal cells are unlikely to survive beyond implantation (Harper and Delhanty, 2000).

Following the work of Jamieson and West (1994) on artificial mouse chimaeras in which tetraploid cells were found only in the trophectoderm, it was expected that abnormal cells in human blastocysts would be most likely found in the trophectoderm and therefore not in the embryo proper. However, after FISH was performed to determine the number of cells and the degree of mosaicism in the ICM of human blastocysts, it was shown that the average degree of aneuploidy in the ICM was similar to that in the overall blastocyst, suggesting that there is probably no selection for a euploid ICM. It was thought selection against the embryos with a high frequency of aneuploid or complex mosaicism would lead to elimination of these embryos prior to blastocyst formation, which would explain the lower frequency of abnormal cells in blastocysts compared to that in the cleavage stage embryos (Evsikov and Verlinsky 1998, Magli et al., 2000).

Sandalinas and co-workers (2001) performed a study to determine which chromosomal abnormalities are compatible with development to the blastocyst stage. Extensive mosaicism was detected in blastocysts and trisomic embryos reached the blastocyst stage with a frequency of 37%. Interestingly only those monosomies compatible with first trimester development (monosomy X and 21) were detected at the blastocyst stage. Conclusively, even though there is a strong selection against chromosomally abnormal embryos, extended culture to day 5 or 6 cannot be used as a reliable tool to select against clinically relevant chromosome abnormalities such as trisomies (Sandalinas et al., 2001).
1.4.5 Effects of IVF Treatment on Chromosomal Abnormalities in Embryos.

Munne et al. (1998e) assessed chromosome abnormalities in embryos obtained from IVF and compared them to embryos resulting from ICSI with consideration of maternal age, embryo morphology, and developmental rate. The study concluded that the same proportion of chromosomal anomalies are found in ICSI embryos as in conventional IVF produced embryos considering general and specific types of abnormalities such as gonosomal aneuploidy and mosaicism, meaning that the injection of the sperm into the cytoplasm is not disturbing the M-II metaphase spindle.

Any influences of IVF treatment on the embryo’s chromosomal status are extremely difficult to quantify due to the number of variables encountered including different drug regimes and culture protocols, and presumably most importantly the diverse patient groups involved. Additional FISH experiments lead to the conclusion that the hormonal stimulation protocols as well as certain culture conditions applied in IVF centres might play a role in the generation of several chromosomal abnormalities (Munne et al., 1997). Mosaicism, was suggested to be caused by impairment of the cytoskeleton and/or mitotic spindle, which is strongly related to temperature control. Moreover, cryopreservation in mouse embryos was considered to be responsible for some chromosomal abnormalities (Bongso et al., 1988; Shaw et al., 1991). Based on these results it is difficult to establish whether the IVF generated surplus embryos used are representative of all human embryos. Embryos form infertile couples might be more likely to be aneuploid because chromosomal errors could represent an underlying cause of infertility (Wilton, 2002). However, some patients who access IVF for PGD testing are fertile. Embryos that are genetically unsuitable for transfer for these patients have been examined for chromosomal errors using FISH and interestingly almost half of them have been found to be aneuploid mosaics (Munne et al., 1994b; Delhanty et al., 1997c). A comparison of retrospective data from four IVF centres by Munné et al., (1997) has found levels of mosaicism to vary greatly between IVF centres (11% to 52%), proposing a relationship between this and different drug and culture conditions. The impact which suboptimal in vitro factors have on post-zygotic chromosomal mosaicism is less clear, though fluctuations in temperature and
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Oxygen tensions have been shown to affect oocyte spindle formation and chromosome segregation (Almeida and Bolton, 1995; Van Blerkom, 1998). It is obvious that some patients do inexplicably produce higher levels of aneuploid (Munné et al., 1996), polyploid (Pergamont et al., 2000a) or chaotic embryos (Delhanty et al., 1997c) than others even when subjected to identical IVF regimes and this patient-specific factor is difficult to allow for when comparing abnormality rates between centres.

1.5 Preimplantation Genetic Diagnosis (PGD).

PGD is a clinical diagnostic procedure that has evolved from the substantial advances both in assisted reproduction technology and molecular genetic analysis. Originally developed as an alternative to prenatal diagnosis it aims to reduce the transmission of severe genetic disease for fertile couples with a reproductive risk, by genetic screening of early human embryos (Handyside et al., 1990). Patients undergo routine IVF procedures and the resulting embryos are biopsied, usually at the cleavage stage, and genetic analysis is carried out on one or two cells depending on the embryonic stage targeted. Only those embryos subsequently diagnosed as unaffected are then selected for transfer to the uterus. In this way if implantation occurs, a pregnancy is begun in the knowledge that it is likely to be normal so removing the uncertainty and anxiety felt by many parents in the early stages of a high risk pregnancy (Raeburn, 1995).

PGD is not a new concept. Sexing of rabbit embryos by using a sex-specific chromatin pattern, in biopsies from blastocyst stage rabbit embryos, was performed by Gardner and Edwards in 1968. Sex-selection of animals using PGD has been also used in animal breeding (Johnson, 1996). Substantial groundwork for the clinical application of PGD was undertaken in the late 1980s with the advent of IVF technology and sensitive diagnostic techniques such as FISH and PCR (Monk et al., 1987; 1988; Handyside et al., 1989; Penketh et al., 1989; Hardy et al., 1990; Monk and Holding, 1990; Verlinsky et al., 1990). In 1990 Handyside and co-workers report the first livebirths following successful application of PGD for the purpose of sexing (Handyside et al., 1990). The first live birth following PGD for Cystic Fibrosis (CF) was reported in 1992, a breakthrough which represented the first successful clinical application of PGD for a single gene disorder (Handyside et al., 1992). Since then PGD has been developed for an increasing variety of other genetic conditions.
including single gene defects, age-related aneuploidy and more recently specific chromosomal rearrangements (ESHRE PGD Consortium, 1999; 2000, 2002).

There are several groups of patients for whom PGD is the preferred option. The first group includes cases of couples at risk of passing on an X-linked disorder for which there is, as yet, no specific molecular diagnosis. The current treatment offered is fetal sex determination and selective abortion of all males, of which half will be unaffected. Secondly, there are couples where one or both partners carries a known gene mutation or chromosomal rearrangement which makes them at high risk of transmitting severe genetic disease to their children. Although couples in this group who are infertile and already require IVF are obviously the ideal candidates for PGD, many couples will be able to conceive naturally but have suffered repeated spontaneous or induced abortion as a result of a series of abnormal conceptions. The advantage of PGD in these latter ‘fertile’ cases is that by using IVF, many embryos can be tested simultaneously in a single reproductive cycle, so increasing the likelihood of selecting unaffected embryos and achieving a normal pregnancy (Handyside, 1993). Certain couples having suffered repeated unexplained IVF failure (Gianaroli et al., 1997) or recurrent spontaneous abortion (Vidal et al., 1997) are now able to undergo PGD based on their reproductive history alone. Moreover, as advanced maternal age is strongly associated with an increased risk of chromosomally abnormal pregnancy infertile couples already requiring routine assisted conception can be referred to undergo preimplantation genetic screening aiming to screen embryos for the common viable aneuploidies such as Down syndrome, to increase their IVF success rate. Finally, there are those with moral or religious objections to pregnancy termination. This group would prefer selection to occur at the preimplantation stage, giving them a reasonable chance of starting with a normal pregnancy.

In some cases, without PGD, couples may avoid pregnancy completely, as they feel unable to risk the birth of an affected child. This is reflected in a number of surveys to measure attitudes of prospective patients to PGD, where most indicate that the main benefits are perceived to be early reassurance and avoidance of the trauma of TOP (Pergamont, 1991; Snowdon and Green, 1997; Chamayou et al., 1998). With the clinical application of PGD has come the realisation that the reasons for referral are far more complex and wide ranging than at first envisigated (Delhanty and Harper, 2000), it is therefore crucial to examine in depth the reasons for referral especially in
view of recent fears that “designer” babies are being selected to meet the desires of the parents (Braude et al., 2002). Ethical and moral obligations have to be considered when dealing with PGD. The challenge will be to regulate the use of PGD technology for medical purposes and to limit or prevent its use for eugenic selection. In the UK all centres offering PGD have to obtain a licence from the Human Fertilisation and Embryology Authority (HFEA) who licence all clinical and research work involving human embryos.

It is vitally important that all PGD patients have genetic counselling and mutation detection before they start a PGD programme to confirm that this treatment is suitable for them and that they are aware of the possible drawbacks to this approach as well as the benefits. Such non-directive counselling is particularly important for this group of patients, many of whom will have had multiple affected pregnancies (Harper, 1998). PGD is now considered as an alternative to prenatal diagnosis procedures such as chorionic villus sampling (CVS) and amniocentesis (Harper and Wells, 1999; Iwarsson et al., 2000; Ruangvutilert et al., 2000b; Fridström et al., 2001; Wells and Delhanty, 2001; Harper and Bui, 2002), carried out on established pregnancies from 10-16 weeks gestation (reviewed in Jauniaux and Rodeck, 1995; Holzgreve et al., 1999). However PGD will not replace prenatal diagnosis as patients have to go through IVF and it is very expensive, but its further development will give parents an alternative way of achieving a healthy family without resorting to abortion (Delhanty & Harper, 2000).

1.5.1 Obtaining embryos for PGD.

Uterine lavage, involving flushing of the uterus following natural conception on day 5-post ovulation, has been a theoretical possibility in obtaining embryos for PGD (Whittingham and Penketh, 1987). However, although a non-invasive method for retrieving embryos, simpler and less expensive than IVF, was never a practical alternative. The technical problems arising involve the uncertainty of removing all the embryos from the uterine cavity, as well as the risks of backflow resulting in ectopic implantation.

All embryos undergoing PGD are produced from IVF treatment protocols. In order to attain an adequate supply of normally developing embryos to improve the chance of detecting and subsequently transferring a normal embryo, patients undergo protocols aimed at producing the maximum number of oocytes, while at the same
time limiting ovarian stimulation to a minimum to avoid hyperstimulation syndromes and to aid collection of better quality oocytes (Vandervost et al., 1998). Limiting extraneous DNA that could lead to contamination jeopardising the diagnosis, is a major concern. Therefore stringent removal of all maternally derived cumulus cells is required. In the case of PCR-based PGD protocols ICSI is employed to avoid sperm contamination (Wells and Sherlock, 1998).

1.5.2 Sampling Strategies for PGD.

Embryo biopsy has been described as a two stage process involving the puncture or removal of a part of the zona pellucida surrounding the oocyte or embryo and subsequent removal of a cell or cells. Theoretically, this can be performed at any developmental stage, including preconception, and different technical approaches have been described to better suit different developmental stages. The advances made in reproductive biology including micromanipulation procedures (Palermo et al., 1992; Van Steirteghem et al., 1993) and the development of sequential media allowing prolonged in vitro culture up to the blastocyst stage, allowed the biopsy of polar bodies, blastomeres, or trophectoderm cells from oocytes, cleavage stage embryos, and blastomeres respectively (De Vos and Van Steirteghem, 2001). The main priority at each of these embryonic stages is where possible to obtain a cell sample for analysis, which is representative of the genetic constitution of the whole embryo, in a way that is not detrimental to subsequent development. The debate over whether taking two cells rather than one is detrimental to the embryo is ongoing (De Vos and Van Steirteghem, 2001; Van de Velde et al., 2000). Several strategies and techniques have been suggested over the years with regard to zona drilling and subsequent removal of blastomeres.

1.5.2.1 Zona Drilling and Removal of Cells.

The first step in the procedure involves making an opening in the zona pellucida through which a cell can be removed and this can be achieved either mechanically involving partial zona dissection (PZD) by direct piercing or cutting with a micropipette (Grifo et al., 1990; Verlinsky et al., 1990; Cieslak et al., 1999), or chemically by the localised application of acid Tyrode's solution (Hardy et al., 1990; Handyside, 1991; Inzunza et al., 1998) which is the most commonly used method for
cleavage or blastocyst stage biopsy. Most recently an extremely precise laser system has been employed (Veiga et al., 1997; Boada et al., 1998; Montag et al., 1998). The use of a laser for zona drilling in cases of PGD is an easier procedure and results in more intact blastomeres. Since similar pregnancy rates are obtained from studies comparing it to the widely used acid Tyrodes technique (De Vos and Van Steirteghem, 2001), it is advantageous to perform laser assisted human embryo biopsy (Joris et al., 2003).

Once the hole is drilled in the zona, there are a number of different methods for removal of blastomeres, but the majority of centres use aspiration where the blastomeres are aspirated by gentle suction using a polished pipette. Cell removal and separation were initially difficult due to increased intracellular contacts established by the cleavage stage embryo via gap and other junctions. Since these junctions are calcium dependent, if the biopsy is performed in calcium/magnesium-free medium, decompaction occurs and cells can be easily removed from the embryo (Dumoulin et al., 1998). So far there are no indications that its use might have an adverse effect on embryo viability and normal fetal development (Dumoulin et al., 1998; Staessen et al., 1999; Vandervost et al., 2000; Kahraman et al., 2000).

1.5.2.2 Polar body biopsy.

The procedure of polar body removal (PBR) has first been described by Verlinsky and Cieslak (1993). The first PB is biopsied optimally within six hours of oocyte retrieval to preserve chromosome morphology (Verlinsky and Cieslak, 1993), whilst the second PB is extruded at fertilisation and can be biopsied sequentially (Kuliev et al., 1998; Strom et al. 1998) or alternatively both polar bodies can be removed in a single procedure (Verlinsky et al., 1998; 1999c).

Polar body biopsy has the advantage that it samples extra embryonic material and is therefore less likely to affect detrimentally subsequent embryonic development. However, this advantage can also be viewed as the main disadvantage as any postzygotic mitotic errors could not be detected. As it provides a longer time for the analysis and is less invasive it might be considered ethically preferable by some. However, polar body biopsy, inferring the genetic status of the oocyte, is an indirect method that can only provide information about the maternal genome and no paternal alleles can be analysed. In addition, if the first polar body is heterozygous, the second polar body will also need to be analysed and unbalanced predivision of chromatids.
will complicate the diagnosis. Both first and second maternal meiotic errors can be excluded if information on both polar bodies is obtained (Angell, 1994a). In fact, where predivision of chromatids or undetected recombination has taken place, a reliable diagnosis might not always be possible (Munne et al., 1998a; Rechitsky et al., 1999). Moreover, this technique has been described as labour intensive as not all of the oocytes analysed will fertilise. Also, even after a successful biopsy, uninformative PBs or a subsequent poor fertilisation rate can have a negative effect on the number of embryos for transfer. The first clinical applications of first polar body analysis for monogenic disorders led to misdiagnosis (Verlinsky et al., 1990). Later simultaneous analysis of first and second polar body was employed for aneuploidy screening (Verlinsky et al., 1996c, 1998, 1999c) or single gene disorders (Verlinsky et al., 1999c; Strom et al., 1998; Kuliev et al., 1999), resulting in healthy live births. Munne et al. used it for translocations of female origin, significantly reducing spontaneous abortion rate (Munne et al., 1998g; 1998h; 2000). So far no detrimental effects from PGD by PBR have been observed on children born after the procedure (Strom et al., 2000a).

1.5.2.3 Cleavage-stage biopsy.

The efficacy and safety of cleavage stage biopsy were first shown in studies using mouse embryos (Liu et al., 1993) and this technique has since been used in many clinical procedures worldwide (De Vos and Van Steirteghem, 2001). The basic technique of cleavage stage biopsy has changed little from the original method (Handyside et al., 1989). The embryo is usually biopsied on the morning of day 3 of development (day 1 being the day of zygote formation) when the embryo is composed of 6 to 8 blastomeres. Fig. 1.9 shows the four stages of cleavage stage embryo biopsy.

Fig. 1.10: Cleavage stage embryo biopsy

Fig. 1.10 illustrating cleavage stage embryo biopsy. (a): Embryo attached to holding pipette and positioned. (b): Acid Tyrodes is used to create a hole in the zona pellucida. (c): A single blastomere is carefully removed. (d): The embryo following the biopsy.
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Early studies to measure the effect of biopsy on human embryonic development showed that two cells could be removed from 8-cell embryos on day three post-insemination without reducing the number blastulating or disturbing cleavage rates (Hardy et al., 1990). It has been shown that at this stage of development blastomeres retain totipotentiality and the embryo can be biopsied successfully even if compacting (De Vos and Van Steirteghem, 2001). Moreover, as the embryonic genome is fully activated after the eight cell stage, it may be beneficial to evaluate embryos at least until after the transition from maternal to embryonic genome, making it possible to identify these embryos with a better developmental potential (De Vos and Van Steirteghem, 2001). Unfortunately, not all embryos reach the 7- or 8-cell stage by the morning of day 3. Six cell embryos might also be included for one or two cell removal. However, biopsies performed at the 4-cell stage may alter the ratio of inner cell mass to trophectoderm cells, if more than one cell is removed, which may be detrimental to embryo development (Tarin et al., 1992). Consequently two cell biopsy procedures should only be carried out on day three post-insemination at the 6-8 cell stage, when up to a quarter of the blastomeres can be removed without disturbing subsequent development (Handyside et al., 1989; Handyside, 1991; Hardy et al., 1990), as the biopsied cells are still undifferentiated (Harper et al., 1996). The safety of this practice has been confirmed in a follow-up study of pregnancies and births resulting from biopsied embryos which found no significant difference compared to those from routine IVF treatment (Soussis et al., 1996a,b).

Cell selection in cleavage stage biopsy plays a very important role, especially in view of multinucleated blastomeres biopsied for PGD analysis (Munne and Cohen, 1993; Staessen and Van Steirteghem, 1998). Each cell selected should be checked for the presence of a single nucleus, as anucleate fragments or blebs can be mistaken for blastomeres (Hardy et al., 1993). Good cell morphology with respect to its size and shape should also be taken into consideration (Harper, Delhanty and Handyside, 2000).

The decision as to whether one or two cells should be removed is controversial; removing two cells reduces the cellular mass and could potentially reduce its developmental capacity (Braude et al., 2002). The accuracy of the diagnosis however, is likely to be enhanced if embryos are replaced when results from both cells are concordant (Lewis et al., 2001; Van de Velde et al., 2000). The phenomenon
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of mosaicism poses the risk that the cell analysed is from a minor cell line and so not representative of the remainder of the embryo transferred, and the existence of haploid cells in normally diploid embryos can lead to problems in the diagnosis of dominant disorders and chromosomal abnormalities (Harper et al., 1996). For this reason many groups prefer to base their diagnosis on the result of two biopsied cells, particularly for chromosomal analysis and dominant disorders (Delhanty et al., 1994; Delhanty and Handyside, 1995; Kuo et al., 1998; Van de Velde et al., 2000; Simopoulou et al., 2003).

1.5.2.4 Blastocyst biopsy.

The feasibility of trophectoderm biopsy at the blastocyst stage was shown in several animal models (rabbit, Gardner and Edwards, 1968; mouse Gardner, 1971, Monk et al., 1988; cattle, Carson et al., 1993; monkey, Summers et al., 1988). These in vivo studies were later confirmed in the in vitro human embryo, showing that removal of some trophectoderm cells from the preimplantation blastocyst shows no adverse effect on further in vitro development (Dokras et al., 1990). Blastocyst biopsy can be performed on day 6 to day 7 post fertilisation when the blastocyst contains approximately 120 cells.

The main advantage of blastocyst biopsy, is that of removing 10-30 cells (Muggleton and Harris, 1995), allowing generous amount of embryonic material for a reliable genetic analysis, overcoming the paucity of material made available with cleavage stage biopsy. As these cells are from the trophectoderm there is no deleterious decrease in the ICM and therefore in the embryo proper. However, as TE is directly involved in implantation of the embryo, although removing some of it will not affect the embryo proper, its potential for implantation might be compromised (Dokras et al., 1990). Although maximizing the number of cells biopsied without harming the embryo proper, which is the focal benefit of blastocyst biopsy, at the same time the trophectoderm might have diverged genetically from the inner cell mass (Delhanty et al., 1994) and consequently fail to represent the embryo proper.

Observations of blastocyst culture show only half or fewer of the IVF generated embryos to reach blastocyst stage (Jones et al., 1998) with most arresting before reaching this stage of in vitro development (Hardy et al., 1993). This is the main drawback of this approach, resulting in a limited number of embryos available for biopsy and diagnosis. Recent work to try and overcome this block has centred on
adding certain amino acids to the culture medium, such as glutamine, but the results have shown no significant increase in blastocyst development (Gardner et al., 1998). Moreover, the time for diagnosis will be severely limited if biopsy is postponed to this later preimplanation stage, posing serious restrictions the time allocated for genetic analysis (De Vos and Van Steirteghem, 2001). However, the recent report of a human livebirth following blastocyst biopsy (De Boer et al., 2002), might encourage the increased use of this technique (Braude et al., 2002).

1.5.3 PGD Of Chromosome Abnormalities.

Diagnostic techniques for PGD need to be sensitive, accurate and rapid enough to allow reliable analysis at the single cell level in a timeframe accommodating IVF procedures. FISH and PCR, following extensive groundwork for their suitability in clinical application, are the methods of choice and now form the basis of most PGD strategies used today for a spectrum of genetic defects ranging from gross chromosomal aberrations to single base-pair mutations. Although classical cytogenetic techniques can also be successfully applied to single blastomeres with a view to PGD, the efficiency with which analysable metaphase preparations can be produced per biopsied cell is notoriously low (Kola and Wilton, 1991). However, issues like that have been addressed with the advent of novel techniques such as interphase chromosome conversion enabling examination of chromosomes by arresting the nucleus in metaphase (Evsikov & Verlinsky, 1999; Willasden et al., 1999). Research towards complete karyotyping has shown CGH to be the most successful technique in interphase cells. Recently, many studies have been performed to assess the efficiency of CGH as a diagnostic tool especially for age related aneuploidy, as a healthy live birth has been reported following the transfer of an embryo from which a single cell has been fully analysed (Wilton et al., 2001).

1.5.3.1 Embryo sexing for X-linked disorders.

Sexing the embryo to avoid X-linked disease was the first application of FISH in this context and remains one of the major indications for PGD (ESHRE PGD Consortium, 1999, 2000 and 2002)). X-linked recessive diseases account for 6-7% of single gene defects and include conditions such as Duchenne muscular dystrophy (DMD), haemophilia, and various mental retardation syndromes. There are over 400 X-
linked diseases (McKusick, 1994) for the majority of which no specific diagnosis is available. Prior to PGD the only option open to such couples was prenatal sex determination and the termination of all male pregnancies, even though 50% of these will be normal. The advent of ICSI has led to another group of patients being referred for this type of PGD. These are patients with sex chromosome aneuploidies, particularly Klinefelter syndrome (Staessen et al., 1996; Reubinoff et al., 1998; Emiliani et al., 2000). In addition, couples who are keen to choose the sex of their baby for purely social reasons have shown interest in this approach, however the ethical implications of this mean that in a number of countries including the UK, such family balancing using PGD is prohibited and remains a controversial issue (Pennings et al., 1996; Pembrey, 1998; Ethics Committee of ASRM, 1999; Sureau, 1999, ESHRE PGD Consortium, 2002).

The first clinical application of PGD was the diagnosis of sex by PCR amplification of a repeat sequence from the long arm of the Y chromosome (Handyside et al., 1990). The embryos for which no amplification was present were diagnosed as female and recommended for transfer. The first misdiagnosis using this approach was reported in 1996 as routine prenatal diagnosis showed one of the seven clinical pregnancies established was in fact male (Handyside et al., 1996). This error presumably arose from amplification failure of a XY blastomere, subsequently shown to occur in 15% of cells tested, although biopsy of an anucleate or haploid blastomere would give the same result (Kontogianni et al., 1996). Amplification failure can be a problem at the single cell level as the major concern is for amplification of contaminating material along with the biopsied cell, as well as the failure to provide any information on chromosome copy number, so that sex chromosome aneuploidy (Turner's and Klinefelter's syndrome) remains undetected. Crucially this means that a monosomy X embryo also at risk of X-linked disease is indistinguishable from a normal XX embryo with potentially disastrous effects (Hassold et al., 1988). Following this, protocols were developed for the simultaneous detection of both X and Y chromosomes, using either combinations of specific primers (Kontogianni et al., 1991; Grifo et al., 1992) or more reliably common primers for homologous sex chromosome sequence such as amelogenin (Nakahori et al., 1991) steroid sulphatase (Liu et al., 1994) or ZFX/ZFY (Chong et al., 1993). However, the use of interphase FISH using an indirectly labelled probe for chromosome Y was developed for embryo sexing (West et al., 1988; Penketh et al., 1989). This was followed by the use of two
indirectly labelled probes detecting both sex chromosomes (Griffin et al., 1991, 1992, 1993) diagnosing female embryos on the presence of two fluorescent signals for chromosome X and distinguishing the male embryos based on the presence of one signal for chromosome X and one signal for chromosome Y. Indirectly labelled probes originally employed were soon replaced by directly labelled probes reducing the time of the FISH procedure from 7 to 2 hours (Harper et al., 1994a; Harper and Delhanty, 1996). Subsequently, the use of at least one autosome probe (usually detecting chromosome 16 or 18) was included providing further information on ploidy status of the blastomere (Delhanty et al., 1997; Staessen et al., 1999; Vandervorst et al., 2000). Therefore, to misdiagnose a normal male embryo as a normal female embryo two errors must occur; the signal for chromosome Y must be lost and an extra signal for chromosome X must be generated. This provides an effective internal check (Braude et al., 2002). A possibility for misdiagnosis can arise in the case when the embryo analysed is chaotic or grossly mosaic and the cells biopsied are not representative of the whole embryo (Kuo et al., 1998). However, only one FISH misdiagnosis occurred among 78 cycles of social sexing as reported to the ESHRE PGD consortium (2002). The efficiency, simplicity and speed characterising FISH have established it as the method of choice for detecting the chromosome constitution of preimplantation embryos (Delhanty et al., 1993).

1.5.3.2 PGD for chromosomal rearrangements.

For patients carrying chromosomal abnormalities FISH has been employed as the method of choice for PGD. These patients may experience some fertility problems, or recurrent miscarriages, and they often do not reach the stage of prenatal diagnosis or if they do a percentage of them undergo termination of pregnancy (TOP). PGD is difficult, as different chromosomes and breakpoints can be involved and hence cannot be standardised for these cases. A number of reports concerning PGD have been published involving Robertsonian and reciprocal translocations, inversions, insertions, microdeletion syndromes and gonadal mosaicism (Conn et al., 1998, 1999; Iwarssson et al., 1998; Reubinoff et al., 1998; Scriven et al., 1998, 2001; Van Assche et al., 1999; Simopoulou et al., 2003). The first stage is to predict the likely behaviour of the rearranged chromosome and the normal homologues at meiosis so a PGD strategy can be devised to detect all the possible segregation outcomes can be noted (Gardner & Sutherland, 1996). In practise, in complex situations it may not be
possible to detect all the theoretically possible outcomes of meiotic segregation and the aim then would be to exclude the more viable unbalanced products.

Blastomere analysis is almost always used for PGD of chromosomal rearrangements. In the case of female carriers polar body biopsy can be applicable but is rarely done. Whole chromosome-specific painting probes, sometimes in combination with α-satellite repeat and locus specific probes, are used to confirm the number of chromatids present (Munne et al., 1998a). This probe approach cannot be used on biopsed blastomeres as the cells are unlikely to be in metaphase. Diagnosis is based on the assumption that the metaphase II oocyte and its first polar body have complementary karyotypes. Following this, polar bodies with an unbalanced chromosome complement imply an unbalanced chromosome complement in the oocyte while polar bodies with a balanced chromosome complement imply a balanced chromosome complement in the oocyte which will give rise to a phenotypically normal offspring.

**Robertsonian translocations** occur repeatedly and are common in the population (1 in 1000-2000). Indications such as recurrent spontaneous abortions, reduced fertility or infertility in couples with Robertsonian translocation carriers may suggest PGD, where the use of FISH analysis on biopsied blastomeres (Conn et al., 1998; Munné et al., 1998c) or polar body analysis in cases of female carriers (Munné et al., 1998g; 1998h) may be proved as the only viable options. To detect Robertsonian translocations chromosome enumerator probes are used to count the chromosomes in the interphase nuclei in the cases of cleavage blastomere biopsy (Conn et al., 1998). Probes can be chosen which bind to any point on the long arm of each chromosome involved in the translocation. Studying infertile couples carrying Robertsonian translocations using FISH, revealed two factors leading to infertility in some of these cases. Firstly the aneuploid segregation of Robertsonian translocations carried by the parent and secondly a factor acting at the post-zygotic level provoking an uncontrolled chromosome distribution in early cleavage stages giving rise to chaotic embryos (Conn et al., 1998).

**Reciprocal translocations** are the commonest form of chromosomal abnormality occurring 1 in every 500 live births. Most can achieve a healthy family with the help of prenatal diagnosis, but PGD is appropriate for those at high
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reproductive risk. Due to the complex nature of reciprocal translocations, each case is usually unique, hence difficult to treat by PGD (Harper and Bui, 2002a). For reciprocal translocations the prevalence of unbalanced gametes is estimated to be between 50-70% (Gardner & Sutherland, 1996). In each case different proportions of possible segregation products are expected, of which there are 32 possible types including those that result from errors at meiosis II. Algorithms for analysis of reciprocal translocations have been published by Jalbert et al. (1980), establishing the most likely viable mode of any such translocation and predicting the more viable pregnancy outcomes. Constructing such a pachytene diagram as suggested by Jalbert can be very helpful in devising a probe strategy.

For cleavage stage blastomere analysis of reciprocal translocations two approaches have been used; the use of flanking (Conn et al., 1998; Munne et al., 1998d) or spanning probes (Munne et al., 1998c). To detect all possible segregation patterns in the case of the flanking probe strategy, ideally four probes should be used, each located on either side of the breakpoint. In the study of Van Assche et al., (1999), the use of four-colour FISH analysis allowed the identification of all the possible segregation modes using commercially available probes. However, due to the limitations in the variety of FISH fluorochromes it proved difficult to obtain the necessary probes in different colours and so three colour FISH has been used (Conn et al. 1998, 1999). The probes should be selected based on the pachytene diagram indicating which three probes would be necessary to ensure an accurate diagnosis. Two probes would be used flanking the breakpoint of one chromosome and the third probe would be specific for the other chromosome. This approach has been used for PGD of reciprocal translocations by several groups (Munné et al. 1998c, Pierce et al. 1998; Conn et al. 1999; Van Assche et al. 1999). Conn and co-workers employed this strategy for studying a chromosome 6; 21 reciprocal translocation, being able to detect all segregation patterns apart from free trisomy and monosomy of chromosome 6, which is not viable (Conn et al., 1998). The disadvantage of using flanking probes is that balanced and normal embryos cannot be distinguished, but in practise there are rarely sufficient embryos to make such a choice valuable.

Scriven and associates (1998), have suggested a generalized strategy involving chromosome specific sub-telomeric probes specific for the subtelomeric regions of the translocated segments (Handyside et al., 1998), combined with
proximal probes in order to provide a fast and reliable approach to PGD for cases of reciprocal, Robertsonian translocations, inversions and other complex chromosomal rearrangements. This approach, which is simply a variant of the flanking probe strategy, is made possible by the commercial availability of sub telomeric probes.

Approaches such as using case-specific probes that span the breakpoints and that are labeled with different reporter molecules, and using case-specific probes that bind close to the breakpoints, have been employed by Munne et al., (1998a). Munne et al., (1998c), used breakpoint-spanning probes for a chromosome 3p;4p translocation case. The advantage of this strategy is that these probes could detect all the potential segregation patterns of this translocation, plus they could discriminate between embryos carrying a balanced translocation and normal ones. A similar strategy was followed by Cassel and colleagues (1997), using breakpoint-flanking probes for studying an inversion of chromosome 6. However, since the breakpoint-spanning probes are specific for each rearrangement, screening available databases for possible clones that need to be identified and optimised is required. Therefore, the method has been described as labour intensive and time consuming although faster techniques for cloning translocation breakpoints are being developed (Fung et al., 1998b).

Interphase conversion (Willasden et al., 1999) has also been used for PGD for reciprocal translocations by Munne and colleagues where polar body analysis had failed. No embryo transfer was carried out in the two reciprocal translocation PGD cases. Although theoretically this new method could be used to assist diagnosis of structural and numerical chromosomal abnormality, its potential as a PGD tool will only become apparent as more data is collected.

The phenomenon of mosaicism could lead to misdiagnosis (Delhanty et al., 1997). For this reason biopsy and analysis of two blastomeres is frequently recommended. The generally low pregnancy rate in this group of patients is due to the very high level of chromosomally abnormal embryos produced, of both pre and post-zygotic origin. As 50-70% of gametes arising from translocations may be unbalanced there is a possibility that the embryo transfer stage is not reached due to the absence of normal or balanced embryos. However, PGD for chromosomal translocation has resulted in the birth of many normal babies (ESHRE PGD Consortium. 2002; Munne et al., 1998c; Scriven et al., 2000; Ogilvie et al., 2001; Simopoulou et al., 2003).
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The vast majority of patients requesting PGD for inversions carry a pericentric inversion with a very large inverted segment. The probe strategy devised for such cases involves a probe for one of the distal segments of the chromosome involved, which can then detect chromosome imbalance from either of the two possible recombinant chromosomes. Additional probes on the same or different chromosomes can be added as a control as reported by Iwarsson et al. (1998b).

PGD for insertions can be rather problematic since depending on whether synapsis takes place or not, several types of meiotic behaviour are possible that have to be taken into consideration while devising a strategy. The complexity of such cases is increased as the orientation of the inverted segment is often impossible to detect. Therefore, the use of a subtelomeric probe along with a locus specific for the inserted segment is essential.

The growing availability of commercial probes for the diagnosis of microdeletion syndromes (Di George, cri du chat, Prader-Willi/Angelman, Wolf-Hirschhorn and Miller Dieker) has allowed their uncomplicated diagnosis for PGD. However, when these dual probes are used on their own without a probe for another chromosome as an internal control check, misdiagnosis would only require a single mistake so in these cases biopsing two blastomeres is advisable (Braude et al., 2002).

The presence of a second aneuploid line in a phenotypically normal individual creates a high-risk situation. In females below the age of 35, repeated conceptions with free trisomy for a certain chromosome are likely to be due to gonadal mosaicism. PGD may be employed in these cases to screen for trisomy of the specific chromosome (Conn et al., 1999).

1.5.3.3 PGD for Aneuploidy Screening

FISH has been employed to screen for certain aneuploidies as a means to improve implantation rates mainly in older aged IVF patients, as it has been demonstrated that aneuploidy in normally developing embryos increases with age (Munne et al., 1995, Wilton et al., 2003a). More than 2000 cycles have been reported for this purpose (Verlinsky et al., 2001), accounting for over half of all PGD cycles carried out to date world-wide (Verlinsky and Kuliev 1998). The couples opting for this test are infertile and undergoing IVF/ICSI to overcome their infertility. There are three particular indications for aneuploidy screening (1) advanced maternal age (AMA) (2) recurrent implantation failure (RIF) (3) and recurrent miscarriage (RM). These patients are
thought to be predisposed to producing aneuploid embryos which would die at or before the time of implantation (Wilton, 2002). Individual embryos are biopsied, and a single cell is examined for numerical chromosomal abnormalities using 5-9 probes.

The first report for PGD-AS was by Verlinsky et al. (1995) using probes for chromosomes X, 18 and/or 13/21 to analyse first and sometimes second polar bodies of oocytes, predicting 23% of them to be normal. Munne et al. (1995b) also analysed first polar bodies in a similar study employing probes for X, Y, 18, and 16 or 13 and 21 concluding that 88% of the oocytes showed a normal segregation pattern. The same study highlighted the importance of immediate analysis of biopsied polar bodies to avoid artefactual false-positive results. Analysis of polar bodies has important limitations as it fails to report on errors arising at second meiotic divisions or during early cleavage divisions of the embryo, as well as for abnormalities deriving from aneuploid male gametes (Wilton, 2002). Therefore cleavage stage biopsy is preferred, as it would be expected to identify both meiotic and post-zygotic chromosome abnormalities.

Many groups worldwide are currently offering PGD-AS and have contributed to data collection on this test (Gianaroli et al. 1997a, b; Kahraman et al., 2000; Munne et al., 1998d; Verlinsky et al., 1999). Gianaroli et al. (1999) found that although there was no significant benefit of PGD-AS for patients with RIF, there was an increased implantation rate and ongoing pregnancy rate in patients with AMA compared to a matched control group. Munne et al. (1999) also found that AMA patients who had embryos screened had an increased ongoing pregnancy rate. In contrast Kahraman et al. (2000) found the ongoing pregnancy rate after PGD-AS to be the same in patients with AMA or RIF. The ESHRE consortium data collection III (2002) showed a 28% pregnancy rate in PGD-AS for AMA (>35 years), but only a 7% pregnancy rate in women with RIF (excluding couples with translocations) indicating that, in the latter group factors other than aneuploid gametes might be likely to be the main cause of infertility.

Most groups use FISH probes to detect chromosomes X, Y, 18, 13 and 21 which together account for 95% of all postnatal chromosome abnormalities. Probes for chromosomes X, Y, 13, 16, 18 and 21 can now be ratio-labelled with three fluorochromes and detected in a single step and have been used in over 55 PGD cycles resulting in pregnancy for 12 patients (Muné et al., 1998d). The most extensive FISH screen reported to date include chromosomes X, Y, 13, 14, 15, 16, 21
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and 22 (Munne et al., 1998) including a re-probing stage. Inclusion of probes for chromosomes 16 and 22 is of particular importance as trisomies of these two chromosomes are the two most common autosomal aneuploidies observed in spontaneous abortions (Wilton, 2002). However, it was in this series that a misdiagnosis of trisomy 21, resulting in an abnormal pregnancy, was reported (Munne et al., 1998). The misdiagnosis may have been due to overlapping signals or disomy/trisomy mosaicism. Errors from mosaicism or overlapping signals will be increased if a diagnosis is made from a single cell (Delhanty and Handyside 1995; Kuo et al. 1998).

The advantages and disadvantages of the PGD-AS procedure are still under debate (Dailey et al., 1996; Egozcue 1996; Reubinoff and Shusan 1996; Verlinsky and Kuliev 1996; Gianaroli et al., 1997, Smith et al. 1998, Munne et al. 1998, Wilton, 2002). As well as being labour intensive, older aged women produce few good quality embryos, PGD reduces the number of embryos available for transfer and the error rate is estimated to be 15% (Munne et al. 1998). Smith et al. (1998) found that so few embryos were diagnosed as normal that undiagnosed and monosomic embryos were transferred. The reasoning is that the FISH error rate is higher than the risk of a monosomic embryo going to term. The addition of every extra probe, combinatorial labelling used by many centres, and sequential FISH rounds, compromise FISH efficiency (Liu et al., 1998). The efficiency of any probe combination should be established using lymphocytes and embryos donated for research. Overlapping signals and hybridisation failure can affect all FISH protocols and so strict scoring criteria have to be followed (Hopman et al. 1988 and Munné et al. 1998d).

Clear benefits of this technique in terms of live birth rate per initiated cycle have yet to be shown in any large-scale prospective controlled study that would properly evaluate this technology and its effects (Braude et al., 2002). An international clinical trial is needed, with a suitably matched control group to determine if this procedure benefits older-aged IVF patients.

The application of CGH for PGD-AS is not straightforward as after embryo biopsy there is only a narrow window of time for the diagnosis to be made. As most protocols for CGH require 72 hours for hybridisation alone, two strategies have been proposed. Embryos can be frozen following biopsy and thawed after the CGH
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analysis has been completed (Wilton et al., 2001) or alternatively Wells et al. (2002) tried an accelerated protocol following polar body biopsy on the day of fertilisation.

CGH following cleavage stage biopsy and subsequent cryopreservation of the embryo until the diagnosis is complete has already been applied, and several ongoing pregnancies, and one healthy child have been reported (Wilton et al., 2001). Unfortunately, the benefit of being able to identify chromosomally normal embryos with the use of CGH has to be offset against the current necessity for cryopreservation. In fact, cryopreservation reduces embryo viability and implantation potential by 30% (Edgar et al., 2000), counterbalancing any improvement in implantation gained by PGD-AS (Wells and Levy, 2003). However, Wilton and colleagues (2002) reported that had PGD-AS been applied employing FISH with a five probe set for chromosomes 13, 16, 18, 21, and 22, only 2 of 12 aneuploidies would have been detected, and most importantly two of four aneuploid embryos would have been misdiagnosed as normal. The major limitation of CGH for PGD-AS is, apart from the fact that it cannot provide any information of the ploidy status of the embryo, the analysis takes at least five days to complete because of the long hybridisation time required and the laborious analysis of template chromosomes. These difficulties will be overcome when the template chromosomes are replaced by microarrays where hundreds or thousands of chromosome specific probes are spotted onto a glass slide (Wilton, 2002).

In a recent study applying an accelerated protocol of CGH on first polar bodies (Wells et al., 2002) nine out of ten first polar bodies from a 40 years old IVF patient contained some chromosomal imbalance, further illustrating the reasoning behind PGD-AS for high risk women of advanced reproductive age. Interestingly, seven of ten unbalanced chromosomes were equal to or smaller in size than chromosome 14. The main limitation of this approach is that imbalances arising during male meiosis could not be detected, although they are infrequent compared with those of maternal origin (Wells and Levy, 2003).

1.5.4 PGD of Single Gene Defects

PGD to avoid single gene defects can be performed either by the use of linked markers or by detection of the causative mutation, in the case that the gene has been cloned. The basis of the molecular methods for the detection of single gene defects is
PCR as an efficient means of amplifying a specific region of the genome. Over 8000 human monogenic disorders have been described to date, but the molecular basis is known for only a fraction of these. Initial work to develop PGD for such cases began with the common disorders for which mutation profiles and prenatal diagnostic tests were already well established such as CF and the haemoglobinopathies (Monk et al., 1988; 1993; Monk and Holding, 1990; Liu et al., 1992). The first single gene defect to be diagnosed was cystic fibrosis. This was accomplished using simple heteroduplex analysis to identify unaffected homozygous normal and heterozygous embryos for transfer (Handyside et al., 1992).

Since then increasingly sophisticated PCR-based protocols have been developed and applied for over twenty other single gene defects on a list which is growing steadily along with patient demand and technological advances (Wells and Delhanty, 2001). These include Tay-Sachs disease (Gibbons et al., 1995), Duchenne muscular dystrophy (Lui et al., 1995), Marfan syndrome, (Harton et al., 1996), spinal muscular atrophy (Dreesen et al., 1998), Lesch-Nyhan syndrome (Ray et al., 1999), sickle cell anaemia (Xu et al., 1999) as well as the triplet repeat expansion disorders Huntingtons disease, myotonic dystrophy, Fragile X (Sermon et al., 1998a,b; 1999; Apessos et al., 2001; Piyamonkol et al., 2001) and inherited cancer syndromes, familial adenomatous polyposis coli (FAPC) (Ao et al., 1998), Li-Fraumeni syndrome (Verlinsky et al., 2001) and neurofibromatosis type-2 (NF-2) (Abdou Sleiman et al., 2002). Once the DNA from the single blastomeres has been amplified to a level where analysis is possible a wide variety of mutation detection approaches are available for PGD. Heteroduplex analysis has been used for the mutation detection for the CFTR gene (Handyside et al., 1992), as well as to offer PGD for Tay Sachs disease (Gibbons et al., 1995). For the Lesch-Nyhan syndrome the mutation has been detected in PCR amplified fragments by enzymatic digestion using restriction endonucleases specific to the mutation sites, followed by electrophoresis to reveal cleaved DNA fragments (Ray et al., 1999). Diagnosis of FAP (familial adenomatous polyposis) was available following whole genome amplification via PEP, by using single strand conformation polymorphism (SSCP) a method capable for detecting a wide range of mutations (Ao et al., 1998). Finally, the use of a linked dinucleotide repeat polymorphism was successful for diagnosing Marfan syndrome (Harton et al., 1996).
The most significant problems that are taken into consideration in the most updated PCR protocols include contamination, amplification failure (AF) and allele dropout (ADO) (Wells and Sherlock, 1998; Sermon, 2002). As cellular contamination is liable to come from culture media, the PCR products present in the laboratory environment, but most importantly from extraneous sperm or from maternal cumulus cells; the latter must be carefully removed prior to biopsy, while the use of ICSI reduces paternal contamination (Wells and Delhanty, 2001). Moreover, single-cell PCR should be set up in a DNA-free environment away from the analysis area, which can reduce the chance of ‘carry over’ contamination. Nested PCR was developed to increase sensitivity and specificity (Monk and Holding, 1990) addressing the problem of “carry over” contamination. Nested PCR is widely used in PGD and is based on the use of two sequential amplification reactions in order to enhance the specificity of PCR and reduce the risk of contamination caused by the accidental amplification of DNA fragments (Wells and Sherlock, 1998). Despite all efforts, paternal and maternal contamination has caused misdiagnosis (Sermon et al. 1998; Harper and Delhanty 2000).

Amplification efficiency in the case of single cells is rather low which accounts for anucleate cells, lost genetic material, and cells being biopsied from arrested, fragmented, or poor morphology embryos (Ciu and Matthews, 1996; Ray et al., 1998). Amplification failure (AF) has an occurrence of around 10% during single cell PCR (Kontogianni et al. 1996). The problem of AF of single cell PCR emerged when a misdiagnosis from the first series of PGD for X-linked disorders was reported (Hardy and Handyside, 1992). Protocols relying on reverse transcription of mRNA molecules and PCR (RT-PCR) have been proposed as means of reducing amplification failures (Eldadah et al., 1995).

The most important problem unique to single cell PCR is that of allele dropout (ADO) which allows only one of the alleles present to be successfully amplified (Ray et al., 1994; Findlay et al., 1995a; Ray and Handyside, 1996; Retchinsky et al., 1996; 1998). ADO may lead to misdiagnosis of heterozygous embryos, being the most significant obstacle to diagnosis of dominant disorders in single cells (Grifo et al., 1994; Harper and Handyside, 1994; Verlinsky, 1996). The incidence of ADO varies, however it has been reported to be higher in blastomeres than in any other cell type (Retchinsky et al., 1998). The cause of ADO still remains a mystery, however, suggestions have been made involving imperfect PCR conditions such as increased
denaturation temperature (Ray and Handyside, 1996) or incomplete cell lysis (Ray and Handyside, 1996; El-Hashemite and Delhanty, 1997). Following several reports of misdiagnosis (Grifo et al., 1994; Kuliev et al., 1998; Sermon et al., 1998a), superior PGD protocols utilising stringent safeguards against such an outcome have been employed by several groups. Further modifications were introduced in an attempt to improve the technique, addressing the problem of ADO. Multiplex-PCR was developed claiming to substantially decrease the possibility of misdiagnosis (Lewis et al., 2001), by providing the added assurance of a partial “fingerprint” of the embryo, confirming that the amplified fragment is of embryonic origin (Findlay et al., 1995). If the polymorphisms tested are also linked to the disease locus, and are informative, then a supplemental diagnostic result can be obtained, assisting in cases where amplification of the mutation site has been compromised by ADO (Kuliev et al., 1998; Rechitsky et al., 1998; Ioulianos et al., 2000; Piamongkol et al., 2001b).

As an alternative to multiplex PCR some PGD centres have attempted methods of whole genome amplification to provide sufficient DNA for confirmatory testing (Kristjansson et al., 1994; Ao et al., 1998; Wells et al., 1999). Currently, the use of F-PCR technology (See section 1.2.3.2) is having a wide impact on the PGD of single gene disorders proving to be more sensitive, reliable, accurate, and fewer cycles are required, thereby reducing the time taken to reach diagnosis (Findlay et al., 1996; Sermon et al., 1998). Also, the ADO rate seems to be lower than in conventional PCR techniques (Sermon et al., 1998). Although QF-PCR reliably detects aneuploidies in prenatal samples (Verma et al., 1998), at the single cell level this procedure can be unreliable in approximately 25% of cases due to PA. This results in artificially skewed ratios of PCR products and the potential for misdiagnosis of trisomic di-allelic cells (Sherlock et al., 1998a; Wells and Sherlock, 1998). Additionally methods have been reported for ‘cell recycling’ using sequential PCR and FISH analysis on the same blastomere fixed to a microscope slide, although resulting efficiencies are reported to be lower than for single test protocols (Thornhill et al., 1994; Thornhill and Monk, 1996; Rechitsky et al., 1996).

1.6 Aims and Outline of Study.

This study is comprised of four projects employing molecular and cytogenetic approaches to the analysis of human chromosomes.
The initial objective of this study was to develop reliable FISH-based protocols for the analysis of chromosome abnormalities in cleavage-stage embryos, which could then be applied to PGD referrals involving chromosomal rearrangements. The couples referred to the PGD Centre presented with a severe reproductive history characterised by recurrent spontaneous or induced abortions, abnormal live births, or even primary infertility, due to the chromosomal abnormality carried by one parent.

During the period of this study the growing availability of commercially prepared probes reduced the previously needed time for developing and evaluating suitable FISH probes for PGD strategies, an extremely time consuming and laborious procedure. This in turn allowed an increase in the number of cases that could be offered PGD.

Optimising conditions and investigating the possibility of different probe combinations, therefore allowing diagnosis of complex chromosomal rearrangement cases to be performed, was an important part of this study.

Interphase FISH was applied to the spare untransferred (abnormal or not biopsied) embryos derived from clinical treatment cycles. In addition to confirming the diagnosis, the research was carried out to gather data regarding segregation patterns during meiosis, as well as the mechanisms involved in the formation of mosaic and chaotically dividing embryos. The main results of this work are described in Chapter 3, describing the progression of the project from the initial preliminary work (Part III) through to the clinical application of PGD (Part IV).

The approach of multiprobe interphase FISH analysis requires development of a specific probe combination for each couple. During the final project of this thesis, the use of comparative genomic hybridisation (CGH) was investigated as an alternative, global strategy. This involved assessing the efficiency of CGH, improving the protocol for optimised use on single cells, and its application as a diagnostic tool on human preimplantation embryos.

Prior to this final stage of research two smaller projects were performed as part of the training required for CGH analysis on single cells. Firstly, to gain proficiency at performing PCR, fluorescent PCR and CGH were employed delineating the involvement of the WHS in a patient presenting with several phenotypic characteristics of the syndrome and the following karyotype; 46,XY,der(4)t(4;20)(p16.3;q11.1),i(20)(q11.1). The patient however, did not present with the typical for the syndrome deletion within the WHSCR as supported by
Introduction

cytogenetic studies performed. Therefore, further, molecular refinement was required to examine this case, and based on the literature, the most common polymorphic markers within the WHSCR were selected. The purpose of this study was to delineate the involvement of the WHS by examining the status of these polymorphic markers. CGH analysis was carried out to eliminate the possible involvement of other chromosomal regions in this patient and to determine the extent, if any, of the deletion of the WHSCR in this case. It was hoped that this information might then provide the basis on which to proceed to molecular studies towards gene isolation.

The second smaller project involved molecular cytogenetic analysis of embryonic DNA samples from fetal tissues obtained from social terminations. The aim of this project was to obtain information on the chromosomal status of the fetuses, prior to storage in an embryonic DNA bank. Both the techniques, FISH and CGH were employed in order to attain information with regard to ploidy status and chromosome copy number respectively. Genomic embryonic DNA was provided for the CGH study, while fetal tissue touch-preps were made available for interphase FISH analysis to be performed.
CHAPTER 2

MATERIALS AND METHODS
2.1 Materials

Detailed composition of reagents and solutions is given in Appendix A.

2.1.1 General Reagents and Equipment.

2.1.1.1 Chemicals.

Unless otherwise stated general laboratory chemicals and reagents were obtained from BDH Chemicals UK, Sigma UK and were of Analar or biochemical grade. Solutions and buffers were prepared as described in A.

2.1.1.2 Enzymes.

DNA polymerase I (5-10U/μl) and DNase I (1μg/ml) were obtained from Promega UK and Boehringer Manheim UK respectively. These enzymes were also supplied as components of nick translation kits from Gibco BRL, Boehringer Mannheim and Vysis UK. E. Coli DNA Polymerase (10U/μl) was obtained from New England Biolabs for the labelling of the DNA subjected to CGH. Lyticase for yeast DNA extraction was obtained in desiccated form (made up to stock concentration 25U/μl, 2A.2.5) from Sigma Chemical Company and in solution (3.5U/μl) from Boehringer Mannheim. PCR amplifications were carried out using SuperTaq DNA polymerase (5U/μl) and SuperTaq plus DNA Polymerase with 10x SuperTaq plus Buffer containing Mg supplied by HT Biotechnologies. Proteinase K (50μg/ml) from Boehringer Mannheim, pepsin (10mg/ml) and RNase H (100μg/ml) both from Sigma Chemical Company, used during FISH, were obtained in desiccated form and aliquoted to the working concentrations shown. All were stored at -20°C.

2.1.1.3 Nucleic acids.

Sonicated herring sperm DNA (10mg/ml) and calf thymus DNA (stock concentration 100μg/ml) were supplied by Sigma Chemical company whilst Cot-1 DNA (1μg/ml) and DNA size standards (1kb ladder) were from Gibco BRL. Oligonucleotide primers for PCR (2A.3.2) were obtained from Oswell DNA Service. Unlabelled deoxynucleoside triphosphates (dNTPs; dATP, dCTP, dGTP, dTTP) were supplied by Pharmacia and labelled dNTPs were from Boehringer Mannheim (Digoxigenin-dUTP), Amersham (Fluorored-12-dUTP, Fluorogreen-12-dUTP) and Vysis UK (SpectrumOrange-dUTP, SpectrumGreen-dUTP). All were stored at -20°C.
The DNA size standard 1KbPlus Ladder (100bp-12Kb ladder) was supplied by Gibco BLR. Genesacn-500 {TAMRA} size standard (1-500bp ladder) with TAMRA fluorochrome labelled for the ABI Prism 310 was purchased from Applied biosystems, Warrington England.

2.1.1.4 Cell culture media and equipment.

Cell culture flasks, glass pipettes and microscope slides were obtained from BDH whilst all microcapillaries (internal diameters 75-200μm) for embryo, oocyte and single cell handling were from Laser. Nunc Nucleon 50x9mm Petri dishes were used for single cell isolation and purchased from Gibco BRL. Reagents used for media preparation as detailed in 1A were obtained from Difco Bacto, Sigma Chemical Company and Gibco BRL. Antibiotics and growth supplements supplied in desiccated form were made up to stock concentrations, filter-sterilised and stored at -20°C. Iscove’s modified Dulbeccos medium for lymphocyte culture was obtained from Imperial Laboratories and colcemid (100μg/ml) was from Sigma Chemical Company, both were stored at 4°C. Fetal calf serum supplement was supplied by Gibco BRL and stored at -20°C.

Four models of thermal cyclers were used for PCR amplification. Hybaid Omnigene and Hybaid Touchdown were manufactured by Hybaid Middlesex, GeneAmp2400 PCR System was made and serviced by Applied Biosystems and Mastercycler Gradient was from Eppendorf-Netheler-Hinz GmbH, Cambridge. The analysis of fluorescent PCR was performed on the ABI Prism 310 using Genescan analysis software. The dissecting microscope used for single cell isolation and oocyte spreading was the product of Nikon as was the phase contrast microscope employed to check slide preparations.

2.1.2 PGD materials

2.1.2.1 Ethical approval

The preliminary work on surplus embryos and the clinical application of PGD were approved by the Research Ethics Committees of the University College London Hospital Trusts, and carried out under licence from the Human Fertilisation and Embryology Authority. Informed written consent was obtained from patients for surplus embryos to be used for research purposes.
2.1.2.2 Patient details

Forty four couples at risk of chromosomally unbalanced pregnancy due to parental chromosome rearrangement were initially referred for PGD; thirty with balanced reciprocal translocations, seven with balanced Robertsonian translocations, four with other structural chromosomal rearrangements, one with suspected mosaicism for a trisomic cell line and two for duplications and deletions. Mean maternal age was 35.2 years (SD ±3.95 years), range 25 to 45 years. Table 2.1 lists the patient referrals. The reproductive histories of these couples are shown in Table 2.2 and summarised in figure 2.1.

Table 2.1 Details of forty four patients referred for PGD of chromosomal abnormalities.

<table>
<thead>
<tr>
<th>CASE</th>
<th>KARYOTYPE</th>
<th>PROBE COMBINATION PROPOSED FOR WORK UP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reciprocal Translocations</td>
</tr>
<tr>
<td>1</td>
<td>46,XX,t(2;21)(q24.3;q21.2)</td>
<td>2 α-sat O<del>2qter R</del>21q22.2 G</td>
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<tr>
<td>2</td>
<td>46,XY,t(1;4)(p36.1;p14)</td>
<td>4 α-sat A<del>1 α-sat R</del>1pter G</td>
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<tr>
<td>3</td>
<td>46,XX,t(X;16)(q25;q22)</td>
<td>X α-sat G<del>16 sat-II O</del>16qterR</td>
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<tr>
<td>4</td>
<td>46,X,t(Y;1)(q.11.23;q12)</td>
<td>1 α-sat G<del>Y satIII A</del>Y α-sat R~1qter R</td>
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<tr>
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<tr>
<td>6-D</td>
<td>46,XY,t(5;19)(p12;p12)</td>
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<tr>
<td>7</td>
<td>46,XX,t(2;17)(q33;p13)</td>
<td>2 α-sat O<del>17 α-sat G</del>2qter R</td>
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<tr>
<td>8</td>
<td>46,XY,t(1;7)(p11;q10)</td>
<td>1 α-sat O~7 α-sat G</td>
</tr>
<tr>
<td>9-J</td>
<td>46,XX,t(12;22)(q24.1;q11)</td>
<td>12 α-sat O<del>22q11 R</del>22q13 G</td>
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<td>46,XX,t(1;18)(q41;q23)</td>
<td>1 α-sat O<del>18 α-sat G</del>1qter R</td>
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<td>11</td>
<td>46,XY,t(10;13)(q22;q10)</td>
<td>10 α-sat G~13qter R</td>
</tr>
<tr>
<td>12-K</td>
<td>46,XY,t(8;9)(q24.3;q21.2)</td>
<td>8 α-sat G<del>9 α-sat O</del>8qter R</td>
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<tr>
<td>13-C</td>
<td>46,XX,t(16;17)(p13.3;p11.1)</td>
<td>16 sat-II O<del>17p12 R</del>17q21.1 G</td>
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<tr>
<td>14</td>
<td>46,XY,t(2;8)(q37.3;q23.1)</td>
<td>2 α-sat O<del>8 α-sat G</del>8qter R</td>
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<tr>
<td>15</td>
<td>46,XY,t(1;4)(p31.2;q31.3)</td>
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</tr>
<tr>
<td>16</td>
<td>46,XX,t(2;14)(q32.1;p15)</td>
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<tr>
<td>17-A</td>
<td>46,XX,t(5;11)(q34;q25)</td>
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<tr>
<td>18-H</td>
<td>46,XY,t(1;18)(p32;q23)</td>
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<tr>
<td>19</td>
<td>46,XX,t(4;11)(q31.1;q21)</td>
<td>4 α-sat A<del>11 α-sat G</del>11qter R</td>
</tr>
<tr>
<td>20-B</td>
<td>46,XX,t(1;12)(q42.1;p23)</td>
<td>2 α-sat G<del>1 α-sat O</del>1qter R</td>
</tr>
<tr>
<td>21</td>
<td>46,XX,t(11;22)(q23.3;q11.2)</td>
<td>11 α-sat O<del>22q11 R</del>22q13 G</td>
</tr>
<tr>
<td>22</td>
<td>46,XY,t(X;1)(q24;q32.3)</td>
<td>X α-sat G<del>1 α-sat O</del>1qter R</td>
</tr>
<tr>
<td>23-G</td>
<td>46,X,X.t(8;12)(q11.2;q12)</td>
<td>12 α-sat O<del>8 α-sat G</del>8qter R</td>
</tr>
<tr>
<td>24</td>
<td>46,XY,t(5;7)(q35.3;q36)</td>
<td>7 α-sat O<del>5p15.2 G</del>7qter R</td>
</tr>
<tr>
<td>25</td>
<td>46,XX,t(6;7)(q27;p12)</td>
<td>7 α-sat O<del>6 α-sat G</del>7qter R</td>
</tr>
<tr>
<td>26</td>
<td>46,XX,t(6;10)(q25.1;q26.13)</td>
<td>10 α-sat O<del>6 α-sat G</del>6qter R</td>
</tr>
<tr>
<td>27</td>
<td>46,XY,t(1;4)(q14.1;p12)</td>
<td>1 α-sat G<del>4 α-sat A</del>1qter R</td>
</tr>
<tr>
<td>28</td>
<td>46,XY,t(14;16)(q13;q11.1)</td>
<td>16 sat-II O<del>14qter R</del>16qter G</td>
</tr>
</tbody>
</table>

Materials and Methods
### Materials and Methods

<table>
<thead>
<tr>
<th>CASE</th>
<th>REPRODUCTIVE HISTORIES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reciprocal Translocations</td>
</tr>
<tr>
<td>1</td>
<td>2 spontaneous abortions</td>
</tr>
<tr>
<td>2</td>
<td>6 spontaneous abortions, 1 normal live birth</td>
</tr>
<tr>
<td>3</td>
<td>1 TOP for chromosome abnormality &amp; 3 spontaneous abortions</td>
</tr>
<tr>
<td>4</td>
<td>Primary infertility</td>
</tr>
<tr>
<td>5</td>
<td>1 spontaneous abortion</td>
</tr>
<tr>
<td>6-D</td>
<td>Primary infertility</td>
</tr>
<tr>
<td>7</td>
<td>1 spontaneous abortion</td>
</tr>
<tr>
<td>8</td>
<td>3 spontaneous abortions</td>
</tr>
<tr>
<td>9-J</td>
<td>1 chromosomally abnormal livebirth</td>
</tr>
<tr>
<td>10</td>
<td>9 spontaneous abortions, 1 normal livebirth</td>
</tr>
<tr>
<td>11</td>
<td>Primary infertility</td>
</tr>
<tr>
<td>12-K</td>
<td>Primary infertility</td>
</tr>
<tr>
<td>13-C</td>
<td>4 spontaneous abortions</td>
</tr>
<tr>
<td>14</td>
<td>2 spontaneous abortions, 2 TOP for chromosome abnormality</td>
</tr>
<tr>
<td>15</td>
<td>1 spontaneous abortion</td>
</tr>
<tr>
<td>16</td>
<td>Primary infertility</td>
</tr>
<tr>
<td>17-A</td>
<td>1 normal livebirth, 1 abnormal livebirth, 2 TOP for chromosome abnormalities</td>
</tr>
</tbody>
</table>

R: Red fluorescence
G: Green fluorescence
O: Orange fluorescence (combination of red and green fluorescence)
A: Aqua fluorescence
All the cases proceeding to PGD are represented by capital letters.

### Table 2.2. Reproductive histories of forty four patients referred for PGD of chromosomal abnormalities.
### Materials and Methods

<table>
<thead>
<tr>
<th>Case</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-H</td>
<td>2 spontaneous abortion</td>
</tr>
<tr>
<td>19</td>
<td>1 normal livebirth secondary infertility</td>
</tr>
<tr>
<td>20-B</td>
<td>2 spontaneous abortions, 1 TOP for social reasons</td>
</tr>
<tr>
<td>21</td>
<td>2 spontaneous abortions, 1 TOP for chromosome abnormalities</td>
</tr>
<tr>
<td>22</td>
<td>Primary infertility</td>
</tr>
<tr>
<td>23-G</td>
<td>5 spontaneous abortions</td>
</tr>
<tr>
<td>24</td>
<td>3 TOP for fetal abnormalities</td>
</tr>
<tr>
<td>25</td>
<td>3 spontaneous abortions</td>
</tr>
<tr>
<td>26</td>
<td>1 TOP (ectopic), 4 spontaneous abortions</td>
</tr>
<tr>
<td>27</td>
<td>8 spontaneous abortions</td>
</tr>
<tr>
<td>28</td>
<td>Primary infertility</td>
</tr>
<tr>
<td>29</td>
<td>1 chromosomally abnormal livebirth</td>
</tr>
<tr>
<td>30</td>
<td>Primary infertility</td>
</tr>
<tr>
<td>31</td>
<td>3 spontaneous abortions</td>
</tr>
<tr>
<td>32-I</td>
<td>1 spontaneous abortion, 1 TOP for chromosome anomaly</td>
</tr>
<tr>
<td>33</td>
<td>Primary infertility</td>
</tr>
<tr>
<td>34</td>
<td>3 spontaneous abortions</td>
</tr>
<tr>
<td>35-E</td>
<td>Primary infertility</td>
</tr>
<tr>
<td>36</td>
<td>1 spontaneous abortion</td>
</tr>
<tr>
<td>37</td>
<td>4 spontaneous abortions</td>
</tr>
<tr>
<td>38</td>
<td>2 chromosomally abnormal livebirths</td>
</tr>
<tr>
<td>39</td>
<td>1 chromosomally abnormal livebirth</td>
</tr>
<tr>
<td>40-F</td>
<td>1 normal livebirth, 1 spontaneous abortion, 1 TOP for chromosome abnormality</td>
</tr>
<tr>
<td>41</td>
<td>Primary infertility</td>
</tr>
<tr>
<td>42</td>
<td>2 chromosomally abnormal livebirths, 1 TOP for trisomy 21</td>
</tr>
<tr>
<td>43</td>
<td>1 normal livebirth, 1 TOP for chromosome abnormality</td>
</tr>
<tr>
<td>44</td>
<td>1 normal livebirth, 2 chromosomally abnormal livebirths</td>
</tr>
</tbody>
</table>

### Robertsonian Translocations

- 31: 3 spontaneous abortions
- 32-I: Primary infertility
- 33: Primary infertility
- 34: 3 spontaneous abortions
- 35-E: Primary infertility
- 36: 1 spontaneous abortion
- 37: 4 spontaneous abortions

### Other Structural Rearrangements

- 38: 2 chromosomally abnormal livebirths
- 39: 1 chromosomally abnormal livebirth
- 40-F: 1 normal livebirth, 1 spontaneous abortion, 1 TOP for chromosome abnormality
- 41: Primary infertility

### Mosaicism for a Trisomic Cell Line

- 42: 2 chromosomally abnormal livebirths, 1 TOP for trisomy 21

### Duplications/Deletions

- 43: 1 normal livebirth, 1 TOP for chromosome abnormality
- 44: 1 normal livebirth, 2 chromosomally abnormal livebirths
2.1.23 Preimplantation embryos

As part of the work up for PGD for chromosomal rearrangements 64 human preimplantation embryos were obtained for analysis from 19 patients undergoing routine IVF/CSI (mean maternal age 35.8 [SD ±3.9] years, range 28 to 45 years). Thirty-five embryos (245 blastomeres) were normally fertilised (presence of two pronuclei observed 18hr: post-insemination) and scored as grade I, II or III on day three post-insemination whilst twenty-nine embryos were either arrested at the 2- to 4-cell stage by late day three post-insemination or had resulted from abnormal fertilisation (presence of one or three pronuclei). An additional one hundred and fourteen embryos resulting from thirteen IVF/ICSI cycles carried out for PGD were analysed to assess the levels of chromosome abnormalities, and to obtain information about meiotic segregation patterns in the patient group studied.

2.1.24 Grading criteria for the embryos.

Preimplantation embryos were graded according to Bolton et al., (1989) as follows;

*Grad 1*  Embryo at the correct stage of in-vitro development with perfect symmetrical and even-sized blastomeres with no fragmentation
Materials and Methods

Grade 1  Embryo at the correct stage of in-vitro development with perfect symmetrical and even-sized blastomeres with less than 10% fragmentation.

Grade 2\textsuperscript{*}  Development with unequally sized blastomeres with less than 20% fragmentation

Grade 2  Retarded development with unequally sized blastomeres with 25%-50% fragmentation.

Grade 3  Retarded development with unequally sized blastomeres with more than 50% fragmentation.

2.1.2.5 Categorisation of chromosomal abnormalities in embryos.

Embryos analysed for the preliminary work and during PGD cycles were categorised after Delhanty et al., (1997) into four groups; normal, uniformly abnormal, mosaic (diploid mosaic or aneuploid mosaic) and chaotic. Embryos were allocated where possible to each group on the basis of the chromosome constitution of the majority of cells present.

*Normal* - Embryo uniformly normal for the chromosomes tested.

*Uniformly Abnormal* - Embryo uniformly abnormal for the chromosomes tested.

*Diploid mosaic* - Majority of embryo euploid but one or a few cells differ (i.e. aneuploid, polyploid or haploid).

*Aneuploid or polyploid mosaic* - Majority of embryo uniformly aneuploid or polyploid but one or a few cells differ.

*Chaotic* - Chromosome constitution varies randomly from cell to cell and status of original zygote cannot usually be determined.

As chaotic embryos were frequently encountered in embryos derived from PGD patients. Three further sub-categories were noted.

*Fully chaotic* - Chromosome constitution varies randomly from cell to cell and no normal/balanced cells or aneuploid cells lines are noted.

*Chaotic/Aneuploid* - Majority of embryo is chaotic but an aneuploid cell group is present.

*Chaotic/Diploid* - The embryo is chaotic but a normal or balanced group of cells is present.
**Materials and Methods**

2.1.2.6 DNA probes.

Details of DNA probes used in this study along with their sources are summarised in Table 2.3. All of the yeast artificial chromosome (YAC) clones as well as plasmid DNA clones for chromosomes 1, 2, 3, 4, 6, 10, 12, 15, 16 were obtained from resource centres as agar stabs. Maxiprep of plasmid and cosmid DNA was carried out with Wizard maxiprep kit from Promega. Probe DNA was labelled via nick translation using either reagents prepared from laboratory stocks or those supplied in kit form (Nick Translation Kit Vysis UK). Commercially obtained labelled α-satellite and locus-specific probes were supplied by Appligene Oncor, Cytocell and Vysis UK. All were stored at -20°C and protected from light.

<table>
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<tr>
<th>PROBE (Label*)</th>
<th>CYTOGENETIC POSITION</th>
<th>SOURCE/REFERENCE</th>
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<tr>
<td>PUC1.77</td>
<td>Satellite-III 1</td>
<td>Cooke et al., (1979)</td>
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<tr>
<td>CEP 1 (SO/SG)</td>
<td>α-satellite 1</td>
<td>Vysis UK</td>
</tr>
<tr>
<td>TelVysion 1q (SO)</td>
<td>1qter</td>
<td>Vysis UK</td>
</tr>
<tr>
<td>TelVysion 1p (SG)</td>
<td>1pter</td>
<td>Vysis UK</td>
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<td>BA193J5</td>
<td>1q24</td>
<td>Frengen et al., (1999)</td>
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<td>α-satellite 2</td>
<td>Appligene Oncor</td>
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<td>α-satellite 2</td>
<td>Rocchi et al., (1990)</td>
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<tr>
<td>pZ311</td>
<td>α-satellite 3</td>
<td>Archidiacono et al., (1995)</td>
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<tr>
<td>TelVysion 2q (SO)</td>
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<td>Vysis UK</td>
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<tr>
<td>TelVysion 1p (SG)</td>
<td>2pter</td>
<td>Vysis UK</td>
</tr>
<tr>
<td>CEP 4 (SO/SA)</td>
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<td>P4n1/4</td>
<td>α-satellite 4</td>
<td>D' Aiuto et al., (1993)</td>
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<td>LSI D5S721/D5S23 (SG) &amp; EGR1 (SO)</td>
<td>5p15.2 &amp; 5q31</td>
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<td>D6Z1 (Dig)</td>
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<td>pEDZ6</td>
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<tr>
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<td>bA179A23</td>
<td>8q12</td>
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<td>pZ1013</td>
<td>α-satellite 10</td>
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### Materials and Methods

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<tr>
<td>CEP 11 (SO/SG)</td>
<td>α-satellite 11</td>
<td>Vysis UK</td>
</tr>
<tr>
<td>TelVysion 11q (SO)</td>
<td>11qter</td>
<td>Vysis UK</td>
</tr>
<tr>
<td>pBR12</td>
<td>α-satellite 12</td>
<td>Baldini et al., (1990)</td>
</tr>
<tr>
<td>bA434C1</td>
<td>12p12</td>
<td>Frengen et al., (1999)</td>
</tr>
<tr>
<td>LSI 13 RBI (SG)</td>
<td>13q14</td>
<td>Vysis UK</td>
</tr>
<tr>
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<td>13qter</td>
<td>Vysis UK</td>
</tr>
<tr>
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<td>14qter</td>
<td>Vysis UK</td>
</tr>
<tr>
<td>CEP 16 (SO/SG)</td>
<td>β- satellite 16</td>
<td>Vysis UK</td>
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<td>16qter</td>
<td>Appligene Oncor</td>
</tr>
<tr>
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<td>17p12 &amp; 17q21.1</td>
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<td>CEP 18 (SA/SG)</td>
<td>α-satellite 18</td>
<td>Vysis UK</td>
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<tr>
<td>b484C16</td>
<td>19q13.2</td>
<td>Frengen et al., (1999)</td>
</tr>
<tr>
<td>pZ20</td>
<td>α-satellite 20</td>
<td>Baldini et al., (1992)</td>
</tr>
<tr>
<td>LSI D21S259, D21S341, D21S342 (SO) &amp; LSI 21q22.13-q22.2 &amp; 13q14</td>
<td>Vysis UK</td>
<td></td>
</tr>
<tr>
<td>bCO67E3</td>
<td>21q11.2</td>
<td>Frengen et al., (1999)</td>
</tr>
<tr>
<td>TEL21q (R/G)</td>
<td>21qter</td>
<td>Appligene Oncor</td>
</tr>
<tr>
<td>LSI N25 D22S75 (SR) &amp; LSI 22q13 (SG)</td>
<td>22q11.2 &amp; 22q13</td>
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<td>pDMX1</td>
<td>α-satellite X</td>
<td>Archidiacono et al., (1995)</td>
</tr>
<tr>
<td>CEP Y DYZ3 (SO)</td>
<td>α-satellite Y</td>
<td>Vysis UK</td>
</tr>
<tr>
<td>CEP Y DYZ1 (SA)</td>
<td>Satellite-III Y</td>
<td>Vysis UK</td>
</tr>
</tbody>
</table>


Rocchi, D’ Aiuto, Baldini, Archidiacono, Cytogenetic Unit, University of Bari, Italy.

Frengen, Children’s Hospital Oakland, Research Institute Oakland, USA

### 2.1.2.7. FISH reagents.

FISH solutions and buffers are given in 2A.5. All stocks were stored at -20°C with short term aliquots stored at 4°C. Tween 20 detergent (polyoxyethylene sorbitan monolaurate), Poly-L-lysine adhesive solution and the DNA counterstain 4',6-diamidino-2-phenylindole (DAPI) were supplied by Sigma Chemical Company and...
the anti-fade mounting medium Vectorshield by Vector Laboratories; all were stored at 4°C.

2.1.2.8. Microscopy and Image analysis.

Dissecting microscopes from Nikon and inverted microscopes from Olympus were used for embryo and oocyte handling as well as slide preparation. Fluorescence microscopy was carried out with the following microscope systems; Reichert Jung Polyvar microscope with single filters for TRITC, FITC and DAPI, Nikon optiphot microscope with Omega dual band-pass TRITC/FITC filter and Zeiss Axioskop microscope with Chroma multi-band pass TRITC/FITC/DAPI filter and single SpectrumAqua filter. Image capture and analysis was carried out using a Zeiss Axioskop microscope equipped with a Photometries KAF 1400 cooled CCD (charged coupled device) camera controlled by Smartcapture software from Vysis, UK.

2.1.3 Materials for The Wolf Hirschhorn Syndrome (WHS) project

2.1.3.1 Patient

The 19 year old male patient referred to the UCLH cytogenetics unit (by Dr. Flyn, Royal Free Hospital), was described as having some features of WHS. The complex phenotype, not corresponding to a particular syndrome involved skeletal anomalies, dysmorphic facial features including laterally upsplanting eyebrows, striking nose, low hairline, and large low set paosteriorly rotated ears. Other congenital abnormalities included a small right kidney and glandular hypospadias. He had moderate learning difficulties but attended a normal school. Growth was normal with a final height on the 25th centile. Certain phenotypic characteristic such as the “Greek helmet” appearance implied the involvement of the WHS. Hence, molecular and cytogenetic work was commenced to identify the anomalies in this case.

Initially, the karyotype that was determined by standard G banding involved a reciprocal translocation between chromosomes 4 and 20. The origin of this abnormality was unknown as both parents had a normal karyotype. DNA was obtained from the patient the patient’s family (mother, father, sister), and from control individuals. Polymorphic markers mapping on 4p were employed to investigate any possible association with the WHS, by determining the extent, if any, of the deletion of the WHSCR. While the molecular studies were in progress, cytogenetic studies carried out by The Royal Free Haematology Department revealed a rare chromosome
Materials and Methods

rearrangement involving pure trisomy 20p resulting from isochromosome formation associated with a non-reciprocal translocation involving 20q and chromosome 4. At that point the patient’s karyotype was identified as 46,XY,der(4)t(4;20)(p16.3;q11.1),i(20)(q11.1).

2.1.3.2 Polymorphic markers and primers.

Three polymorphic markers were selected (D4S169, D4S43, D4S127) all physically mapping to 4p16.3 which encompasses the WHCR, in order to determine whether there is a deletion within the WHCR in the patient. D4S169 is a dinucleotide repeat, highly variable and likely to be informative being the most distal marker. D4S43 is a variable nucleotide tandem repeat (VNTR) and is situated within the critical region for WHS. Finally, D4S127 is a dinucleotide repeat polymorphism which according to several WHS cases that have been reported can either flank or be included in the WHCR. In this case it was selected as a marker that is at the proximal end of the WHCR. Therefore, two of the markers were likely to flank the WHCR (D4S127, D4S169) and one was situated within it (Gusella et al., 1992).

The primers required were obtained from PE- Applied Biosystems UK and were already published (Pritchard et al., 1991; Horn et al., 1991; Taylor et al., 1992). The forward primer for each marker was 5’-end fluorescently labelled. Table 2.4 shows the sequence the chromosome location and the optimal annealing temperature for each primer.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Sequence of primers</th>
<th>Chrom. location</th>
<th>Annealing Temp.</th>
<th>Fluorescent label</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4S169(F)</td>
<td>5’-GAATTCCA GTTTAGC GTAGCTAG G-3’</td>
<td>4p</td>
<td>65</td>
<td>FAM-Blue</td>
<td>Pritchard et al., 1991</td>
</tr>
<tr>
<td>D4S169(R)</td>
<td>5’-GAATTCAG TCGACTGA GAATCCCTTT -3’</td>
<td>4p</td>
<td>65</td>
<td></td>
<td>Pritchard et al., 1991</td>
</tr>
</tbody>
</table>
Materials and Methods

2.1.4 CGH materials

2.1.4.1 The Wolf Hirschhorn Syndrome Project

DNA obtained from the patient used in the molecular refinement study was also used to perform CGH as the final part of this project to detect any chromosome abnormalities related to this case, and to confirm the formation of isochromosome 20p.

2.1.4.2 Molecular Cytogenetic analysis of Fetal DNA samples (ICH Project)

In collaboration with the Institute of Child Health, (London) DNA and touch preparations derived from fetal tissues from terminations of pregnancies for non-medical reasons were provided for cytogenetic analysis prior to inclusion in an embryonic tissue bank. The collection of all fetal tissues for this study had maternal consent and local ethical committee approval.

- Touch preparations

Fetal tissues from different organs were directly dabbed on a clean slide using the cut surface. The cells were fixed on the slides using methanol and acetic acid. A total of 58 slides (from 58 aborted fetuses) were made available and all were subjected to FISH employing the Vysis UK AneuVysion set screening for

<table>
<thead>
<tr>
<th>D4S43 (F)</th>
<th>5'- GACCACAG AGAGCTTA GTGGAGCT T-3'</th>
<th>WHCR</th>
<th>55</th>
<th>TET-Green</th>
<th>Horn et al., 1991</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4S43(R)</td>
<td>5'- GACCACCTT CACTGACA TCCCATCT TTT-3'</td>
<td>WHCR</td>
<td>55</td>
<td></td>
<td>Horn et al., 1991</td>
</tr>
<tr>
<td>D4S127(F)</td>
<td>5'- CCTCTGT TT GCAATCCA TTT-3'</td>
<td>WHCR-end</td>
<td>52.8</td>
<td>TET-Green</td>
<td>Taylor et al., 1992</td>
</tr>
<tr>
<td>D4S127(R)</td>
<td>5'- GTCCCTTGC ATGCCCTG GCT-3'</td>
<td>WHCR-end</td>
<td>52.8</td>
<td></td>
<td>Taylor et al., 1992</td>
</tr>
</tbody>
</table>
chromosomes X(SG), Y(SO), and 18(SA) in order to assess the ploidy status of the samples. To ensure probe efficiency a normal male lymphocyte culture was prepared, to be used in each experiment as described in a following section.

-**Extracted DNA**

A total of 46 DNA samples of known concentration were made available from the Institute of Child Health and subjected to CGH in order to obtain copy number information on all the chromosomes of the respective fetal tissues.

### 2.1.4.3 Buccal cells, Frozen Fibroblasts and Human Blastomeres

Genomic DNAs extracted from frozen trisomic fibroblasts were employed as positive controls in assessing genomic CGH protocol efficiency. Single cell isolation was performed for buccal cells and fibroblasts to provide practise, and the positive controls in assessing single cell CGH protocol efficiency. Prior to performing the CGH study on the embryos of the selected couples undergoing PGD, three day 3 spare embryos were donated from an infertile couple undergoing an IVF cycle with frozen embryos at the Assisted Conception Unit (ACU) of University College Hospital (UCH) London. These embryos were biopsied and their blastomeres were tubed and subjected to CGH to provide practise at CGH of single blastomeres.

The surplus embryos chosen for the main CGH study were donated by couples carrying a chromosomal rearrangement undergoing PGD (Table 2.1 A-case 17 3\textsuperscript{rd} cycle; E-case 35; F-case 40; G-case 2; and H-case 18). The patient reproductive histories, karyotypes, and fertility status and outcome of the cycles performed at the ACU of UCH London are given in Table 3.44. Following PGD and embryo transfer the spare embryos were again biopsied based on morphological criteria and a further 1-2 blastomeres were retrieved. The remainder of each embryo was spread and subjected to FISH.

### 2.1.4.4 Microscopy and image analysis for CGH.

Metaphase chromosome preparations were photographed using a Zeiss Axioscope microscope equipped with a Photometrics KAFF 1400 cooled CCD camera, and SmartCapture software (Vysis Richmond,UK). Image analysis was performed using Vysis Quips CGH software. Green:Red fluorescence ratios of >1.2:1 indicated gain of genetic material, while ratios of <0.8:1 was indicative of deletions.
2.2 Methods

2.2.1 Cell Culture and Cytogenetic Preparation

2.2.1.1 Peripheral lymphocyte culture and harvest.

A standard synchronised culture method employing thymidine was used to produce extended chromosome preparations from peripheral lymphocytes of controls, patients and their partners. Large scale cultures with 1ml of blood were set up to ensure sufficient material. Harvest was performed employing 0.075M KCL hypotonic solution and 3:1 methanol:acetic acid to fix the cells.

2.2.1.2 Harvest of skin fibroblasts.

In the early part of this study the probes were additionally tested on triploid XXY fibroblast cells to assess efficiency of trisomy detection. Two triploid fibroblast cell cultures were available. Following colcemid treatment one was treated with hypotonic solution prior to fixing cells allowing visibility of metaphase spreads. The second culture was treated with PBS, which resembled the embryo treatment during spreading employing 1% Tween. The aim was to compare efficiency of detection in nuclei treated by the different methods.

For the CGH study, in addition to single cells and clumps of trisomic cells isolated to provide the positive controls for the CGH experiments, skin fibroblast cultures were obtained from fetuses with normal karyotypes as well those with trisomy 13, 14, 18, 21 and 22. These were used to prepare DNA for use in the preliminary work performed for the single cell CGH study.

In the DNA preparation, the cell culture medium was poured off, and the cells were washed with 3-5 ml of Hanks medium (Gibco BRL #14170-070), prior to adding 3-5 ml of trypsin/versene solution (Gibco BRL #15040-033) to detach the monolayer. When most of the cells were detached 5 mls of PBS (2A.1.1) were added, and the cells were transferred to centrifuge tubes and spun at 6,000g for 5 minutes, and then washed 2-3 times using PBS. The cell suspension could be used for single cell isolation (see section 2.2.6.1) or for DNA extraction (see section 2.2.5.1).

2.2.1.3 Slide preparation of Blastomeres.

Slides of embryonic nuclei were prepared from whole day three and/or four post-insemination embryos and biopsied blastomeres using the method developed by
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Harper et al., (1994) and Coonen et al., (1994a). Adhesive coated slides were used in this air-drying method to ensure cells remain attached to the slide until lysis and air-drying are complete. These were prepared by immersing clean slides in a coplin jar of tissue adhesive solution (0.01% Poly-L-Lysine in deionised water) for 5 minutes at room temperature before air-drying overnight, after which storage was at 4°C. The embryo or biopsied blastomere was washed in phosphate buffered saline (PBS) for 2 minutes to remove traces of medium or oil before transfer to a small drop (approximately 10-20μl) of spreading solution (0.01N HCl, 0.1% Tween 20) on a Poly-L-Lysine coated slide, the position marked on the underside beforehand with a diamond pen. The slide was then transferred to an inverted microscope allowing the embryo to be constantly observed whilst a microcapillary primed with spreading solution was used to gently agitate the spreading solution drop. This was continued until the zona pellucida had dissolved (whole embryos), blastomeres had lysed and all nuclei were clear of cytoplasm. Slides were air-dried, washed in PBS for 5 minutes and dehydrated through an ethanol series (70%, 90%, 100% 5 minutes each). Slides were stored covered at room temperature for a maximum of two weeks prior to analysis. Finally, the location of nuclei was recorded using an England Finder (Optech, UK), and the slides were stored for up to 2 weeks at room temperature.

2.2.1.4 Slide preparation of unfertilised oocytes.

Unfertilised oocytes were cytogenetically prepared 48 h post insemination. The oocytes were kept in a 37°C 5%CO₂ incubator. The method used was a modified version of the Tarkowski (1996) protocol. Under an inverted microscope and using a microcapillary the oocyte was transferred from medium and incubated for 6 minutes in a small petri dish containing 1% sodium citrate hypotonic solution. The oocyte was placed on a slide marked using a diamond marker in a drop (2μl) of freshly prepared fixative I (5:4:1 bidistilled water:methanol:acetic acid). The oocyte was incubated for 5 seconds until the zona pellucida dissolved. When fixative I nearly evaporated but not dried out, a drop (13 μl) of fixative II (3:1 methanol:acetic acid) was gently dropped using a Gilson pipette from a height of one centimetre in order to ensure good chromosome spreads minimising any cell breakage. Following the spreading and retraction of the fixative II another drop was added from 3 cm above the slide. The same process was repeated 4-5 times to conclude the fixation process. Slides were dehydrated through an ethanol series (70%, 90%, 100% 5 minutes each) air-
dried and stored at 4°C. Prior to FISH performed on the slides DAPI staining was performed to distinguish analysable metaphases. Oocytes were located using a fluorescent microscope and co-ordinates were taken with the use of the England Finder.

2.2.1.5 Slide preparation of Sperm.

The samples were washed with 5ml of PBS and centrifuged for 10 minutes at 3000g. The supernatant was removed and the pellet was washed twice as described above. Finally, the supernatant was removed and 10 ml of fresh fix (fix: methanol 3: Acetic acid 1) was added and left to fix the cells at room temperature for 10 minutes. The samples were centrifuged at 3000g for 10 minutes and stored at 4°C or -20°C. The slides were prepared as for standard lymphocyte slides (reference) and dehydrated through an ethanol series. The slides underwent decondensation treatment in order for the sperm heads to swell making the DNA more accessible to the probes. They were incubated at room temperature for 30 minutes in 50ml of fresh 10mM DTT (Sigma) + 0.05 M Tris solution. This incubation was followed by 30 minutes in 2XSSC, and dehydration through an ethanol series. The slides could be stored at this stage at room temperature. Standard FISH procedure was carried out, excluding the pepsin and paraformaldehyde steps.

2.2.1.6 G-banding.

G-banding of peripheral lymphocyte chromosome preparations from couples referred for PGD was carried out in cases where no previous cytogenetic reports were available. Karyotyping was carried out by the Clinical Cytogenetics Department, UCH. Karyotypes were re-analysed in cases where chromosomal breakpoints mapped by FISH differed from those stated on the supplied cytogenetic reports.

2.2.2 Preparation and selection of DNA probes

2.2.2.1 Bacterial culture

Plasmid and cosmid DNA for FISH probes was amplified in recombinant bacterial strains and extracted using either miniprep or maxiprep techniques. For both approaches a small starter culture was initiated by inoculation with a single bacterial colony into 4 ml of 2xTY medium (1A.2), supplementing the appropriate antibiotic
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(100μg/ml ampicillin or 25μg/ml kanamycin). The culture was left overnight in a shaking incubator at 37°C. The following day this culture was re-inoculated into 200 ml of 2xTY medium supplemented with the appropriate antibiotic, and was again incubated overnight as above before DNA extraction. Glycerol stocks (1A.3) were prepared at this time and stored at -70°C.

2.2.2.2 Miniprep extraction of plasmid/cosmid DNA

The extraction techniques used relied on controlled alkaline lysis, neutralisation and SDS treatment to selectively denature bacterial chromosomal DNA, which was then precipitated along with proteins and RNA. Circularised plasmid or cosmid DNA present in the cleared lysate was then isolated. For miniprep extraction, a 10ml bacterial culture in a universal tube was centrifuged at 5,000g for 7 minutes before the pellet was resuspended in 300μl of GTE (Appendix 2A.2.1). The cell suspension was transferred into 2.2ml Eppendorf tubes and mixed by inverting several times after adding 600 μl of denaturation solution (Appendix 2A.2.2) freshly made at room temperature. The appearance of the lysate should appear viscous and should not be lysed more than 5 minutes. Five hundred μl of 7.5M ammonium acetate was added and mixed immediately by inverting several times. The suspension was left on ice for 10 minutes and inverted several times during the incubation period. The suspension was spun at 13000rpm for 20 minutes and the supernatant was poured into fresh 2.2ml Eppendorf tubes. A second centrifugation at 10,000g for 10 minutes was required for the supernatant to be clear and poured into fresh 2.2ml Eppendorf tubes before 700μl of isopropanol was added and mixed by inversion. The suspension was centrifuged at 10,000g for 20 minutes, the supernatant was discarded and the pellet washed with 500μl of 70%EtOH before it was centrifuged again at 10,000g for 5 minutes. The supernatant was discarded and the pellet not allowed to dry but was instantly resuspended in 100μl of TE by tapping the tubes. Treatment with RNAse at 37°C for 30 minutes (100ug/ml) followed before the DNA was precipitated with 1/10th volume of NaAc and 3 volumes 100% ethanol. The samples were incubated at -20°C for 20 minutes and centrifuged at 10,000g for 15 minutes. Finally, the pellet was washed with 70% ethanol prior to being resuspended in the appropriate volume of TE (2A.1.4)
2.2.2.3 Maxiprep extraction of plasmid/cosmid DNA

A larger scale maxiprep extraction of plasmid and cosmid DNA was performed using a commercial maxiprep kit (Wizard; Promega, USA) according to the manufacturers instructions. A 200ml overnight bacterial culture was pelleted by centrifugation at 5,000g for 10 min. The supernatant was discarded and the cells resuspended into 15 ml of cell resuspension solution (Appendix 2A.2.3). Subsequently, 15 ml of denaturation solution were added and mixed gently but thoroughly, by stirring or inverting. When the cellular mixture became clear 15 ml of neutralization solution (Appendix 2A.2.4) were added, and immediately mixed by gently inverting the centrifuge bottle several times. The suspension was centrifuged at 10,000g for 15 minutes at 22-25°C in a room temperature rotor. The supernatant was filtered through blotting paper and transferred into a 100 ml graduated cylinder. After its volume was measured, the supernatant was transferred to a new centrifuge bottle. Half a volume of isopropanol was added to this supernatant, and mixed by inversion. The suspension was centrifuged at 10,000g for 15 minutes before the supernatant was discarded and the DNA pellet resuspended in 2 ml TE buffer. The DNA purification was achieved with the use of the Wizard resin and vacuum pump.

2.2.2.4 Yeast culture and miniprep extraction of yeast DNA method 1.

YAC DNA for FISH probes was obtained from total genomic yeast DNA that was amplified and extracted from appropriate recombinant yeast strains. The YAC was seeded in 5-10 mls of YPD (Appendix 1A.4) (50 µg/l ampicillin) and incubated under aseptic conditions for 48 hours at 30°C in a stirrer. The yeast cultures in universal tubes were centrifuged for 5 minutes at 3,000g, the supernatant was discarded and the pellet was resuspended in 0.5mls 1M sorbitol-0.1M EDTA (pH7.5) and transferred in an Eppendorf tube to which 7 µl of lyticase (10,000U/ml) had been added. The mixture was incubated at 37°C for 30 minutes or until 80-90% of the cells were spheroplasts, the timing dependent on the yeast strain. The percentage of spheroplasts was assessed by spreading 10µl of culture onto a microscope slide and checking under a phase contrast microscope. Dark specks indicated the appearance of spheroplasts whilst intact cells appeared translucent. The suspension was centrifuged at 10,000g for 1 minute, the supernatant removed and the pellet was resuspended in 0.5 ml of 50mM Tris-HCl (pH7.4)-20mM EDTA. Fifty µl of SDS 10% were added and mixed at 65°C for 30 minutes before 0.2 mls of 5M potassium acetate were added.
and the mixture was placed on ice for an hour. the tubes were again centrifuged at 10,000g for 5 minutes and the supernatant was transferred into a fresh microcentrifuge tube. An equal volume of isopropanol was added, gently mixed and left for 5 minutes at room temperature. The tubes were spun briefly at 10,000g for 10 seconds, the supernatant was discarded, the pellet was left to air dry and subsequently resuspended in 300μl of 1xTE. The DNA suspension was treated with 15μl of RNase A 1mg/ml at 37°C for 30 minutes after which 30μl of sodium acetate were added and mixed. An equal volume of phenol was added and mixed vigorously before tubes were spun at 1200rpm for 5 minutes. The upper aqueous layer containing the DNA was transferred to a fresh microcentrifuge tube and a second phenol extraction was performed using the same method. An equal volume of chloroform was added and mixed vigorously before tubes were spun at 3,000g for 5 minutes. The upper layer was transferred to a fresh tube to which a 2.5 volume of 100% ethanol was added mixed vigorously and the tubes were placed at -70°C for one hour. DNA was recovered by centrifuging at 10,000g for 10 minutes, the supernatant was discarded and the pellet resuspended in 100μl 1xTE buffer and stored at -20°C.

2.2.2.5 Yeast culture and miniprep extraction of yeast DNA method 2.

If discrete colonies were not available, SD agar plates (Appendix 1A.5) were prepared supplemented with 100μg/ml ampicillin and inoculated from glycerol stock suspension or confluent yeast growth, under aseptic conditions. Plates were incubated inverted at 30°C for 48 hours or until discrete colonies developing a pink coloration were identified. The colour change indicates the disruption of a yeast insert acting as a marker for clones containing an intact YAC. A single pink recombinant colony was used to inoculate a 4ml volume of SD medium (Appendix 1A.6) supplemented with 100μg/ml ampicillin in a 10ml universal tube. Cultures were incubated at 30°C with constant agitation for 48 hours before DNA extraction. Glycerol stocks were prepared at this time and stored at -70°C. A sterile needle was used to scrape some of the frozen stock and inoculate it into 10 ml of S.D. medium (Appendix 1A.6). The liquid cultures were incubated in a shaker at 30°C for three days.

The DNA extraction was performed with the use of the “nucleon extraction and purification kit” for yeast DNA extraction (Amersham LIFE SCIENCE, UK). The protocol was as follows. Each of the 10 ml cultures was separated into 1.5 ml Eppendorf tubes, which were spun for 30 seconds at 10,000g. The supernatant was
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discarded, and the cells were resuspended by mixing with 540 µl of Solution A. When each of the pellets were completely diluted 60 µl of Solution B were added and mixed thoroughly by rapid inversion. These tubes were incubated at 70°C for 10 minutes before 300 µl of Solution C were added and mixed by shaking. The tubes were placed on ice for 3-5 minutes. The samples were centrifuged at 10,000g for 5 minutes. The supernatant was collected and 600 µl of isopropanol was added to precipitate the DNA. Again the samples were centrifuged at 10,000g for 5 minutes, the supernatant was discarded, and 200 µl of cold 70% ethanol was added. The samples were again centrifuged at 10,000g for 5 minutes, and each tube was drained and allowed to dry by incubation at 70°C for 3 minutes. Finally, 50 µl of Solution D was added and the Eppendorfs were placed at 70°C. The samples were mixed periodically, until the DNA was completely resuspended. The resuspended DNA was stored at 4°C.

2.2.3.6 Fluorometry

The concentration of plasmid, cosmid and YAC DNAs was measured by using a Hoefer Scientific Instruments TKO 100 fluorometer. The fluorometer was calibrated using 100ng/ml calf thymus DNA as a standard and a fluorometry solution containing an intercalating DNA dye (0.1µg/ml Hoechst 33258 dye in 1xTNE buffer). Readings for sample DNA concentrations were converted to ng/µl.

2.2.2.7 Alu-PCR

To selectively amplify the human insert of the YAC vector from the total genomic yeast DNA extracted from yeast cells, PCR was used with human specific Alu oligonucleotide primers (Alu-PCR). PCR amplifications were set up on ice using dedicated Gilson pipettes in a laminar flow cabinet to limit extraneous DNA contamination. A standard 50µl reaction as described by Romana et al., (1993) was set up in a microcentrifuge tube containing 100ng total genomic yeast DNA template, 25pM of each Alu primer (Appendix 2A.3.2), 0.2mM dNTPs (dATP, dGTP, dCTP, dTTP), 5µl 10x PCR Buffer (Appendix 2A.3.1) and 0.5 units SuperTaq polymerase, the volume adjusted with sterile water. This mix was overlaid with mineral oil and PCR cycling was carried out using a Hybaid Omnigene PCR machine. Two different sets of cycles were used for the amplification and these were the following: Initial denaturation at 95°C for 5 minutes, and then 30 cycles of PCR with denaturation at
95°C for 1 minute, annealing at 65°C for 1 minute, and extension at 72°C for 4 minutes. At the end of the last cycle a 10 minute extension took place at 72°C. The second set of PCR cycles involved an initial denaturation at 96°C for 1 minute, and then 30 cycles of PCR with denaturation at 96°C for 1 minute, annealing at 40°C for 30 seconds, and extension at 72°C for 6 minutes. At the end of the last cycle a 10-minute extension at 72°C took place. DNAs were stored at -20°C.

2.2.2.8 Agarose gel electrophoresis.

Products of DNA amplification were visualised by agarose gel electrophoresis to assess PCR efficiency. Agarose gels were prepared by melting 2% agarose in 1xTBE buffer (Appendix 2A.1.3) to which was added 1μg/ml ethidium bromide. The molten agar was poured into a gel tank with gel-slot formers and left to set at room temperature before immersing in 1xTBE buffer. DNA samples were prepared in 10μl volumes and mixed with 1μl loading buffer (Appendix 2A.1.6) before loading into individual gel wells. Electrophoresis was carried out at 50V for 30 minutes after which gels were viewed via ultra-violet trans-illumination.

2.2.2.9 Labelling of probe DNA for FISH

DNA probes for FISH were labelled via nick translation. During this process DNase I introduces random nicks in DNA which are used as priming sites for DNA polymerase I to synthesis new DNA, incorporating a labelled nucleotide as it degrades the old strand. The procedure enables the incorporation of about 20% of the fluorescent-labelled nucleotide into the DNA, generating in this way a clear bright signal during hybridization. Ethanol precipitation removes the unincorporated nucleotides.

Using reagents provided by the Vysis, UK nick translation kit and according to the manufacturers instructions, a 50μl nick translation reaction was set up in a microcentrifuge tube on ice with 1μg probe DNA, 0.2mM each dATP, dCTP, dGTP, 0.1mM dTTP, 0.2mM Spectrum Green-dUTP or SpectrumRed-dUTP, 5μl 10x nick translation buffer (Appendix 2A.4.2) and 10μl nick translation enzyme (Appendix 2A.4.3) This mix was incubated at 15°C for two hours before the reaction was stopped by the addition of 30mM EDTA. Labelled DNAs were stored protected from light at 4°C.
2.2.2.10 Probe preparation for FISH

Labelled DNA was ethanol precipitated in the presence of x50 fold excess of herring sperm carrier DNA, 1/10 volume of 3M sodium acetate and 2.5 volumes of 100% cold ethanol in a microcentrifuge tube. For locus-specific probes, x100 fold excess of unlabelled human Cot-1 competitor DNA was also included to block non-specific repetitive sequence in the probe DNA. The tubes were placed at -70°C for one hour, before centrifugation at 10,000g for 10 minutes. The supernatant was discarded and the pellet was freeze dried for 10 minutes before resuspending in hybridisation mix (Appendix 2A.5.3, 2A.5.4). Locus-specific probes were resuspended in 50% formamide, 10% dextran sulphate in 2xSSC whilst α-satellite probes were resuspended in 60% formamide, 10% dextran sulphate in 2xSSC to give stock concentrations of 80ng/μl and 20ng/μl respectively. Working concentrations per slide were 20ng/μl for locus-specific probes and 5ng/μl for α-satellite probes. All probes were stored at -20°C.

2.2.3 Fluorescent In Situ Hybridisation Procedures

2.2.3.1 Slide pretreatments

Slide pretreatment included proteolytic enzyme to increase the accessibility of probes to nuclei as well as an additional fixation. The method of FISH performed was described previously by Harper et al (1994b). Slides were first dehydrated through an ethanol series (70%, 90%, 100% 5 minutes each) and air-dried. Slides were incubated in 100μg/ml pepsin in 0.01N HCl at 37°C for 20 minutes and rinsed briefly in bi-distilled water followed by PBS. Cells were fixed in 1% paraformaldehyde in PBS at 4°C for 10 minutes and briefly rinsed in PBS followed by two rinses in bi-distilled water, before dehydrating and air-drying as before.

In order to restore the chromosome morphology following the treatments, the slides were incubated in 50 ml of 2x SSC (5ml 20x SSC {Appendix 2A.1.2}, 45 ml H2O) (20x SSC: 3M sodium chloride, 0.3M tri sodium citrate, pH 7.2) at 37°C for 30 min, after the dehydration following the paraformaldehyde incubation. The slides were dehydrated again and left to dry.
2.2.3.2 Probe preparation slide denaturation and hybridisation

Simultaneous denaturation

A 5 μl probe mix was prepared according to the source and the type of the probes. The hybridisation mix consisted of different hybridisation buffers and volumes depending on the types of probes used. The hybridisation buffers suggested to use for commercial probes were the CEP, and LSI buffer (Vysis, UK). The hybridisation buffer used for the YAC probes was cosmix (50% deionized formamide, 2x SSC and 10% dextran sulphate) and for laboratory prepared satellite probes cosmix with 60% formamide concentration was employed. The probe combinations in the hybridisation mix were directly applied to the pre-treated dehydrated slides in a final 5μl volume under a 13mm diameter coverslip before denaturing both simultaneously at 75°C for 5 or for 3 minutes or at 73°C for 5/3 minutes. These were hybridised at 37°C in a moist chamber as described previously by Harper et al. (1994). Minimum hybridisation times were approximately 45 minutes for centromeric probes and 16 hours for locus-specific probes. If the hybridisation time exceeded two hours the coverslips were sealed with rubber cement to prevent drying.

Separate denaturation

Using the separate denaturation method, denaturation of nuclear DNA on the slide was achieved by applying 70% formamide in 2xSSC under a coverslip at 75°C for 5 minutes, after which coverslips were removed and slides were immediately immersed in a pre-chilled coplin jar containing 70% ethanol at -20°C for 5 minutes. Slides were dehydrated through an ethanol series (70%, 90%, 100% 5 minutes each) and air-dried preserving the single-stranded DNA conformation. Locus-specific probe DNA in hybridisation mix was denatured separately in a microcentrifuge tube at 75°C for 5 minutes and left to pre-anneal at 37°C for a minimum of 30 minutes with the Cot^{-1} DNA. This occurred as locus specific probes contain high amounts of Cot^{-1} DNA, which blocks the repetitive sequences and prevents cross-hybridisation. The incubation at 37°C for 30-60 min. activates the Cot^{-1} DNA and makes the probes more specific. When co-hybridisation of locus-specific and centromeric probes was required, the centromeric probe was denatured in the same way in a separate microcentrifuge tube but without a pre-annealing step and placed on ice. Probes were combined just prior to being applied to the slide in a final 5μl or 10μl volume under a coverslip (13mm or 22 mm respectively) and left to hybridise at 37°C in a moist
chamber. Minimum hybridisation times were approximately 45 minutes for centromeric probes and 16 hours for locus-specific probes. If the hybridisation time exceeded two hours the coverslips were sealed with rubber cement to prevent drying.

2.2.3.3 Post-hybridisation.

The formamide concentration, salt concentration and temperature were the variants according to which the stringency of probe binding was controlled during the post-hybridisation washes. Therefore conditions were dependent on probe type. All washes were carried out in 50ml volume coplin jars with those containing formamide restricted to a laminar flow cabinet and slides were protected from light at all stages in cases where fluorochrome labelled probes were used. After hybridisation any rubber cement was removed and coverslips were gently floated off by immersing briefly in the first wash solution. For combinations of probes including locus-specific probes, slides were treated at 45°C with 3 x 3 minutes washes in 50% formamide in 2xSSC and then 3 x 3 minutes washes in 2xSSC followed by a 5 minute wash in SSCT (Appendix 2A.5.5) at room temperature. When only repetitive probes were used the stringency was raised by increasing the formamide concentration to 60% in the 2xSSC solution. All other washes were as above.

According to the Vysis, (UK) protocol any unbound probe was removed by washing the slides for 10 minutes at 45°C in 70% formamide/2xSSC pH 5.3 (three times), 10 minutes in 2xSSC pH 7.0 at 45°C and then 5 minutes in 0.05% 2xSSC/0.1% NP40 while at 45°C.

An alternative rapid hot wash formamide-free protocol was employed in some cases allowing stringency to be controlled without the use of formamide. Slides including locus-specific probes were treated post-hybridisation by one wash in 0.4xSSC/0.3%NP-40 at 73°C± 2°C for 2 minutes followed by one wash in 2XSSC/0.1%NP-40 for 3 minutes at room temperature. As this method is highly temperature sensitive a maximum of four slides were processed simultaneously to avoid a drop in temperature associated with non-specific probe binding. For this reason the formamide method being less sensitive to temperature fluctuations was preferred for post-hybridisation washes during PGD.

The slides were left to dry in the dark and mounted in Vectorshield antifade medium (Appendix 2A.5.6) and stored covered at 4°C.
2.2.3.4 Reprobing of slides

The coverslip was removed with care by two 10 minute washes in SSCT. The slide was washed for another 10 minutes in PBS and finally dehydrated through an ethanol series (70%, 90%, 100% 5 minutes each) and air-dried. No additional slide pre-treatment was carried out before the new probe was applied to the slide. All subsequent FISH steps remained the same.

2.2.3.5 Scoring criteria and FISH analysis.

The examination and scoring of the slides was carried out using a fluorescent Olympus (BX-40) microscope, which was fitted with a photometrics cooled CCD camera utilising pathVysion software (Digital Scientific, Cambridge, UK). Two hundred interphase nuclei were scored in order to calculate probe efficiency as part of the PGD work-up. All embryonic blastomeres analysed were located using an England Finder.

FISH probe signals in interphase nuclei were scored following Hopman et al., (1988) such that two signals closer than a signals diameter apart were considered a single split signal and those further apart than this were considered two separate signals.

2.2.4 PGD

2.2.4.1 PGD consultation

Prior to commencing treatment all patients were fully informed regarding the limitations of PGD. The requirement for IVF treatment, the risk of misdiagnosis, the problems caused by mosaicism, the expected implantation and pregnancy rates, were all outlined during two thorough IVF/PGD consultations.

2.2.4.2 IVF treatment and manipulation of embryos.

The patients underwent routine IVF procedures as described previously (Ranieri et al., 2001). Following ovarian stimulation follicles were aspirated and fertilisation was evaluated 24h after insemination. Oocytes and embryos were cultured in IVF medium (Cook Australia).

2.2.4.3 PGD performed

PGD was performed for nine patients; A-case 17 (three cycles), B-case 20, C-case 13, D-case 6, E-case 35 (two cycles), F-case 40, G-case 23, H-case 18 and I-case
32 (two cycles). On day 3 embryos were biopsied in Ca\textsuperscript{2+} Mg\textsuperscript{2+}-free embryo biopsy medium (Medicult UK), using Research Instrument micromanipulators. Zona drilling was performed using acid Tyrode’s solution as described previously (Piyamongkol et al., 2001a). One or two blastomeres were aspirated according to the developmental stage and morphology of the embryos. In general two cells were biopsied from embryos consisting of 6 or more cells. For the first four PGD cycles prior to biopsy embryos were subjected to 10 minutes pre-incubation in Ca\textsuperscript{2+} Mg\textsuperscript{2+}-free embryo biopsy medium. For the following cycles performed no such pre-incubation took place. Biopsied cells and untransferred embryos were spread according to Harper et al (1994). As described in section 2.2.1.3 untransferred embryos were spread on day 4 or 5 and analysed with the diagnostic probe set to assess the accuracy of the original diagnosis and to obtain information on mosaicism and meiotic segregation.

**Methods relevant to CGH study**

**2.2.5 DNA Extraction**

**2.2.5.1 DNA extraction from Frozen fibroblasts**

Several cell lines (Trisomy 13, 14, 18, 21, 22 and normal) were subjected to DNA extraction to be subsequently used as positive controls for preliminary studies to assess efficiency of the genomic CGH protocol. Cell suspensions were centrifuged at 1000rpm for 5 minutes, and the supernatant containing glycerol was removed. The cells were washed with Hank’s medium (Sigma) and centrifuged at 6,000g for 5 minutes. The supernatant was removed and 2.5ml of lysis buffer I (Appendix 2A.6.1) warmed at 37°C was added and the cells were left at 37°C for 30 minutes. An equal volume of isopropanol was added in order to precipitate the DNA which was “hooked out” and dissolved in water.

**2.2.5.2 DNA extraction from blood.**

To obtain DNA from blood the following protocol was used (Laird et al., 1991). The blood was collected in sodium EDTA tubes. 5ml of blood was placed in a centrifuge tube and 5ml of low salt buffer TKM1 (Appendix 2A.6.2) was added and 125µl of Novidet P-40 was added to lyse the cells. The cells were mixed by inversion and centrifuged at 1,000g for 10 minutes. The supernatant was slowly poured off and the small nuclear pellet was saved. The pellet was washed repeatedly as before until the redness of the pellet was reduced. The pellet was resuspended in a drop of TKM1,
0.8ml of TKM2 (Appendix 2A.6.3) was added together with 50μl of 10% SDS and mixed thoroughly. The tube was firmly sealed with nescofilm (BDH, UK) and incubated at least 20 minutes at 55°C until “lumps” have disappeared. 300μl of 6MNaCl was added and well mixed. The cells were spun at 10,000g for 5 minutes, the supernatant was saved and the precipitated protein pellet was discarded. TwoVolumes of 100% ethanol was added to the supernatant at room temperature and the tube was inverted until the DNA was precipitated. The precipitated strands were removed with an inoculation loop and 1ml of ice-cold ethanol 70% was added and they were microfuged at 10,000g for 5 minutes. The pellet was dried and resuspended in 200μl of H₂O of TE.

2.2.6 Single cell isolation

2.2.6.1 Single cell isolation, tubing and lysis for buccal and fibroblast cells.

Isolation of cell clumps and single cells isolation was performed for normal buccal cells and fibroblasts to provide practice prior to handling embryos, as well as to obtain positive controls for the single cell CGH study. Several fibroblast cell lines were available, trisomy 13, 14, 18, 21 and normal male and female.

Fifty μl of the original concentrated cell suspension was placed on a 5cm petri dish. Another Petri dish was used to dilute this original concentrated sample until the cell isolation was achieved. A minute volume of cell suspension of approximately 5μl was taken through a series of PBS/0.1%PVA drops that were placed in the Petri dish. The transfer to an adjacent drop of PBS/0.1%PVA (Sigma, UK) was performed using a pulled glass micropipette, while visualising under a dissecting microscope. The single cells, once isolated, were transferred in and out of at least three fresh PBS drops to wash away any contaminants. The single cells were transferred into an individual thin-wall microcentrifuge tubes containing 3μl lysis buffer II containing proteinase K (Appendix 2A.6.4) that was prepared prior to the single cell isolation. Once the single cell was transferred into the lysis buffer, the mixture was covered with a drop of light mineral oil to prevent contamination and evaporation before closing the lid. A clump of 30-50 cells was taken as a positive control for each PCR. Also, 2μl of the last drop was taken as a blank (negative control) for each single cell.
The proteinase K was activated at 37°C for 1h, and inactivated by incubating at 99°C for 15 min. The lysed cells were stored at -70°C.

2.2.6.2 Isolation and tubing of single human blastomeres

Human blastomeres were provided following informed patient consent, from day 3 or day 4 donated spare embryos from certain PGD and standard IVF/ICSI cycles. All spare embryos of PGD patients were biopsied and 1-2 blastomeres were obtained prior to spreading the remainder of the embryo for FISH analysis. The blastomeres biopsied were selected based on good morphological appearance. All other embryos included in the CGH study were disaggregated. Using a pulled micropipette, the embryo to be disaggregated was placed in a drop of acid Tyrodes until the zona disappeared. The embryo was transferred to a prepared Petri dish with PBS drops. Using a pulled microcapillary of smaller diameter than the one employed to transfer the embryo the embryo was mechanically disaggregated while pipetting in and out. The single blastomeres derived from desegregation or biopsy were subsequently washed through several drops to remove excess contaminants such as cumulus cells or sperm, before they were transferred into individual thin-wall microcentrifuge tubes (0.2μl Eppendorf tubes, Eppendorf, UK) containing 3μl lysis buffer II containing proteinase K (Appendix 2A.6.4). 2μl of the last wash drop was taken as a blank for each single blastomere. The lysed cells were stored at -70°C.

2.2.7 Comparative Genomic Hybridisation procedures

2.2.7.1 Comparative Genomic Hybridisation (CGH)- METHOD 1

2.2.7.1.1 Degenerate oligonucleotide primed PCR (DOP-PCR I)

Two 50μl mixtures were prepared; one for the normal (reference DNA) and the other for the test DNA. The PCR mix included 5μl of 10X buffer (HT Biotechnology), 0.2mM deoxynucleoside triphosphates (dATP, dGTP, dCTP, dTTP), 2.0μM DOP primer CCGACTCGAGNNNNTGGGG (Telenius et al., 1992) and 2.5U Taq polymerase (HT Biotechnology). The mixture was overlaid with oil and denatured at 94°C for 9 minutes. This preceded 8 cycles of 94°C for 1 minute, 30°C for 1.5 minutes and 72°C for 3 minutes followed by 25 cycles of 94°C for 1 minute,
Materials and Methods

62°C for 1 minute and 72°C for 1.5 minute. Finally, an 8 minute extension was undertaken at 72°C.

2.2.7.1.2 DOP II (Labelling of probe DNA for CGH)

5 µl of the reaction product of DOP I was transferred to a fresh microfuge tube containing 5 µl of PCR buffer, 0.2 mM dNTPs, 2.0 µM DOP primer and 2.0 Taq polymerase in a total volume of 50 µl. If labelling the DNA for CGH only 0.1 mM dTTP was included in the reaction mixture and 2.5 µl of fluorescein-11-dUTP or rhodamine-4-dUTP (Amersham) was added. After overlaying with oil the mixture was heated to 94°C for 4 minutes followed by 25 cycles of 94°C 1 minute, 62°C 1 minute, 72°C 1.5 minutes and a final incubation at 72°C for 8 minutes.

2.2.7.1.3 Preparation of labelled DNA for CGH

The chromosomally normal (reference) and the aneuploid (test) DNA samples, labelled in different colours, were precipitated together in the presence of Cot-1 DNA and 10 µg of Salmon sperm DNA. The DNA pellet was dried before being resuspended in 10 µl of hybridisation mixture (50% deionised formamide, 20% w/v dextran sulphate, 2XSSC, 0.1 mM EDTA, pH8, 0.2 mM tris-HCl). Labelled DNA samples dissolved in hybridisation mixture were denatured at 75°C for 10 minutes and allowed to cool at a 37°C water bath. Probes were maintained at this temperature for at least 30 minutes before being applied to denatured chromosome spreads.

2.2.7.1.4 CGH slides

Normal male metaphase spreads were prepared according to standard protocols and aged for approximately three days at room temperature.

2.2.7.1.5 Prehybridisation treatment of metaphase chromosome spreads.

Slides were washed in PBS for 5 minutes at room temperature, dehydrated through an ethanol series (70%, 90%, 100% ethanol for 5 minutes each) and air-dried. The slides were treated with 100 µg/ml RNase A in 2XSSC and incubated for an hour at 37°C in a humidified chamber. The RNase A was removed with two washes in 2XSSC at room temperature, each lasting 5 minutes. Slides were washed in proteinase K buffer (2A.7.1) at 37°C for 5 minutes, before a 7 minute treatment again at 37°C with proteinase K (50 ng/ml in proteinase K buffer). Following a brief immersion in
Materials and Methods

magnesium chloride buffer (2A.7.2) the slides were fixed with paraformaldehyde
magnesium chloride buffer (2A.7.3) for 10 minutes at room temperature. The slides
were washed in PBS, sent through an ethanol series as before and left to dry.

2.2.7.1.6 Denaturation of metaphase chromosome spreads and probe
hybridisation

Denaturation of the slides was achieved by applying 100 µl of denaturation
solution (2A.7.4) under a coverslip, and heating the slides in an oven at 75°C for 5
minutes. Immediately after denaturation the coverslips were removed and the slides
were washed in 70% ethanol chilled to -20°C. This preceded passage through another
alcohol series and drying. Finally the probe prepared and denatured as previously
described was added to the slides under a coverslip, and the coverslip sealed with
rubber cement. Hybridisation of the probe proceeded over 24 or 72 hours during
which time the slides were kept in a humidified chamber at 37°C.

2.2.7.1.7 Post-hybridisation washing

After hybridisation the slides were given three 10 minute washes in 50%
formamide/2XSSC at 45°C. These were followed by two washes in 2XSSC at the
same temperature and one wash in 2XSSC at room temperature. The slides were
placed in TNT (2A.7.5) with 0.1% Tween 20 detergent for 10 minutes before being
washed in distilled water for another 10 minutes. The slides were dehydrated by 5
minute washes in a series of ethanol and air dried. Finally, they were mounted in anti­
fade medium to counterstain chromosomes and nuclei.

2.2.7.2 Comparative Genomic Hybridisation (CGH)- METHOD 2

2.2.7.2.1 Degenerate oligonucleotide primed PCR (DOP-PCR I)

For the 50 µl PCR reaction 46 µl of a stock solution were added to achieve a
final concentration of 2.5IU SuperTaq Plus (Enzyme technologies Ltd, Cambridge,
UK), 2 µM DOP primer CCGACTCGAGNNNNNTGGGG (Telenius et al.,
1992), 1X SuperTaq Plus buffer (containing magnesium) and 0.2mM dNTPs. The
thermal conditions were as follows: 94°C for 4.30 minutes; eight cycles of 95°C for
30 seconds, 30°C for 1 minute, and 72°C for 3 minutes at a ramp of 1.0; 35 cycles of
95°C for 30 seconds, 56°C for 1 minute, 72°C for 1.30; and finally 72°C for 8 minutes.
Two μl of the DOP-PCR amplified DNA were run on an agarose gel to assess the efficiency of DOP-I and to exclude any incidence of contamination.

2.2.7.2.2 Labelling of probe DNA for CGH-METHOD 2

The DNA product of DOP-I was ethanol precipitated redissolved in water and labelled by nick translation. The 50μl reaction contained 5μl of 10X reaction buffer mix (2A.7.6), 5μl A4 Nucleotide mixture (2A.7.7), 1μl of 1mM Hapten/fluorochrome-dUTP, 2.5μl DNA Pol (10U/μl) and 1μl DNase a different range of DNase concentrations (0.01-0.1U/μl) were tried to see which gives the best fragment size on an agarose gel. The time for the nick translation was 60 minutes at 15°C. The reaction was stopped with a 10 minute incubation at 72°C.

2.2.7.2.3 Preparation of labelled DNA for CGH- METHOD 2

A 1% agarose gel was run to check the fragment sizes. Optimal sizes for CGH were estimated between 300-2000bp. If fragment sizes were too long a further incubation at 15°C with DNase I was performed. The reference DNA was labelled with Spectrum Red (Vysis, UK) and the test DNA was labelled with spectrum Green (Vysis, UK). The appropriate DNA to be hybridised against the single blastomere was a group of 3 buccal cells that had undergone the same treatment as the single blastomeres and were amplified during the same DOP-I-PCR experiment. Labelled reference and test DNA were mixed with 30μg Cot-1 DNA and ethanol precipitated and washed with 70% ethanol and air-dried. The pellet was resuspended in 6μl Hybridisation mix (50% formamide; 2XSSC; 10%Dextran Sulphate) and dissolved by 20 minutes incubation at 37°C. The probe was denatured at 75°C for 10 minutes and cooled at 37°C by incubation for 2 minutes at room temperature in the dark before being applied to the denatured normal chromosome spreads as described below.

2.2.7.2.4 CGH slides

Prepared as described for method 1.

2.2.7.2.5 Prehybridisation treatment of metaphase chromosome spreads.

Prepared as described for method 1.
2.2.7.2.6 Denaturation of metaphase chromosome spreads and probe hybridisation

Denaturation of the slides was performed by a 5 minute incubation of the slides in a coplin jar with denaturation solution (70% formamide; 2XSSC pH 7.5) prewarmed at 73°C. Immediately after denaturation the slides were put through an ice-cold (chilled at -20°C) ethanol series (70%, 85%, 100%) and dried. The probe was applied to the slide and covered with a coverslip sealed with rubber cement and hybridised in a moist chamber at 37°C for 72 hours.

2.2.7.2.7 Post-hybridisation washing

The post hybridisation washes consisted of 5 minutes in 2X SSC at 72°C, 5 minutes in 4X SSC at 37°C, 5 minutes in 4X SSC + 0.1% Triton-X (Sigma, UK) at 37°C, 5 minutes in 4X SSC at 37°C, and 5 minutes in 2X SSC at room temperature followed by dipping of the slides in double-distilled water. The slides were put through an ethanol series 3 minutes each, air-dried and mounted in anti-fade medium (Vector Labs, Ptereborough, UK) containing diamidinophenylindole (DAPI) to counterstain the chromosomes and nuclei.

2.2.7.2.8 Karyotyping

Basic karyotyping skills as well as knowledge of human cytogenetic nomenclature were acquired. The different cells karyotyped had been stained with G-banding techniques, and the exercise involved the recognition of both normal and chromosomally abnormal cells, carrying insertions, isochromosomes, ring chromosomes and reciprocal translocations.

2.2.7.2.9 Microscopy and image analysis

Metaphase chromosome preparations were captured using a Zeiss Axioscope microscope equipped with a Photometrics KAFF 1400 CCD camera, and SmartCapture software (Vysis Richmond, UK). Image analysis was performed using Vysis Quips CGH software. Green:Red fluorescence ratios of >1.2:1 indicated gain of genetic material, while ratios of <0.8:1 was indicative of deletions.
2.2.8 Methods relevant to the Wolf Hirschorn Syndrome project

2.2.8.1 Fluorescent Polymerase Chain Reaction (F-PCR)

The technique of labelling the primers used in the amplification reaction on the 5’ end with the fluorescent dyes allowed detection of PCR products on an automated laser fluorescence sequencer. Primers were tagged with 6’FAM TET or HEX fluorochromes for the ABI prism. The PCR was performed in a 25µl reaction volume. This consisted of 0.2 mM dNTP’s, 2.5µl 10x Taq polymerase buffer, 1-2U Taq polymerase, the amount of the primers added varied according to the concentration of the stock solutions, and the amount of genomic DNA added varied according to the concentration of the DNA mix. Deionised sterile water was added to make up the mix to 25 µl. If a “cold” start was performed, the components of the reaction mix were assembled on ice and transferred to the thermal block. In the case of a “hot” start and in order to prevent unspecific elongations the DNA was denatured in the absence of Taq, which was added to the mix after the first denaturation cycle. The reaction mix was overlaid with a drop of sterile mineral oil (Sigma, UK) in order to avoid evaporation.

The number of cycles, and thermal cycling parameters were determined according to the primers and DNA content and were changed numerous times in order for the optimisation of each reaction to be achieved. The following table (Table 2.5) shows the PCR conditions that were considered to be optimal for each set of primers.

Table 2.5 Optimal PCR conditions for each primer.

<table>
<thead>
<tr>
<th>Polymorphic markers</th>
<th>Temperature</th>
<th>Stage</th>
<th>Duration in min.</th>
<th>No of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4S169</td>
<td>95 C</td>
<td>Denaturation</td>
<td>4’ 30’’</td>
<td>1</td>
</tr>
<tr>
<td>D4S169</td>
<td>95 C</td>
<td>Denaturation</td>
<td>45’’</td>
<td>35</td>
</tr>
<tr>
<td>D4S169</td>
<td>65 C</td>
<td>Annealing</td>
<td>45’’</td>
<td>35</td>
</tr>
<tr>
<td>D4S169</td>
<td>72 C</td>
<td>Extention</td>
<td>45’’</td>
<td>35</td>
</tr>
<tr>
<td>D4S169</td>
<td>72 C</td>
<td>Final extention</td>
<td>10’</td>
<td>1</td>
</tr>
<tr>
<td>D4S43</td>
<td>94 C</td>
<td>Denaturation</td>
<td>4’ 30’’</td>
<td>1</td>
</tr>
<tr>
<td>D4S43</td>
<td>94 C</td>
<td>Denaturation</td>
<td>20’’</td>
<td>28</td>
</tr>
<tr>
<td>D4S43</td>
<td>55 C</td>
<td>Annealing</td>
<td>20’’</td>
<td>28</td>
</tr>
<tr>
<td>D4S43</td>
<td>74 C</td>
<td>Extention</td>
<td>20’’</td>
<td>28</td>
</tr>
</tbody>
</table>
### 2.2.5.2 Fluorescent PCR analysis using ABI Prism 310.

The analysis of PCR products using primers labelled with fluorescent dyes was carried out on the ABI prism 310 employing a capillary electrophoresis system with a special polymer (Performance Optimised Polymer 4, POP-4). The amplified products were loaded on to the mediator and electrophoresed through it. During electrophoresis, a laser stream passed through the mediator. When the fluorescent PCR fragments pass through the laser, the fluorochromes attached to the fragments were excited and emitted photons of specific wavelength. The signal was picked up by the CCD (charged couple device) detector and interpreted by the computer software. A mixture of 1 μl of each fluorescent PCR product, mixed with 12 μl of deionised formamide and 0.3 μl of size standards (Genescan 500-TAMRA) were prepared in a 0.5ml sample tube without a lid. The tube was capped with rubber septa, denatured at 95°C for 5 minutes and loaded on to a 48-tube sample rack in the ABI prism 310 sequencer. The denatured sample was subjected to capillary electrophoresis using Performance Optimised Polymer 4 (POP-4; 5 sec injection time, 15,000V, 60°C, 24 minutes). The data was analysed by Genescan analysis software.

### 2.2.5.3 Agarose gel electrophoresis.

Products of DNA amplification were visualised by agarose gel electrophoresis to assess PCR efficiency. Agarose gels were prepared by melting 2% agarose in 1xTBE buffer to which was added 1μg/ml ethidium bromide. The molten agar was poured into a gel tank with gel-slot formers and left to set at room temperature before immersing in 1xTBE buffer. DNA samples were prepared in 10μl volumes and mixed with 1μl loading buffer before loading into individual gel wells. Electrophoresis was
carried out at 50V for 30 minutes after which gels were viewed via ultra-violet trans-illumination.
CHAPTER 3
RESULTS
Chapter 3

Results – Part I PGD preliminary work
3.1 Selection and Evaluation of DNA Probes for FISH.

The majority of the probes used in this study were commercially available including centromeric probes, locus specific probes targeting specific chromosomal regions, as well as subtelomeric probes. However, for some of the PGD cases, a number of locus specific and α-satellite probes were laboratory prepared (from plasmids and YAC inserts), allowing the possibility of labelling probe combinations as desired according to the PGD strategies devised. All the FISH probe details including label, cytogenetic position, and source, are listed on Table 2.3. Combining probes that map at the same cytogenetic position labelled with different fluorochromes such as SO (red fluorescence) and SG (green fluorescence) results in a signal of orange fluorescence when probes are mixed in the same ratio (1:1). All probes employed in PGD cases were tested separately as well as in appropriate combinations on control lymphocytes and 200 interphase nuclei were scored to obtain information on hybridisation efficiency and specificity. Table 3.1 lists the probes tested on lymphocytes and their efficiencies.

Table 3.1. Evaluation of FISH probes in lymphocyte control interphase nuclei.

<table>
<thead>
<tr>
<th>Probe Reference</th>
<th>Cytogenetic Position</th>
<th>% Probe Efficiency* Control Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUC1.77</td>
<td>Satellite-III 1</td>
<td>97%</td>
</tr>
<tr>
<td>CEP 1 (SO/SG)</td>
<td>α-satellite 1</td>
<td>95%</td>
</tr>
<tr>
<td>TelVysion 1q (SO)</td>
<td>1qter</td>
<td>86%</td>
</tr>
<tr>
<td>TelVysion 1p (SG)</td>
<td>1pter</td>
<td>75%</td>
</tr>
<tr>
<td>D2Z1 (Dig/Bio)</td>
<td>α-satellite 2</td>
<td>87%</td>
</tr>
<tr>
<td>PBS4D</td>
<td>α-satellite 2</td>
<td>94%</td>
</tr>
<tr>
<td>pZ311</td>
<td>α-satellite 3</td>
<td>N/A (Non-specific binding)</td>
</tr>
<tr>
<td>TelVysion 2q (SO)</td>
<td>2qter</td>
<td>83%</td>
</tr>
<tr>
<td>TelVysion 1p (SG)</td>
<td>2pter</td>
<td>78%</td>
</tr>
<tr>
<td>CEP 4 (SO/SA)</td>
<td>α-satellite 4</td>
<td>91%</td>
</tr>
<tr>
<td>P4n1/4</td>
<td>α-satellite 4</td>
<td>89%</td>
</tr>
<tr>
<td>LSI D5S23 (SG)</td>
<td>5p15.2</td>
<td>81%</td>
</tr>
<tr>
<td>LSI D5S721/D5S23 (SG) &amp; EGR1 (SO)</td>
<td>5p15.2 &amp; 5q31</td>
<td>88%</td>
</tr>
<tr>
<td>D6Z1 (Dig)</td>
<td>α-satellite 6</td>
<td>80%</td>
</tr>
<tr>
<td>pEDZ6</td>
<td>α-satellite 6</td>
<td>92%</td>
</tr>
<tr>
<td>TelVysion 6q (SO)</td>
<td>6qter</td>
<td>79%</td>
</tr>
<tr>
<td>D7S486 (SO) &amp; D7S522 (SG)</td>
<td>7q11.23 &amp; 7q31</td>
<td>78%</td>
</tr>
<tr>
<td>TelVysion 7q (SO)</td>
<td>7qter</td>
<td>81%</td>
</tr>
<tr>
<td>TelVysion 7p (SO/SG)</td>
<td>7pter</td>
<td>84%</td>
</tr>
<tr>
<td>CEP 8 (SG)</td>
<td>α-satellite 8</td>
<td>91%</td>
</tr>
</tbody>
</table>
**Results**

<table>
<thead>
<tr>
<th>Probe Description</th>
<th>Chromosome</th>
<th>Location</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>TelVysion 8q (SO)</td>
<td>8qter</td>
<td>8qter</td>
<td>84%</td>
</tr>
<tr>
<td>CEP 9 (SG/SO)</td>
<td>α-satellite 9</td>
<td>90%</td>
<td></td>
</tr>
<tr>
<td>pZ1013</td>
<td>α-satellite 10</td>
<td>97%</td>
<td></td>
</tr>
<tr>
<td>CEP 11 (SO/SG)</td>
<td>α-satellite 11</td>
<td>89%</td>
<td></td>
</tr>
<tr>
<td>TelVysion 11q (SO)</td>
<td>11qter</td>
<td>89%</td>
<td></td>
</tr>
<tr>
<td>pBR12</td>
<td>α-satellite 12</td>
<td>97%</td>
<td></td>
</tr>
<tr>
<td>LSI 13 RBI (SG)</td>
<td>13q14</td>
<td>97%</td>
<td></td>
</tr>
<tr>
<td>TelVysion 13q (SO)</td>
<td>13qter</td>
<td>95%</td>
<td></td>
</tr>
<tr>
<td>TelVysion 14q (SO)</td>
<td>14qter</td>
<td>90%</td>
<td></td>
</tr>
<tr>
<td>PZ15C</td>
<td>Satellite-III 15</td>
<td>N/A-Poor hybridisation efficiency</td>
<td></td>
</tr>
<tr>
<td>CEP 16 (SO/SG)</td>
<td>β-satellite 16</td>
<td>84%</td>
<td></td>
</tr>
<tr>
<td>pZ16A</td>
<td>β-satellite 16</td>
<td>97%</td>
<td></td>
</tr>
<tr>
<td>TEL16q-G</td>
<td>16qter</td>
<td>91%</td>
<td></td>
</tr>
<tr>
<td>LIS1 (SO) &amp; RARA17q21.1 (SG)</td>
<td>17p12 &amp; 17q21.1</td>
<td>95%</td>
<td></td>
</tr>
<tr>
<td>CEP 18 (SA/SG)</td>
<td>α-satellite 18</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td>b484C16</td>
<td>19q13.2</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td>pZ20</td>
<td>α-satellite 20</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>LSI D21S259, D21S341, D21S342 (SO) &amp; LSI RB1 (SG)</td>
<td>21q22.13-q22.2 &amp; 13q14</td>
<td>95%</td>
<td></td>
</tr>
<tr>
<td>TEL21q (R/G)</td>
<td>21qter</td>
<td>90%</td>
<td></td>
</tr>
<tr>
<td>LSI N25 D22S75 (SR) &amp; LSI 22q13 (SG)</td>
<td>22q11.2 &amp; 22q13</td>
<td>95%</td>
<td></td>
</tr>
<tr>
<td>pDMX1</td>
<td>α-satellite X</td>
<td>95%</td>
<td></td>
</tr>
<tr>
<td>CEP Y DYZ3 (SO)</td>
<td>α-satellite Y</td>
<td>89%</td>
<td></td>
</tr>
<tr>
<td>CEP Y DYZ1 (SA)</td>
<td>Satellite-III Y</td>
<td>96%</td>
<td></td>
</tr>
</tbody>
</table>

* FISH probe efficiency in control lymphocyte nuclei (n=200); percentage of interphase cells showing the expected number of signals.

Plasmid DNAs for centromeric regions of chromosomes 1, 2, 3, 4, 6, 10, 12, 15, 16 and 20 were transformed into a competent E. coli strain JM 101 before amplification and isolation of DNA for use as FISH probes. All centromeric probes excluding those for chromosome 3 and 15 showed good specificity and intense signals on lymphocyte controls with 97%, 94%, 89%, 92%, 97%, 97%, 95% & 94% respectively of interphase nuclei showing the expected number of two signals (n=200). The remaining two probes were evaluated and rejected at this stage for further use. The probe for chromosome 3 was presumed to bind non-specifically showing signals on an additional chromosome to that expected even under conditions of high stringency. Examining metaphase spreads with the centromeric probe for chromosome 15 revealed a fainter signal on one of the chromosome 15 centromeres or complete absence of the expected signal in some cells, even though the probe was...
Results

tested on control lymphocytes obtained from three individuals. Only 35% of nuclei (n=200) showed the expected two signals. This probe was considered unsuitable for further analysis due to the polymorphic variation in signal intensity demonstrated.

Mapping of probes to specific chromosomal regions was also required for individual PGD cases involving chromosomal rearrangements. With this aim, five large insert YAC and cosmid clones for chromosomes 1, 8, 12, 19 and 21 were analysed. However, it was only the clone for chromosome 19 (b484C16) that successfully amplified as required for case 6-D. This probe showed good specificity and minimal background fluorescence in metaphase chromosomes of lymphocyte controls with relatively large discrete signals, easily scored in interphase nuclei. The expected number of two signals were observed in 98% of lymphocyte control nuclei and the probe was considered suitable for use as diagnostic probe for embryo analysis and PGD.

Some of these probe combinations were also tested on triploid fibroblast cell lines to assess efficiency of detection of trisomic chromosomes. The two triploid fibroblast cell cultures both (XXY) were treated differently. One culture was pretreated with hypotonic solution producing more swollen cells with a few metaphases, while the other cell line was pretreated with PBS to resemble the embryonic cells treatment prior to fixation. The second treatment (with PBS) produced nuclei that were of smaller size and the signals of FISH on them were more compact as expected for embryonic nuclei. Nine probe combinations developed for PGD cases were tested on these two differently treated triploid fibroblast cultures and the efficiency of the probes was assessed by scoring 200 interphase nuclei as illustrated in Table 3.2. The hybridisation efficiency was marginally but consistently higher in the cells treated with PBS, compared to the conventionally pretreated cell line. This supports the view that this alternative fixation technique is associated with more accurate FISH diagnosis in fibroblasts.
Table 3.2 Evaluation of nine combinations of probes selected for PGD cases in lymphocyte control and triploid fibroblasts (PBS or hypotonic solution treated).

<table>
<thead>
<tr>
<th>Case &amp; Probe combination</th>
<th>% Probe Efficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>9-J</td>
<td></td>
</tr>
<tr>
<td>22q11.2 &amp; LSI 22q13 (SG)</td>
<td>91%</td>
</tr>
<tr>
<td>9-J</td>
<td></td>
</tr>
<tr>
<td>12-K</td>
<td></td>
</tr>
<tr>
<td>TelVysion 8q(8qter) (SO), CEP 8 (8 cen) (SG)</td>
<td>87%</td>
</tr>
<tr>
<td>13-C</td>
<td></td>
</tr>
<tr>
<td>CEP 16 (SO/SO), LIS1 17p12 (SO) &amp; RARA17q21.1 (SG)</td>
<td>92%</td>
</tr>
<tr>
<td>14</td>
<td></td>
</tr>
<tr>
<td>pBS4D (2 cen) (SO+SG), CEP 8 (SG), TelVysion 8q (SO)</td>
<td>90%</td>
</tr>
<tr>
<td>17-A</td>
<td></td>
</tr>
<tr>
<td>LSI D5S723 (5p15.2) (SG); CEP 11 (11 cen) (SO+SG), TelVysion 11q (11qter) (SO)</td>
<td>84%</td>
</tr>
<tr>
<td>20-B</td>
<td></td>
</tr>
<tr>
<td>pUC1.77 (1 cen) (SO+SG), TelVysion 1q (1qter) (SO), D2Z1 (2 cen) (Dig/Bio)</td>
<td>86%</td>
</tr>
<tr>
<td>21</td>
<td></td>
</tr>
<tr>
<td>11 CEP (11 cen) (SO+SG), LSI N25 D22S75 22q11.2 (SR) &amp; LSI 22q13 (SG)</td>
<td>87%</td>
</tr>
<tr>
<td>32-1</td>
<td></td>
</tr>
<tr>
<td>LSI RBl 13q14 (SG), TelVysion 14q (SO)</td>
<td>92%</td>
</tr>
<tr>
<td>40-F</td>
<td></td>
</tr>
<tr>
<td>Elastin Probe LSI Elastin 7q11.23 SO/D7S486,D7S522 control 7q31 SG</td>
<td>78%</td>
</tr>
<tr>
<td>9 cases</td>
<td>Average hybridisation efficiency</td>
</tr>
</tbody>
</table>

* FISH probe efficiency in control lymphocyte nuclei (n=200); percentage of interphase cells showing the expected number of signals; percentage of triploid cells showing 3 signals.

3.2 FISH Analysis of Donated IVF Embryos.

Combinations of selected FISH probes for prospective PGD cases were tested on a total of 64 normally or abnormally fertilised IVF embryos between days two and four post-insemination to evaluate hybridisation efficiency and assess levels of
mosaicism for individual autosomes. Embryos were donated for research by 19 patients undergoing routine IVF (mean maternal age 35.8 years). Embryos from the same donor are listed with the same reference letters. Results are presented using ISCN FISH nomenclature (ISCN, 1995). Table 3.3 shows the number of embryos & blastomeres analysed for 12 FISH probe combinations as part of the work-up for PGD. Of the 64 available embryos that were tested 35 were normally fertilised, while the remainder 29 were either abnormally fertilised or normally fertilised embryos that showed retarded or arrested in vitro development, ranging from 2-10 cells. Results were used to categorise embryos as: uniformly normal for the chromosome tested, uniformly abnormal, diploid mosaic, aneuploid mosaic or chaotic (2.1.2.5). Allocation to each group was on the basis of the chromosome constitution of the majority of cells present, with the chaotic group representing the most highly mosaic embryos where the original zygote chromosome constitution was unclear.
Table 3.3 Numbers of blastomeres tested from 64 cleavage-stage embryos, in the evaluation of 12 FISH probe combinations developed for prospective PGD cases. Probe combination efficiency in lymphocyte control interphase nuclei also shown.

<table>
<thead>
<tr>
<th>Case: Parental Karyotype</th>
<th>Probe Combination Selected for PGD</th>
<th>FISH Efficiency on lymphocytes</th>
<th>Normally Developed Embryos Analysed*</th>
<th>Abnormally Developed Embryos Analysed**</th>
</tr>
</thead>
<tbody>
<tr>
<td>32-I: 45,XY,der(13;14)(q10;q10)</td>
<td>LSI RBI 13q14 (SG), TelVysion 14q (SO)</td>
<td>92%</td>
<td>a1, a2, j1, j2, g1-5 (35)</td>
<td>r1, r2, r3, r4, g2, g3, g4, j3, j4, s1, s2-11</td>
</tr>
<tr>
<td>44: 46,XY,del(22)(q11.2q11.2)</td>
<td>LSI N25 D22S75 22q11.2 (SR) &amp; LSI 22q13 (SG)</td>
<td>95%</td>
<td>m1, m2-2 (16)</td>
<td>m3, m4-2</td>
</tr>
<tr>
<td>6-D: 46,XY, t(5;19)(p12;p12)</td>
<td>LSI D5S721/D5S23 (SG) 5q31,EGR1 5p15.2 (SO), 19q13.2 (SO+SG)</td>
<td>96.75%</td>
<td>n1, n2-2 (13)</td>
<td>n3, n4-2</td>
</tr>
<tr>
<td>40-F: 46,XX,ins(7)(p22q31.1q32)</td>
<td>LSI Williams Syndrome Region, Elastin Probe LSI Elastin 7q11.23 SO/D7S486,D7S522 control 7q31 SG</td>
<td>78%</td>
<td>f1, f2, f3, f4-4 (27)</td>
<td>f5-1</td>
</tr>
<tr>
<td>9-J: 46,XX,t(12;22)(q24.1;q11)</td>
<td>LSI N25 D22S75 (SR) 22q11.2 &amp; LSI 22q13 (SG), pBR12(12 cen) (SO+SG)</td>
<td>91%</td>
<td>b1, b2-2 (15)</td>
<td>-</td>
</tr>
<tr>
<td>12-K: 46,XY,t(8;9)(q24.3;q21.2)</td>
<td>TelVysion 8q(8qter) (SO), CEP 9 (SG/SO)(9 cen), CEP 8 (8 cen) (SG)</td>
<td>87%</td>
<td>a1, a2, a3-3 (20)</td>
<td>o4-1</td>
</tr>
<tr>
<td>13-C: 46,XX,t(16;17)(p13.3;p11.1)</td>
<td>CEP 16 (SO/SG), LIS1 17p12 (SO) &amp; RARA17q21.1 (SG)</td>
<td>92%</td>
<td>i1, i2, i3-3 (17)</td>
<td>i4-1</td>
</tr>
<tr>
<td>14: 46,XY,t(2;8)(q37.3;q23.1)</td>
<td>pBS4D (2 cen) (SO+SG), CEP 8 (SG), TelVysion 8q (SO)</td>
<td>90%</td>
<td>k1, k2, k3-3 (24)</td>
<td>k4-1</td>
</tr>
<tr>
<td>17-A: 46,XX,t(5;11)(q31;q23)</td>
<td>LSI D5S723 (5p15.2) (SG); CEP 11 (11 cen) (SO+SG), TelVysion 11q (11qter) (SO)</td>
<td>84%</td>
<td>d1, d2, d3-3 (23)</td>
<td>-</td>
</tr>
<tr>
<td>19: 46,XX,t(4;11)(q31.1;q21)</td>
<td>4 CEP (4 cen) (SA), TelVysion 11q (11qter) (SO), 11 CEP (11 cen)(SG)</td>
<td>88%</td>
<td>c1-1 (9)</td>
<td>-</td>
</tr>
<tr>
<td>20-B: 46,XX,t(1;2)(q42.1;p23)</td>
<td>pUC1.77 (1 cen) (SO+SG), TelVysion 1q (1qter) (SO), D2Z1(2 cen) (Dig/Bio)</td>
<td>86%</td>
<td>e1-1 (7)</td>
<td>e2, e3, p1, p2, p3-5</td>
</tr>
<tr>
<td>21: 46,XX,t(11;22)(q23.3;q11.2)</td>
<td>11 CEP (11 cen) (SO+SG), LSI N25 D22S75 22q11.2 (SR) &amp; LSI 22q13 (SG)</td>
<td>87%</td>
<td>h1, h2, l1, l2, l3, h3, q1, q2, q3, l5-5</td>
<td>l4-6 (39)</td>
</tr>
</tbody>
</table>

* FISH probe hybridisation efficiency, percentage of lymphocyte interphase nuclei showing the expected number of clear signals (2 red, 2 green, 2 orange/2 red, 2 green, 2 aqua).
* Embryos normally fertilised and scored as grade I or II on day three post-insemination.
** Embryos which resulted from abnormal fertilisation (a single pronucleus or more than two pronuclei observed 18 hours post-insemination) or were normally fertilised but showed retarded or arrested in vitro development. Ranging from two to ten cells.
3.2.1 FISH Analysis of Chromosome Abnormalities in Normally Developing Day Three Post-insemination Embryos.

Thirty-five normally developing cleavage-stage embryos (245 blastomeres) were obtained for analysis from 15 donors [mean age 30.8 (+3.2) years]. All were normally fertilised and scored as grade II, or III on day three post-insemination. In the main, embryos obtained from each donor were solely employed to test probes selected for a certain case, meaning that each group of donated embryos deriving from one patient was allocated to test probes selected for one case. However, probe combinations selected for cases involving chromosomes 13, 14, and 11, 22 were tested on embryos that derived from two or more patients. The reason for selecting embryos deriving from more than one patient for these cases was the fact that some of the autosomes involved in these translocations are strongly associated with aneuploidy and significant chromosomal imbalances. Overall, 35 normally developing embryos from 15 patients yielded 245 blastomeres all of which were analysed and detailed results of the FISH analysis are outlined in Tables 3.4-3.15.

Table 3.4- Dual colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 32-I. LSI RBI 13q14 (SG), TelVysion 14q (SO).

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 13/14 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>a1</td>
<td>nuc ish 13q14(RB1x2),14q(D14S308x2) [6]</td>
<td>normal</td>
</tr>
<tr>
<td>a2</td>
<td>nuc ish 13q14(RB1x2),14q(D14S308x2) [7]</td>
<td>normal</td>
</tr>
<tr>
<td>g1</td>
<td>nuc ish 13q14(RB1x1),14q(D14S308x2) [6]</td>
<td>abnormal</td>
</tr>
<tr>
<td>j1</td>
<td>nuc ish 13q14(RB1x2),14q(D14S308x2) [7]</td>
<td>normal</td>
</tr>
<tr>
<td>j2</td>
<td>nuc ish 13q14(RB1x2),14q(D14S308x2) [7]</td>
<td>normal</td>
</tr>
</tbody>
</table>

Two embryos from patient a and two from patient j at 6 and 7 cell stage were analysed with the probes selected for case 32-I and all four embryos were normal. Embryo g1 was uniformly monosomic for chromosome 13.
Table 3.5- Dual colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 44. LSI N25 D22S75 22q11.2 (SR) & LSI 22q13 (SG).

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 22 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1</td>
<td>nuc ish 22q11.2(D22S75x2), 22q13 (22q13x2) [8]</td>
<td>normal</td>
</tr>
<tr>
<td>m2</td>
<td>nuc ish 22q11.2(D22S75x2), 22q13 (22q13x2) [6]</td>
<td>diploid/mosaic</td>
</tr>
</tbody>
</table>

The locus specific dual probe Di George, selected for case 44 was tested on two 8-cell embryos. Embryo \( m1 \) carried two copies of chromosome 22, but \( m2 \) was classified as diploid/mosaic with six cells normal and two showing tetrasomy for chromosome 22 indicating tetraploidy.

Table 3.6 Triple colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 6-D. LSI D5S721/D5S23 (SG) 5q31,EGR1 5p15.2 (SO), 19q13.2 (SO+SG).

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 5 &amp; 19 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>n1</td>
<td>nuc ish 19q13.2 (b484C16x2), 5q31 (D5S721/D5S23 x2), 5p15.2 (EGR1x2) [7]</td>
<td>normal</td>
</tr>
<tr>
<td>n2</td>
<td>nuc ish 19q13.2 (b484C16x1), 5q31 (D5S721/D5S23 x2), 5p15.2 (EGR1x1) [1]</td>
<td>chaotic</td>
</tr>
</tbody>
</table>

The probe combination selected for case 6-D was tested on two embryos. Embryo \( n1 \) was balanced for both chromosomes 5 & 19. However, embryo \( n2 \) had a chaotic chromosomal complement with two nuclei showing partial monosomies and 4 nuclei showing nullisomy for chromosome 19.
### Results

Table 3.7- Dual colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 40-F. LSI Williams Syndrome Region, Elastin Probe LSI Elastin 7q11.23 SO/D7S486,D7S522 control 7q31 SG.

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 7 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>fl</td>
<td>nuc ish 7q31(D7S486/D7S522 x2), 7q11.23 (LSI Elastin x2) [8]</td>
<td>normal</td>
</tr>
<tr>
<td>f2</td>
<td>nuc ish 7q31(D7S486/D7S522 x2), 7q11.23 (LSI Elastin x2) [8]</td>
<td>normal</td>
</tr>
<tr>
<td>f3</td>
<td>nuc ish 7q31(D7S486/D7S522 x2), 7q11.23 (LSI Elastin x2) [6]</td>
<td>normal</td>
</tr>
<tr>
<td>f4</td>
<td>nuc ish 7q31(D7S486/D7S522 x2), 7q11.23 (LSI Elastin x2) [5]</td>
<td>normal</td>
</tr>
</tbody>
</table>

All four spare embryos tested for the selection of probes for case 40-F had two copies of chromosome 7.

Table 3.8- Dual colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 9-J. LSI N 25 D22S75 (SR) 22q11.2 & LSI 22q13 (SG), pBR12(12 cen) (SO+SG).

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 12 &amp; 22 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>b1</td>
<td>nuc ish 22q11.2(D22S75x1), 22q13 (22q13x1), 12 p11.1-q11 (pBR12x1) [7]</td>
<td>abnormal (haploid)</td>
</tr>
<tr>
<td>b2</td>
<td>nuc ish 22q11.2(D22S75x2), 22q13 (22q13x2), 12 p11.1-q11 (pBR12x2) [7]</td>
<td>normal</td>
</tr>
</tbody>
</table>

Of the two embryos analysed for chromosome 12 and 22, one was normal and the other was uniformly abnormal showing either monosomy for both chromosomes or haploidy.
### Table 3.9- Triple colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 12-K. TelVysion 8q(8qter) (SO), CEP 9 (SG/SG)(9 cen), CEP 8 (8 cen) (SG).

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 8 &amp; 9 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>o1</td>
<td>nuc ish 8p11.1-q11.1(8q22x2), 8p11-q11(9q34x2), 8qter (yRM2053x2) [7]</td>
<td>normal</td>
</tr>
<tr>
<td>o2</td>
<td>nuc ish 8p11.1-q11.1(8q22x2), 8p11-q11(9q34x2), 8qter (yRM2053x2) [7]</td>
<td>normal</td>
</tr>
<tr>
<td>o3</td>
<td>nuc ish 8p11.1-q11.1(8q22x2), 8p11-q11(9q34x2), 8qter (yRM2053x2) [4]</td>
<td>diploid/Mosaic</td>
</tr>
<tr>
<td></td>
<td>nuc ish 8p11.1-q11.1(8q22x3), 8p11-q11(9q34x3), 8qter (yRM2053x3) [2]</td>
<td>normal</td>
</tr>
</tbody>
</table>

Two embryos tested for the probe combination employed for case 12-K were disomic for the chromosomes examined. However, the third embryo was diploid mosaic with the majority of the blastomeres being disomic for chromosomes 8 and 9 and two cells showing trisomy for both chromosomes which indicates double trisomy or triploidy.

### Table 3.10 Triple colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 13-C. CEP 16 (SO/SG), LIS1 17p12 (SO) & RARA17q21.1 (SG).

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 16 &amp; 17 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>i1</td>
<td>nuc ish 16q11.2(16q11x2), 17p12 (LSI1 x2), 17q21.1 (RARAX2) [6]</td>
<td>normal</td>
</tr>
<tr>
<td>i2</td>
<td>nuc ish 16q11.2(16q11x2), 17p12 (LSI1 x2), 17q21.1 (RARAX2) [6]</td>
<td>normal</td>
</tr>
<tr>
<td>i3</td>
<td>nuc ish 16q11.2(16q11x2), 17p12 (LSI1 x2), 17q21.1 (RARAX2) [5]</td>
<td>normal</td>
</tr>
</tbody>
</table>

All three embryos tested for the probes employed for case 13-C were found to be normal for chromosomes 16 and 17.
Table 3.11 Triple colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 14. pBS4D (2 cen) (SO+SG), CEP 8 (SG), TelVysion 8q (SO).

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 2 &amp; 8 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>k1</td>
<td>nuc ish 2p11.1-q11.1(pBS4D x2), 8p11.1-q11.1(D8Z2x2), 8qter (yRM2053x2) [9]</td>
<td>normal</td>
</tr>
<tr>
<td>k2</td>
<td>nuc ish 2p11.1-q11.1(pBS4D x2), 8p11.1-q11.1(D8Z2x2), 8qter (yRM2053x2) [8]</td>
<td>normal</td>
</tr>
<tr>
<td>k3</td>
<td>nuc ish 2p11.1-q11.1(pBS4D x3), 8p11.1-q11.1(D8Z2x2), 8qter (yRM2053x2) [3]</td>
<td>aneuploid mosaic</td>
</tr>
<tr>
<td></td>
<td>nuc ish 2p11.1-q11.1(pBS4D x1), 8p11.1-q11.1(D8Z2x2), 8qter (yRM2053x2) [4]</td>
<td></td>
</tr>
</tbody>
</table>

Two of the embryos tested for the combination of probes selected for case 14 were normal however, the third embryo was classified as aneuploid mosaic with a cell line showing trisomy for chromosome 2 and the other one showing monosomy for the same chromosome.

Table 3.12 Triple colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 17-A. LSI D5S723 (5p15.2) (SG); CEP 11 (11 cen) (SO+SG), TelVysion 11q (11qter) (SO).

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 5 &amp; 11 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>d1</td>
<td>nuc ish 5p15.2 (D5S723 x2), 11p11.1-q11.1(D11Z1x2), 11qter (VIJ2072x2) [8]</td>
<td>normal</td>
</tr>
<tr>
<td>d2</td>
<td>nuc ish 5p15.2 (D5S723 x2), 11p11.1-q11.1(D11Z1x2), 11qter (VIJ2072x2) [8]</td>
<td>normal</td>
</tr>
<tr>
<td>d3</td>
<td>nuc ish 5p15.2 (D5S723 x2), 11p11.1-q11.1(D11Z1x2), 11qter (VIJ2072x2) [7]</td>
<td>normal</td>
</tr>
</tbody>
</table>

All three embryos tested for the probes employed for case 17-A were found to be normal for chromosomes 5 & 11.
Results

Table 3.13- Triple colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 19. 4 CEP (4 cen) (SA), TelVysion 11q (11qter) (SO), 11 CEP (11 cen)(SG).

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 4 &amp; 11 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>cl</td>
<td>nuc ish 4p11-q11(α-sat x 2), 11p11.1-1q11(D11Z1x2), 4qter(D4S2930x2) [9]</td>
<td>normal</td>
</tr>
</tbody>
</table>

A single embryo tested for the probes employed for case 19 was found to be normal for chromosomes 4 & 11.

Table 3.14- Triple colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 20-B. pUC1.77 (1 cen) (SO+SG), TelVysion 1q (1qter) (SO), D2Z1(2 cen) (Dig/Bio).

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 1 &amp; 2 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>el</td>
<td>nuc ish 1p11.1-q11(pUC1.77 x2), 2p11.1-q11(D2Z1x2), 1qter (D1S3738x2) [7]</td>
<td>normal</td>
</tr>
</tbody>
</table>

A single embryo tested for the probes employed for case 20-B was found to be normal for chromosomes 2 & 8.
Table 3.15- Triple colour FISH analysis of normally developing cleavage-stage embryos with probes selected for case 21. 11 CEP (11 cen) (SO+SG), LSI N25 D22S75 22q11.2 (SR) & LSI 22q13 (SG).

<table>
<thead>
<tr>
<th>Embryo Reference</th>
<th>FISH Analysis Result [No of Blastomeres]</th>
<th>Chr 11 &amp; 22 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>h1</td>
<td>nuc ish 11p11.1-q11 (D11Z1x2), 22q11.2(D22S75x1), 22q13 (22q13x0) [1]</td>
<td>Chaotic</td>
</tr>
<tr>
<td></td>
<td>nuc ish 11p11.1-q11 (D11Z1x1), 22q11.2(D22S75x2), 22q13 (22q13x1) [1]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>nuc ish 11p11.1-q11 (D11Z1x2), 22q11.2(D22S75x2), 22q13 (22q13x0) [1]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>nuc ish 11p11.1-q11 (D11Z1x1), 22q11.2(D22S75x1), 22q13 (22q13x1) [2]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>nuc ish 11p11.1-q11 (D11Z1x2), 22q11.2(D22S75x4), 22q13 (22q13x4) [2]</td>
<td>Chaotic</td>
</tr>
<tr>
<td></td>
<td>nuc ish 11p11.1-q11 (D11Z1x1), 22q11.2(D22S75x2), 22q13 (22q13x2) [1]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>nuc ish 11p11.1-q11 (D11Z1x3), 22q11.2(D22S75x3), 22q13 (22q13x3) [1]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>nuc ish 11p11.1-q11 (D11Z1x2), 22q11.2(D22S75x2), 22q13 (22q13x0) [2]</td>
<td></td>
</tr>
<tr>
<td>l1</td>
<td>nuc ish 11p11.1-q11 (D11Z1x2), 22q11.2(D22S75x2), 22q13 (22q13x2) [6]</td>
<td>normal</td>
</tr>
<tr>
<td>l2</td>
<td>nuc ish 11p11.1-q11 (D11Z1x2), 22q11.2(D22S75x2), 22q13 (22q13x2) [7]</td>
<td>normal</td>
</tr>
<tr>
<td>l3</td>
<td>nuc ish 11p11.1-q11 (D11Z1x2), 22q11.2(D22S75x2), 22q13 (22q13x2) [7]</td>
<td>normal</td>
</tr>
<tr>
<td>l4</td>
<td>nuc ish 11p11.1-q11 (D11Z1x2), 22q11.2(D22S75x2), 22q13 (22q13x2) [8]</td>
<td>normal</td>
</tr>
</tbody>
</table>

Both spare embryos donated from patient h were classified as chaotic, while all four embryos from patient l were found to be normal for the chromosomes examined.

In total, from analysis of all thirty-five embryos using dual and triple colour FISH with twelve different combinations of chromosome specific probes, 77% (27/35) of embryos were normal for the chromosomes examined and 23% (8/35) were...
abnormal. Abnormalities were categorised as 6% diploid mosaic, 6% uniformly abnormal 3% aneuploid mosaic and 8% chaotic (Table 3.16).

Table 3.16- Combined results of dual and triple colour FISH analysis of thirty-five normally developing cleavage-stage embryos.

<table>
<thead>
<tr>
<th>Chromosome(s) Tested</th>
<th>Uniformly Normal</th>
<th>Uniformly Abnormal</th>
<th>Diploid Mosaic</th>
<th>Aneuploid Mosaic</th>
<th>Chaotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome 13 &amp; 14-</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Case 32-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosome 22-</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Case 44</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosome 5 &amp; 19-</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Case 6-D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosome 7-Case 40-F</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chromosomes 12 &amp; 22-</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Case 9-J</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosomes 8 &amp; 9-</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Case 12-K</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosomes 16 &amp; 17-</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Case 13-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosomes 2 &amp; 8-</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Case 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosomes 5 &amp; 11-</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Case 17-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosomes 4 &amp; 11-</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Case 19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosomes 1 &amp; 2-</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Case 20-B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosomes 11 &amp; 22-</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Case 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total 35 (100%) 27 (77%) 2 (6%) 2 (6%) 1 (3%) 3 (8%)

*Normal for the chromosome(s) tested.
3.2.2 FISH Analysis of Chromosome Abnormalities in Abnormally Developing Cleavage-stage Embryos.

Twenty-nine abnormally developing cleavage-stage embryos ranging from two to ten cells were obtained for analysis from fourteen donors [mean maternal age 36.1 (±3.9) years]. Embryos were included in this group, which resulted from abnormal fertilisation (a single pronucleus or more than two pronuclei observed 18 hours post-insemination) or were normally fertilised but showed retarded or arrested in vitro development. These embryos were used to test FISH probes prior to PGD, embryos were analysed as they became available and consequently a variety of probe combinations were applied to embryos of varying morphology.

Tables 3.17 summarises the results of dual and triple colour FISH analysis. In total for all twenty-nine embryos analysed seven (24%) were normal for the chromosome(s) detected and twenty-two (76%) were abnormal, categorised as four (14%) diploid mosaic, 10 (34.5%) aneuploid mosaic, and eight (27.5%) chaotic. No embryos were observed in the uniformly aneuploid category. These results show that the majority of abnormally developing or abnormally fertilised embryos in this small sample group are chromosomally abnormal, mainly scored as aneuploid mosaic.

Table 3.17- Combined results of FISH analysis of twenty-nine abnormally developing preimplantation embryos, grouped according to day post-insemination and number of chromosomes detected.

<table>
<thead>
<tr>
<th>Chromosome(s) Tested</th>
<th>Uniformly Normal*</th>
<th>Uniformly Aneuploid</th>
<th>Diploid Mosaic</th>
<th>Aneuploid Mosaic</th>
<th>Chaotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome 13 &amp; 14-Case 32-I</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Chromosome 22-Case 44</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chromosome 5 &amp; 19-Case 6-D</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Results
### Results

<table>
<thead>
<tr>
<th>Chromosome 7-Case 40-F</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomes 8 &amp; 9-Case 12-K</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chromosomes 16 &amp; 17-</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Case 13-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosomes 2 &amp; 8-Case 14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chromosomes 1 &amp; 2-Case 20-B</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Chromosomes 11 &amp; 22-Case 21</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

**Total 29 (100%)**

|         | 7 (24%) | 0 (0%) | 4 (14%) | 10 (34.5%) | 8 (27.5%) |

* Normal for the chromosome(s) tested.

### 3.3 Details of Forty-four Prospective PGD Referrals.

Over a four-year period forty-four prospective PGD patients were referred due to chromosomal abnormality in a carrier parent. These included thirty couples with balanced reciprocal translocations, seven with balanced Robertsonian translocations, three with insertions, one with an inversion, one with a deletion, one with a duplication, and one with suspected gonadal mosaicism for a trisomic cell line. Mean maternal age was 35.2 years (SD ±3.95 years), range 25 to 45 years. Fig 2.1 shows the combined reproductive histories of this group (listed individually in Table 2.2) showing a total of 93 previous pregnancies resulted in 6 (7%) healthy live births, eight (9%) chromosomally abnormal live births, thirteen (14%) terminations of pregnancy (TOP) and 63 (70%) spontaneous abortions.

The parental chromosomal abnormality was in most cases already well characterised, as the majority of couples were referred by clinical geneticists. Strategies for the respective referrals were initially discussed and agreed with the entire FISH PGD team. Prediction of the likely behaviour of the rearranged chromosome and the normal homologue at meiosis is the first stage in designing a PGD strategy so that all the possible segregation outcomes could be detected. A
combination of probes was then devised which could accurately distinguish the normal from the predicted abnormal chromosome constitutions. The position of these probes was confirmed in parental lymphocyte metaphases before a minimum number of probes were chosen to detect the maximum number of segregation outcomes. Where possible, α-satellite or other probes detecting repetitive sequences were chosen as these show the greatest hybridisation efficiency and the largest signals. In practice, where it was not possible to detect all the theoretical possible outcomes of meiotic segregation, the aim was to exclude the more viable unbalanced products. The ease with which suitable probe combinations were developed was dependent on the chromosomes involved and the position of breakpoints. In cases where patients lost contact with the Centre, preliminary work did not progress beyond the planning step. Table 2.1 lists the patients' karyotype and probe combination employed for work-up, while specific probe details involving mapping position and source are listed in Table 2.3.
3.3.1 Reciprocal Translocations.

Balanced reciprocal translocations made up the largest group of referrals, comprising 50% (15) male, 50% (15) female translocation carriers (mean maternal age 34.3 years). To detect all possible meiotic segregations for a reciprocal translocation, a strategy was developed involving the use of two FISH probes flanking the breakpoint on one translocation chromosome and a third probe specific for the other chromosome (located in any position). These ‘flanking probes’ can be any distance away from each other as long as one is proximal and the other is distal to the breakpoint. The criteria for probe selection being that each of the four chromosomes involved in the translocation (two normal and two derivatives) show a distinct combination of signals with these three probes easily identifiable in interphase nuclei (Fig. 3.1).

Fig. 3.1 FISH detection strategies for reciprocal translocations.

<table>
<thead>
<tr>
<th>a Flanking probe strategy</th>
<th>b Spanning probe strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>A derA derB B</td>
<td>A derA derB B</td>
</tr>
</tbody>
</table>

Diagrams illustrating the two FISH strategies which can be used for PGD for reciprocal translocation carriers based on a flanking probes (Conn et al., 1995) or b spanning probes (Munné et al., 1998b). In both cases each of the four chromosomes involved in the translocation (two normal and two derivatives) shows a distinct combination of signals, however only strategy b allows normal and balanced constitutions to be differentiated.

(Reproduced from Harper, Delhanty, Handyside, 2000).

Probe selection was significantly simplified towards the end of this study period as a greater number of commercially available sub-telomeric, locus specific, and dual probes probes began to be released providing suitable probes for the vast
majority of translocation cases. The approach of using either a single sub-telomeric probe together with two centromeric probes (1, 2, 3, 4, 5, 7, 8, 10, 11, 12-K, 14, 15, 18-H, 19, 20-B, 22, 23-P, 25, 26, 27, and 30) or two sub-telomeric probes and a single centromeric probe (16 and 28), was chosen for 23 cases. Three commercially available dual probes designed for clinical cytogenetics purposes, aiming to detect syndromes such as Cri Du Chat, Miller Dieker and Di George were used as sources of flanking probes for five translocation cases (6-D, 9-J, 13-C, 21 and 29). However, devising a strategy for case 6-D required a suitable locus-specific probe to be developed. Therefore, for this case a chromosome 19 specific YAC probe mapping to 19q13.2 (Frengen et al., 1999), was selected and successfully developed to add to the combination of probes suitable for this case. The commercial availability of locus specific probes enabled the possibility of a strategy comprising of a centromeric, a locus specific and a sub-telomeric probe as employed for cases (9, 17-A, and 24). Plasmid DNAs for chromosomes 1, 2, 4, 6, 10, 12, 16 obtained from resource centres as agar stabs provided suitable centromeric probes to be used for diagnostic purposes following optimisation of the respective FISH protocols. However, many of the centromeric probes included in the strategies devised for the reciprocal translocation cases were commercially available.

Potentially suitable FISH strategies were devised for all thirty cases. In practise suitable probe combinations were available only for twelve (6-D, 9-J, 12-K, 13-C, 14, 15, 17-A, 18-H, 19, 20-B, 21 and 32-G) of the thirty cases (40%) as shown in Table 3.18 IVF treatment was commenced for eight couples, two of which were cancelled due to unsuitability of the embryos to be biopsied (12-K and 9-J) and six cases reached the embryo biopsy stage of PGD (6-D, 13-C, 17-A, 18-H, 10-B and 32-G). Diagnostic work-up was completed for a further four couples (14, 15, 19 and 21) who are currently about to begin treatment.
Table 3.18- FISH probe combinations selected for twelve PGD referrals involving reciprocal translocation and the outcome of these cases.

<table>
<thead>
<tr>
<th>Case: Parental Karyotype</th>
<th>Triple Probe Combination Selected for PGD</th>
<th>FISH Efficiency*</th>
<th>Outcome of Case</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-D: 46,XY,t(5;19)(p12;p12)</td>
<td>LSI D5S721/D5S23 (SG) 5q31,EGR1 5p15.2 (SO), 19q13.2 (SO+SG)</td>
<td>96.75%</td>
<td>PGD (see 3.4.4)</td>
</tr>
<tr>
<td>9-J: 46,XX,t(12;22)(q24.1;q11)</td>
<td>LSI N25 D22S75 (SR) 22q11.2 &amp; LSI 22q13 (SG), pBR12(12 cen) (SO+SG)</td>
<td>91%</td>
<td>Cancelled PGD (see 3.7.2)</td>
</tr>
<tr>
<td>12-K: 46,XY,t(8;9)(q24.3;q21.2)</td>
<td>TelVysion 8q(8qter) (SO), CEP 9 (SG/SO)(9 cen), CEP 8 (8 cen) (SG)</td>
<td>87%</td>
<td>Cancelled PGD (see 3.7.1)</td>
</tr>
<tr>
<td>13-C: 46,XX,t(16;17)(p13.3;p11.1)</td>
<td>CEP 16 (SO/SG), LSI 17p12 (SO) &amp; RARA17q21.1 (SG)</td>
<td>92%</td>
<td>PGD (see 3.4.3)</td>
</tr>
<tr>
<td>14: 46,XY,t(2;8)(q37.3;q23.1)</td>
<td>pBS4D (2 cen) (SO+SG), CEP 8 (SG), TelVysion 8q (SO)</td>
<td>90%</td>
<td>Awaiting treatment</td>
</tr>
<tr>
<td>15: 46,XY,t(1;4)(p31.2;q31.3)</td>
<td>pUC1.77 (1 cen) (SO+SG); CEP 4 (SG)(4 cen) (SG), TelVysion 1p(1pter) (SO)</td>
<td>86%</td>
<td>Awaiting treatment</td>
</tr>
<tr>
<td>17-A: 46,XX,t(5;11)(q31;q23)</td>
<td>LSI D5S723 (5p15.2) (SG); CEP 11 (11 cen) (SO+SG), TelVysion 11q (11qter) (SO)</td>
<td>84%</td>
<td>PGD (see 3.4.1)</td>
</tr>
<tr>
<td>18-H: 46,XY,t(1;18)(p32;q23)</td>
<td>pUC1.77 (1 cen) (SO+SG), 18 CEP (18 cen) (SA), TelVysion 1q (1qter) (SO)</td>
<td>91%</td>
<td>PGD (see 3.4.6)</td>
</tr>
<tr>
<td>19: 46,XX,t(4;11)(q31.1;q21)</td>
<td>4 CEP (4 cen) (SA), TelVysion 11q (11qter) (SO), 11 CEP (11 cen)(SG)</td>
<td>88%</td>
<td>Awaiting treatment</td>
</tr>
<tr>
<td>Chromosome</td>
<td>Abnormality</td>
<td>Status</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-------------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>20-B</td>
<td>46,XX,t(1;2)(q42.1;p23) pUC1.77 (1 cen) (SO+SG), TelVysion 1q (1qter) (SO), D2Z1(2 cen) (Dig/Bio)</td>
<td>86% PGD (see 3.4.2)</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>46,XX,t(11;22)(q23.3;q11.2) 11 CEP (11 cen) (SO+SG), LSI N25 D22S75 22q11.2 (SR) &amp; LSI 22q13 (SG)</td>
<td>87% Awaiting treatment</td>
<td></td>
</tr>
<tr>
<td>32-G</td>
<td>46,X X,t(8;12)(q11.2;q12) CEP 8 (8 cen)(SG), TelVysion 8q (8qter)(SO); pBR12(12 cen) (SO+SG)</td>
<td>87.5% PGD (see 3.4.5)</td>
<td></td>
</tr>
</tbody>
</table>

* FISH probe hybridisation efficiency, percentage of lymphocyte interphase nuclei showing the expected number of clear signals (2 red, 2 green, 2 orange/2 red, 2 green, 2 aqua).
Results

Of note are cases 4 and 8 where there were certain reservations as to the nature and the positions of the breakpoints involved in both cases. Initial work-up was discontinued following proof that the probes selected as part of the strategy devised for each case failed to flank the breakpoints. In fact when tested on both patients' lymphocytes it was apparent that the probes in question (7 α-satellite selected for case 8 & Y satellite-III or 1 satellite II/III selected for case 4) mapped to the actual translocation breakpoints and consequently presented signals on both derivative chromosomes in each case, as well as the normal chromosomes involved in the translocations (Fig. 3.2, 3.3, 3.4). As a result both cases (4 & 8) were classified as technically too difficult as no suitable probe combination were available at the time.

The remaining sixteen cases did not proceed to further work-up (1, 2, 3, 5, 7, 10, 11, 16, 22, 24, 25, 26, 27, 28, 29, and 30) for various reasons. Case 3 lacking one or more probes flanking breakpoints, was classified as technically too difficult as with the case for patient 24 due to both breakpoints being too distal. Two couples (5, 26) achieved a naturally conceived normal pregnancy, whilst on the waiting list. Work was discontinued for the remaining 11 couples, as four of these were lost to follow up after losing contact with the Centre (7, 15, 16, 29), six decided against PGD (1,10, 22, 27, 28, and 30), and three opted for prenatal diagnosis (2, 11 and 25).
FISH analysis of patient lymphocyte metaphase nucleus, showing alpha-satellite probe for chromosome 7 binding on chromosome 7 and the two derivative chromosomes of the translocation excluding the possibility of its use for PGD for this couple.
Fig. 3.3 PGD preliminary work-up for case 4 46,X,t(Y;1)(q11.23;q12)

(a), (b): Potential strategy devised for PGD for case 4 45,X, t(Y;1)(q11.23;q12)
(c) FISH analysis on control lymphocyte metaphase nucleus showing where the alpha-satellite and the satellite-III probes for chromosome Y bind. (d) FISH analysis on patient lymphocyte metaphase nucleus using the same probes, showing that they bind on both derivative chromosomes and therefore excluding the possibility of their use for PGD for this couple.
Fig. 3.4 PGD preliminary work-up for case 46,XY,t(Y;1)(q11.23;q12)

(a) Potential strategy devised for PGD for case 46,XY,t(Y;1)(q11.23;q12)
(b) FISH analysis on patient lymphocyte metaphase nucleus showing the satellite-II/III probe for chromosome 1 binding on both derivative chromosomes as well as chromosome 1, therefore excluding the possibility of its use for PGD for this couple.
3.3.2 Robertsonian Translocations.

Balanced Robertsonian translocations, were the second most common referral, including six cases of 45,XY,der(13;14)(q10;q10) and one 45,XY,der(13;21)(q10;q10) (mean maternal age 35.4 years). All cases involved a paternal translocation and required ICSI due to oligozoospermia.

To detect Robertsonian translocations involving chromosomes 13 & 14 in blastomere interphase nuclei required a minimum of two differentially labelled locus-specific probes, one on each of the acrocentric chromosomes involved in the translocation. A dual colour FISH protocol was developed for PGD for these six cases using a locus specific probe LSI 13 (13q14, SG) combined with a subtelomeric probe for chromosome 14, TelVysion 14q (14qter, SO). Preliminary testing of this dual probe combination showed strong discrete signals in both lymphocyte control and blastomere nuclei showing the expected number of signals in 92% lymphocyte interphase nuclei. However for the case involving chromosomes 13 and 21 it was decided to develop a triple probe combination to increase the accuracy of detection for chromosome 21, involved in the most viable aneuploid syndrome. The protocol was developed in this case used the Vysis locus specific probe cocktail for chromosomes 13, LSI 13 (13q14, SG), and 21, LSI21 (21q22.13-q22.2, SO) together with an additional chromosome 21 probe (21qter, Oncor, directly labelled 50:50 Red;Green). Preliminary testing of this probe combination showed strong discrete signals in both lymphocyte control and blastomere nuclei showing the expected number of signals in 94% lymphocyte interphase nuclei.

Of these seven cases, two reached the embryo biopsy stage of PGD (32-1 and 35-E) (see section 3.5), two couples remain on the list awaiting PGD (33 and 37), one opted for gamete donation (31) and two couples lost contact with the Centre (36 and 37).

3.3.3 Mosaicism for a Trisomic Cell Line.

One phenotypically normal couple (42) was referred for PGD due to suspected maternal gonadal mosaicism for a trisomic cell line, i.e.46,XX/47,XX,+21. Both parental lymphocyte karyotypes were in fact normal. The couple had experienced two livebirths with a trisomy 21 and a TOP for the same. Maternal age was 36 years.
Results

For this case a triple FISH probe combination was selected to increase the accuracy of PGD for this couple at very high risk of Downs’ syndrome pregnancy. This combination included LSI21 SO, LSI13 SG, together with a laboratory prepared bac clone bCO67E3 (21q11.2) (50:50 SG:SO) (Frengen et al., 1999). After preliminary work-up of these probes it was decided not to proceed to clinical application with this probe as the bac clone could not be successfully amplified following repeated attempts. No further work-up took place as the couple deferred PGD for family reasons.

3.3.4 Intrachromosomal Insertions.

Couple (40-F) were referred for PGD having an intrachromosomal between-arm insertion involving chromosome 7, 46,XX,ins(7)(p22q31.1q32). The couple’s reproductive history listed in Table 2.2 involves a normal live birth carrying the balanced insertion, one spontaneous abortion and one TOP both karyotyped as duplicated for the inserted segment. The female partner aged 31 years has two siblings who carry the same rearrangement, one also having experienced two pregnancies involving duplicated ins(7) karyotypes. See Fig 3.5 for pedigree.

For such an insertion if incomplete synapsis occurs during meiosis, the four possible gamete outcomes will be; normal, balanced, duplicated and deleted, all easily detected with a probe for the inserted segment. However, complete synapsis achieved by the formation of a double insertion loop results in a variety of meiotic outcomes depending upon in which loop crossing-over occurs and if the insertion is direct or inverted relative to the centromere. See Fig. 3.6 for PGD strategy for insertions, considering all possible recombinant chromosomes in both cases of a direct or an inverted insertion. Unfortunately the orientation of the inserted fragment could not be detected by cytogenetic analysis increasing the complexity of any approach to PGD for this case. To cope with both eventualities an approach was developed including a sub-telomeric probe incorporated along with a locus-specific probe for the inserted segment. Possible recombinant chromosomes resulting from both direct and inverted insertions could then be detected.
Results

The couple underwent a single PGD cycle (Section 3.6). A commercial chromosome 7 dual-probe (Williams microdeletion probe D7S486, D7S522) was selected containing a control probe (D7S522) mapping to the inserted segment at 7q31, together with a chromosome 7p sub-telomeric probe, as the orientation of this inversion was unclear from the G-banded karyotype. However, preliminary testing of this probe combination showed that optimal results could only be obtained by excluding the 7pter probe. Efficiency of the Williams microdeletion probe was shown to be 78% (based on scoring 200 interphase nuclei). Consequently, it was decided in consultation with the patient to exclude the sub telomeric probes, avoiding ambiguous results potentially leading to misdiagnosis.
Fig 3.6 Shows the six possible recombinant types which may arise for an intrachromosomal between-arm insertion of unknown orientation. (1) duplicated insertion, (2) deleted insertion, (3*) duplicated p/deleted q, (4*) deleted p/duplicated q, (3**) dicentric, (4**) acentric. For direct insertions 1, 2, 3*, and 4* apply, whilst for inverted insertions 1, 2, 3** and 4** apply. A theoretical triple probe combination to detect all outcomes is illustrated.
3.3.5 Chromosomal Deletions and Duplications.

Two cases were referred carrying a partial duplication or deletion of an autosome, both phenotypically normal. The first case involved a female with a partial duplication of region 8q11.2→q13 with a karyotype 46,XX,dup(8)(q11.2;q13). The couple had previously experienced a TOP for chromosome abnormality. The PGD work-up involving a centromeric probe on chromosome 8 (CEP 8-SG) as well as a locus specific YAC (bA179A23)-SO was discontinued as the couple opted for PND. A second couple was referred as the apparently phenotypically normal male partner carried a partial deletion of chromosome 22 with a karyotype 46,XY,del(22)(q11.2q11.2). The carrier exhibited a mild form of DiGeorge syndrome. The couple’s reproductive history listed in Table 2.2 involved a normal livebirth, followed by two chromosomally abnormal live births. The first child died due to congenital heart disease, while the second child died post-operatively following an attempt to repair an atrioventricular septal defect. The PGD protocol devised included a dual Di George microdeletion probe LSI N25 D22S75 22q11.2 (SR) & LSI 22q13 (SG). The preliminary work-up for this couple was successfully concluded. The probe selection was tested on lymphocyte nuclei showing an efficiency of 95%. The couple are awaiting treatment.

3.3.6 Outcome of PGD Referrals for Chromosomal Abnormalities

During the course of this study, from a total of 44 cases initially referred for PGD of chromosomal abnormalities, nine (20.5%) reached the embryo biopsy stage of PGD (detailed in Sections 3.4-7), with a further seven (16%) awaiting treatment. Of the remaining twenty-eight (63.5%) cases, two couples had a naturally conceived normal pregnancy before PGD work up was complete whilst the remainder were withdrawn from further treatment for a variety of reasons, summarised in Table 3.19.
## Results

Table 3.19- Summary of the outcome of forty-four PGD referrals for chromosomal abnormalities over a four-year period.

<table>
<thead>
<tr>
<th>Cases Reaching the Embryo Biopsy Stage (%)</th>
<th>9 (20.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases Awaiting Treatment (%)</td>
<td>7 (16%)</td>
</tr>
<tr>
<td>Cases Discontinued (%)</td>
<td>28 (63.5%)</td>
</tr>
</tbody>
</table>

**Reasons for Discontinuing**

- PGD cancelled due to poor *in vitro* embryo development: 2
- Naturally conceived normal pregnancy: 2
- Elected for prenatal diagnosis in preference to waiting for PGD: 4
- Elected for oocyte donation in preference to waiting for PGD: 2
- Decided against PGD for other reasons*: 7
- Technically too difficult: 6
- Lost contact with Centre: 5

*Total No of Referrals: 44

*Reasons included advanced maternal age, ethical objections, family circumstances or were unknown.*
3.4 PGD for Six Couples with Reciprocal Translocations.

Protocols were developed for six couples referred for PGD with balanced reciprocal translocations. In all cases a protocol was developed using triple colour FISH based on the flanking probe strategy described previously in section (3.3.1), with two probes flanking the breakpoint on one translocation chromosome and one probe specific for the other chromosome. As shown in table 2.1 most of the probes employed were commercially available. A locus specific YAC probe and an α-satellite cosmid probe were developed for cases (6-D) and (20-B) respectively. All probes were directly labelled with fluorochromes with the exception of one indirectly labelled probe used for case (20-B).

3.4.1 Case 17-A: Reciprocal Translocation 46,XX,t(5;11)(q34;q25)

This couple was referred for PGD due to secondary infertility. The female partner, 35 years old, was a carrier of a reciprocal translocation 46,XX, t(5;11)(q34;q25). Her husband was found to be karyotypically normal but presented with oligozoospermia. Their first natural pregnancy was terminated following CVS diagnosis, which detected severe hydrocephalus in the fetus, caused by an abnormal chromosome 11, this being an unbalanced derivative of the maternal translocation. The fetal karyotype was 46,XY,-11,+der(11),t(5;11)(q31;q23)mat. After several years of infertility, at the second IVF attempt a twin pregnancy was achieved with one normal twin and the other affected by the same unbalanced translocation as the previous pregnancy. The pregnancy was allowed to go to term and the abnormal twin died soon after birth. Finally, the same unbalanced derivative as seen before was detected in the fetus of a natural conception. The couple underwent 3 PGD cycles.

The strategy chosen for this case relies on three probes to detect all possible unbalanced translocation products in the embryo. Two of the probes α-satellite DNA/D11Z1 11p11.1-q11.1, [combined Spectrum Orange (SO) and Spectrum Green (SG)] & subtelomeric probe 11qtel (SO) flank the breakpoint on chromosome 11 and the third probe, LSI D5S23/D5S721 L SG maps to chromosome 5p15.2. All probes were from Vysis U.K. Using this FISH probe combination, 84% control lymphocyte nuclei (n=200) showed three clear signals per nucleus. Before clinical application these probes were also tested on blastomeres from day 3 post-insemination embryos.
Results

showing discrete FISH signals easily scored in all nuclei (see Table 3.12). Fig. 3.7 shows the PGD strategy employed for this case tested on patient lymphocytes and three rounds of FISH on a biopsied blastomere at metaphase from the third PGD cycle the couple underwent.

In the first ICSI cycle nine oocytes were collected and seven were fertilised (77.7%) resulting in six embryos suitable for biopsy. Two cells were biopsied on day three post-insemination from four embryos (1, 3, 4 and 6). Embryos 1 and 4 were 8-cells, embryo 3 was a 4-cell while embryo 6 was a 6-cell. A single cell biopsy was possible from embryo 5, which was at 4-cell stage, whilst three blastomeres were biopsied from embryo 2 that was an 8-cell. FISH analysis of the biopsied blastomeres showed two embryos (2 and 3) that were balanced based on all biopsied cells. Embryo one was trisomic for region 5pter→q34 and trisomic for 11q25→qter based on one cell, embryo four was trisomic for 11pter→q25 and monosomic for 11q25→qter according to one cell analysed, and embryo six was trisomic for both regions 5pter→q34 and 11pter→q25 based on both biopsied cells. No results were obtained from the one biopsed cell from embryo five. The two embryos found to be balanced were transferred on day four post-insemination but did not result in a pregnancy.

This diagnosis was confirmed by analysis of the remainder of four of the biopsied untransferred embryos (embryos 1, 4, 5 and 6). Embryo one and six were found to be fully chaotic, with all the blastomeres having different chromosomal complement. Results on embryo five were based on the analysis of a single nucleus which was found to be balanced, whilst embryo four showed trisomy for region 5q34→qter and monosomy for region 11q25→qter based on all eight blastomeres analysed and therefore was classified as mosaic aneuploid as a different aneuploidy was detected on the biopsied cell.

Based on the FISH analysis the possible segregation patterns of the oocytes were deduced. These segregations were based on the simplest outcome assuming a normal paternal gamete contribution, with minor cell lines treated as of post-zygotic origin. Three embryos derived from an alternate segregation pattern (embryos 2, 3, and 5), embryo four was a product of adjacent-1 segregation pattern and embryo 1 could have arisen from a 3:1 tertiary segregation pattern, Table 3.20 summarises this cycle of treatment.
(a), (b): Strategy devised for PGD performed from case A 46,XX,t(5;11)(q34;q25).
(c) FISH analysis of patient lymphocyte metaphase nucleus showing the normal and derivative chromosomes.

(i): The same probe combination applied to a blastomere at metaphase stage biopsied from a day 3 post-insemination embryo during PGD for this couple, showing disomy for both chromosomes 5 and 11. (ii): The same metaphase after a second round of FISH, using only the subtelomeric probe for chromosome 11 confirming the diagnosis. (iii): The same metaphase subjected to a third round of FISH using probes for chromosomes X (SG), Y (SR), and 18(SA), showing a male disomic for chromosome 18 blastomere.
Table 3.20. Classification of embryos from the 1st PGD cycle of patient 17-A. Karyotype 46,XX,t(5;11)(q34;q25)

<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; NO OF CELLS</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>RESULTS FROM SPARE EMBRYO ON D4 ACCORDING TO FISH PERFORMED WITH PROBES SELECTED FOR THE CASE</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION &amp; COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 2+ 8-cell</td>
<td>2 cells biopsied. Results obtained from one nucleus only. Nucleus 1: Tertiary trisomy for 5pter→q34 &amp; for 11q25→qter</td>
<td>4-5 cell stage- 11 nuclei analysed (multinucleated cells) Fully chaotic. All cells had different chromosomal complements.</td>
<td>3:1 Tertiary (5, 11, der5)</td>
<td>Fully chaotic</td>
</tr>
<tr>
<td>2 Grade 2+ 8-cell</td>
<td>3 cells biopsied. Nucleus 1, 2, 3: balanced</td>
<td>Embryo was transferred</td>
<td>Alternate</td>
<td>Balanced</td>
</tr>
<tr>
<td>3 Grade 2 4-cell</td>
<td>2 cells biopsied. Nucleus 1, 2: balanced</td>
<td>Embryo was transferred</td>
<td>Alternate</td>
<td>Balanced</td>
</tr>
<tr>
<td>4 Grade 2+ 8-cell</td>
<td>2 cells biopsied. Results obtained from one nucleus only. Nucleus 1: Trisomy for 11pter→11q25 &amp; monosomy for 11q25→qter</td>
<td>8 cell stage. 8 nuclei analysed Uniformly abnormal: Trisomy for 5q34→qter &amp; monosomy for 11q25→qter</td>
<td>Adjacent-1 (5, der11)</td>
<td>Mosaic aneuploid</td>
</tr>
</tbody>
</table>

Aneuploid
<table>
<thead>
<tr>
<th>Grade</th>
<th>oocytes collected</th>
<th>fertilised</th>
<th>Abnormalities</th>
<th>Chromosomal Abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>9</td>
<td>7</td>
<td>1 cell biopsied. No results were obtained</td>
<td>8 cell stage- results obtained from one nucleus only:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No Result</td>
<td>Balanced</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td></td>
<td>2 cells biopsied</td>
<td>Morula-did not fully lyse. Degenerated chromatin. 3Nuclei analysed.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nucl.eus 1 &amp;2: Trisomy for 5pter→q34 &amp; for 11pter→q25.</td>
<td>Chaotic</td>
</tr>
</tbody>
</table>

9 oocytes collected, 7 oocytes fertilised. 6 embryos were biopsied. All biopsied embryos were analysed on follow up.
Results

In the second ICSI/PGD cycle 12 oocytes were collected, 10 fertilised normally and 8 embryos were found suitable for biopsy. Two cells were biopsied on day three post-insemination from two 6-cell embryos (2 and 5). Two cells were also obtained from embryo 3 (a 7-cell) and embryo 7 (a 4-cell). Interestingly the cell biopsied from embryo 2(a) was binucleate. A single cell biopsy only was possible from embryos four and six, at the 5-cell stage, and also from embryos eight and ten which were at the 4-cell stage. FISH analysis of the biopsied blastomeres showed three embryos (6, 8, and 10) that were balanced based on the biopsied cells. Embryo two showed tetrasomy for 11pter→q25 and pentasomy for 11q25→qter while the second nucleus of the same cell showed tetrasomy for region 11pter→q25 and heptasomy for region 11q25→qter. Both blastomeres from embryo 3 showed monosomy for region 11q25→qter and trisomy for region 5q34→qter. Blastomeres biopsied from embryo five were trisomic for 5pter→q34 and monosomic for 11pter→q25. The first cell of embryo seven showed interchange trisomy for chromosome 5. Interestingly the second biopsied cell was balanced for the chromosomes examined. The three embryos found to be balanced were transferred on day four post-insemination but no pregnancy resulted.

This diagnosis was confirmed by analysis of the remainder of five of the biopsied untransferred (embryos 2, 3, 4, 5 and 10). Embryos 1 and 9 that were not suitable for biopsy were also analysed. Embryo two (a) was aneuploid/balanced mosaic with one cell line balanced (7 cells) and the other (16 cells) showing trisomy for 5pter→q34 and monosomy for 5q34→qter. Embryo three reached the blastocyst stage by day four post-insemination but results were only recovered from two nuclei, the first showing trisomy for region 11pter→q25 and monosomy for regions 11q25→qter and 5q34→qter and the second showing trisomy for 5q34→qter & monosomy for 11q34→qter. Embryo four (b) which was compacting was classified as aneuploid/balanced (mosaic) with 2 cells balanced, 6 cells showing trisomy for region 5pter→q34 and monosomy for region 5q34→qter, 1 cell tetraploid and 4 cells chaotic. Embryo five also compacting was chaotic/aneuploid with no balanced cells detected. Embryo seven was uniformly abnormal with all seven cells showing interchange trisomy for chromosome 5. No results were available for embryo 9, which showed developmental arrest (on day four it was a four-cell stage) and spreading was unsuccessful due to persistant cytoplasm. Based on the FISH analysis the possible segregation patterns of the oocytes were deduced. Three embryos derived from an
alternate segregation pattern (6, 8, and 10), 3 embryos were a product of an adjacent-1 segregation pattern (2, 3, and 4), one embryo was a product of adjacent-2 segregation pattern (embryo 5) and one embryo could have arisen from a 3:1 interchange segregation pattern (embryo 7). Table 3.21 summarises this cycle of treatment.
Table 3.21. Classification of embryos from the 2nd PGD cycle of patient 17-A. Karyotype 46,XX,t(5;11)(q34;q25)

<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; NO OF CELLS</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>RESULTS FROM SPARE EMBRYO ON D5 ACCORDING TO FISH PERFORMED WITH PROBES SELECTED FOR THE CASE</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION &amp; COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 2- 2-cell</td>
<td>Embryo not suitable for biopsy</td>
<td>Embryo lysed.</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>2 Grade 2- 6-cell</td>
<td>1 binucleate cell biopsied</td>
<td>Morula stage-23 nuclei. Mosaic with one cell line balanced (7 cells) and the other (16 cells) trisomy for 11q25→qter &amp; monosomy 5q34→qter</td>
<td>Adjacent-1 (11, der5)</td>
<td>Aneuploid/balanced mosaic. The embryo apparently became mosaic by losing the terminal region of 5.</td>
</tr>
</tbody>
</table>

*Embryo a***

Aneuploid/Mosaic

Aneuploid/Balanced
<table>
<thead>
<tr>
<th>Grade</th>
<th>Cells</th>
<th>Results</th>
<th>Adjacent</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>7-cell</td>
<td>2 cells biopsied</td>
<td>Aneuploid</td>
<td>Adjacent-1 (5, der11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nucleus 1 &amp; 2: Trisomy 5q34→qter &amp; monosomy for 11q34→qter</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blastocyst. Results were obtained from two nuclei</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nucleus 1: showing trisomy for 11p→q25 &amp; monosomy for both 11q→qter &amp; 5q→qter (loss of der5, &amp; duplication of der11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nucleus 2: Trisomy 5q34→qter &amp; monosomy for 11q34→qter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5-cell</td>
<td>1 cell biopsied: balanced</td>
<td>Balanced</td>
<td>Adjacent-1 (11, der5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Compacting (13 nuclei analysed)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 nuclei: balanced, 6 nuclei: trisomy for 11q25→qter &amp; monosomy 5q34→qter</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 nucleus: tetraploid. 4 Nuclei: chaotic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6-cell</td>
<td>2 cells biopsied.</td>
<td>Aneuploid/Balanced</td>
<td>Adjacent-2 (5, der 5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nucleus 1 &amp; 2: Trisomy 5pter→q34 &amp; monosomy for 11pter→q25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Compacting (8 nuclei) Chaotic-No normal cells found</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Embryo b***
<table>
<thead>
<tr>
<th></th>
<th>Aneuploid</th>
<th>Chaotic/Aneuploid</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Grade 2+ 5-cell</td>
<td>1 cell biopsied.</td>
<td>Embryo transferred</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nucleus 1: balanced</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Balanced</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Grade 2+ 4-cell</td>
<td>2 cells biopsied</td>
<td>7 cell stage (7 nuclei analysed) All showed interchange trisomy 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nucleus 1: balanced</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nucleus 2: Interchange trisomy 5 nuc, ish, + der(5)(q34), q25), der(11)(q34)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Balanced/Aneuploid</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aneuploid</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Grade 2+ 4-cell</td>
<td>1 cell biopsied.</td>
<td>Embryo transferred</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nucleus 1: balanced</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Balanced</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Grade 2- 2-cell</td>
<td>Embryo not suitable for biopsy</td>
<td>4 cell stage. Persistent cytoplasm not analysable</td>
</tr>
</tbody>
</table>
12 oocytes were collected, 10 oocytes were fertilised. 8 embryos were biopsied. 10 embryos were analysed. All biopsied embryos gave results. Classification of the two non-biopsied embryos was not possible, as results were not obtained.

***Embryos classified as abnormal with a balanced cell line present
**Results**

In the third ICSI/PGD cycle 13 oocytes were collected, 12 oocytes were fertilised normally and 8 embryos were found suitable for biopsy. Two cells were biopsied on day three post-insemination from three 7-cell embryos (embryos 3, 7 and 11). A single cell biopsy only was possible from five embryos (embryos 2, 8, 9, 10 and 12) at the 5-cell stage. FISH analysis showed two embryos (3 and 12) that were balanced based on the biopsied cells (cell from embryo 12 was in metaphase and therefore more reliable). The biopsied cells of the third cycle were re-FISHed using the chromosome 11 subtelomeric probe (11qtel, SO) as this is the least reliable, and the diagnosis was confirmed for all the biopsied blastomeres. Blastomeres biopsied from embryos 7, 8, and 11 failed to provide results. Embryo 2 was trisomic for chromosome 11 and monosomic for chromosome 5. Embryos 9, and 10 showed monosomy for 11q25—qter and trisomy for 5q34—qter. The two embryos diagnosed as balanced (3 and 12) were transferred on day four and resulted in a normal live birth. The karyotype of the fetus revealed a male carrier of the translocation.

This diagnosis was confirmed by analysis of the remainder of six of the biopsied untransferred embryos (embryos 2, 7, 8, 9, 10, and 11). Embryos 1, 4, 5 and 6 that were not suitable for biopsy were also subjected to analysis. Furthermore, embryos 1, 2, 6, 8, 10 and 11 were biopsied on day 4 to obtain 1-2 blastomeres for CGH analysis. The untransferred embryos were originally FISH-ed with the chosen probe combination to confirm the diagnosis. A second round of FISH was performed employing the Vysis UroVysion Multicolcor Probe [3 Spectrum Red (SR), 7(SG), 17 Spectrum Aqua (SA), 9 Spectrum Gold] for the purpose of obtaining more information as to the chromosomal complement of the spare embryos. Fig. 3.8 shows FISH performed on spare embryos using the same probes as for the diagnosis and the same blastomeres following a second round of FISH using the UroVysion probe set. Finally, a third round was performed using probes from the Vysis AneuVysion Prenatal Kit (X SG, Y SO, 18 SA). Embryos 1, 5 and 6 were fully chaotic. Embryo 4 was aneuploid/chaotic for the translocation and diploid/chaotic for the UroVysion panel chromosomes. Embryo 7 was balanced for the translocation and diploid/chaotic for chromosomes 3, 7, 9 and 19. Embryo 9 was uniformly abnormal showing monosomy 11q25—qter & trisomy 5q34—qter. Embryos 10 and 11 were classified as aneuploid and aneuploid/balanced for the translocation respectively, and were both diploid/aneuploid for the chromosomes examined in the second round of FISH.
Fig. 3.8 FISH analysis on spare embryos from the third PGD cycle for case 17-A 46,XX,t(5;11)(q34;q25).

(i), (ii), (iii) a: FISH analysis employing the same probe combination as used for the PGD performed for couple A showing (i): Trisomy 5 and monosomy 11, (ii): Monosomy 5pter->5q34, nullisomy 11(iii): Trisomy 5pter->5q34. (i'), (ii'). (iii') b: A second round of FISH performed on the same blastomeres employing probes for chromosomes 3, 7, 9, and 17, showing: (i'): Trisomy 7 (ii'): Trisomy 7, monosomy 3, (iii'): Trisomy 7, monosomy 3.
Results

Based on the FISH analysis the possible segregation patterns of the oocytes were deduced. The majority of the embryos derived from an alternate segregation pattern (embryos 2, 3, 7, 11 and 12) while embryos 4, 8, 9 and 10 were products of an adjacent-1 segregation pattern. Finally for embryos 1, 5, and 6 it was not possible to deduce the segregation pattern. Table 3.22 summarises this cycle of treatment.
Table 3.22. Classification of embryos from the 3rd PGD cycle of patient 17-A. Karyotype 46,XX,t(5;11)(q34;q25)

<table>
<thead>
<tr>
<th>EMBRYO NO</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>RESULTS FROM SPARE EMBRYO ON D4 ACCORDING TO FISH PERFORMED WITH PROBES SELECTED FOR THE CASE</th>
<th>RESULTS FROM SPARE EMBRYO ON D4 ACCORDING TO SECOND ROUND OF FISH PERFORMED WITH THE VYSIS UROVYSIS PANEL PROBES</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION &amp; COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Grade 2+</td>
<td>Chaotic (no normal cells)</td>
<td>Chaotic</td>
<td>Unknown.</td>
<td>Fully chaotic (no normal cells)</td>
</tr>
<tr>
<td>2</td>
<td>Grade 2+</td>
<td>1 cell biopsied.</td>
<td>4 cells. 4 Nuclei analysed 2 nuclei: balanced</td>
<td>Alternate</td>
<td>Balanced/chaotic mosaic</td>
</tr>
<tr>
<td></td>
<td>5-cell</td>
<td></td>
<td>2 nuclei: balanced</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>**</td>
<td></td>
<td>2 nuclei: nullisomy 11, monosomy 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*** embryo c</td>
<td></td>
<td><strong>Aneuploid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Grade 2+</td>
<td>2 cells biopsied.</td>
<td>Embryo transferred</td>
<td>Alternate</td>
<td>Balanced for the translocation</td>
</tr>
<tr>
<td></td>
<td>7-cell</td>
<td></td>
<td><em>Balanced</em>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>Grade</td>
<td>Embryo not suitable for biopsy</td>
<td>Nuclei Analysed</td>
<td>Results</td>
<td>Mosaic Status</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>-------------------------------</td>
<td>-----------------</td>
<td>---------</td>
<td>---------------</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>8 nuclei analysed. 5 nuclei: monosomy 11q25→qter &amp; trisomy 5q34→qter. 1 nucleus: Balanced</td>
<td>Diploid/Chaotic</td>
<td>The 5 adjacent-1 products were normal for the chromosomes examined the rest were abnormal</td>
<td>Aneuploid/Chaotic mosaic One cell balanced for the translocation in aneuploid embryo</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2 nuclei analysed. Both chaotic</td>
<td>Chaotic</td>
<td>Both nuclei chaotic</td>
<td>Unknown</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>5 nuclei analysed. All chaotic</td>
<td>Chaotic</td>
<td>All nuclei chaotic</td>
<td>Unknown</td>
</tr>
<tr>
<td>7</td>
<td>2+</td>
<td>2 cells biopsied. No results were obtained</td>
<td>Balanced</td>
<td>4 nuclei were diploid for the chromosomes examined. The other 3 were chaotic</td>
<td>Alternate</td>
</tr>
</tbody>
</table>

**Note:** The table provides a summary of the results from the analysis of embryos, including the number of cells biopsied, whether the embryos were suitable for biopsy, the number of nuclei analysed, and the results for each cell. The mosaic status is also indicated for each case.
<table>
<thead>
<tr>
<th>Grade</th>
<th>Cell Number</th>
<th>Aneuploid/Balanced</th>
<th>Aneuploid/Diploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>5-cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>**</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*** embryo d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 cell biopsied-No results were obtained</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 cells. 7 nuclei analysed</td>
<td>3 cells including 2 classified as balanced for the translocation were normal for the chromosomes examined. The rest were aneuploid for chromosomes 3 &amp; 17 showing trisomy and monosomy of the chromosomes. All nuclei were diploid for 7 &amp; 9.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 nuclei: balanced</td>
<td>Adjacent-1 (11, der5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 nuclei: monosomy for 5q34→qter &amp; trisomy for 11q25→qter</td>
<td>Aneuploid/balanced mosaic. Embryo started off as adjacent-1; 3 cells were normal for the UroVysion panel. The monosomy and trisomy exhibited for chromosomes 3 &amp; 17 was due to mitotic nondisjunction</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>9</th>
<th>5-cell</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 cell biopsied. Nucleus 1: Monosomy 11q25→qter &amp; trisomy 5q34→qter.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 cells. 9 nuclei analysed</td>
<td>Nuclei could not be found</td>
</tr>
<tr>
<td></td>
<td></td>
<td>all showed monosomy 11q25→qter &amp; trisomy 5q34→qter. Embryo uniformly abnormal</td>
<td>Adjacent-1 (5, der11).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aneuploid</td>
</tr>
</tbody>
</table>

Aneuploid/Balanced

Aneuploid

Aneuploid/Diploid

Aneuploid
<table>
<thead>
<tr>
<th>Grade</th>
<th>Cells</th>
<th>Biopsied</th>
<th>Analysed</th>
<th>Chromosome Details</th>
<th>Segregation Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5-cell</td>
<td>1 cell</td>
<td>6 cells</td>
<td>Nucleus 1: Monosomy 11q25→qter &amp; trisomy 5q34→qter.</td>
<td>Adjacent-1 (5, der11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>biopsied</td>
<td>6 nuclei</td>
<td>Uniformly abnormal</td>
<td>D1ploid/Aneuploid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 nuclei</td>
<td>Diploid for all the chromosomes examined. 2 nuclei: Diploid for 3, 7 &amp; 9 but one trisomic and the other monosomic for chromosome 17</td>
<td>Balanced/Aneuploid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Alternative</td>
</tr>
<tr>
<td>11</td>
<td>7-cell</td>
<td>2 cells</td>
<td>5 nuclei</td>
<td>Balanced/ Aneuploid</td>
<td>Balanced/aneuploid mosaic with one cell line balanced (3 cells) and the other chromosomally abnormal with chromosome 5 having lost der11. The embryo was mosaic for trisomy 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>biopsied</td>
<td></td>
<td></td>
<td>Alternate</td>
</tr>
<tr>
<td>12</td>
<td>5-cell</td>
<td>1 cell</td>
<td></td>
<td>Balanced</td>
<td>Normal/balanced</td>
</tr>
<tr>
<td></td>
<td></td>
<td>biopsied</td>
<td></td>
<td></td>
<td>Alternate</td>
</tr>
</tbody>
</table>

13 oocytes were collected, 12 oocytes were fertilised. 8 embryos were biopsied. All 8 biopsied embryos were analysed plus 4 embryos not found suitable for biopsy.

** Embryos were biopsied on D4 to obtain single blastomere for CGH analysis

***Embryos classified as abnormal with a balanced cell line present
Taking into account all the investigations performed with the diagnostic probes, in total there were 28 embryos with 10 classed as balanced, four as aneuploid, 5 as fully chaotic, while the remaining nine were at least partly chaotic. Seven balanced embryos were transferred. Further investigation of chromosomes 3, 9, 7, and 17 on spare embryos generated from the third cycle confirmed that this instability affected chromosomes other than those involved in the translocation. Of particular note was the finding in two embryos from cycle 2 and three from cycle 3 of balanced and aneuploid cells in the same embryo (Table 3.39). In the first example (embryo a)(Table 3.42) the biopsied cell was a binucleate aneuploid. On day 5 the embryo contained 23 cells, 16 were consistent with being the product of adjacent 1 segregation while seven cells were apparently balanced with two signals only for the chromosome 11 sub-telomere probe. Similarly in embryo d from the third cycle, 4 cells were consistent with being the product of adjacent-1 segregation while three cells were apparently balanced. In embryo b, the biopsied cell was balanced; on day 5 the embryo consisted of 13 cells, two apparently balanced, six cells the product of adjacent-1 segregation and the remainder with other abnormalities suggesting a chaotic component. The difference again revolved around the chromosome 11 sub-telomere probe; the six partially aneuploid cells had three copies while the balanced cells had two only. In embryos c and e, from cycle 3, two and three cells respectively were balanced, while the mechanism of chromosome loss lead in the case of embryo c to nullisomy for chromosome 11 and monosomy for chromosome 5 in two cells, and in the case of embryo e, to two cells having lost a derivative chromosome 5 and a chromosome 11.

Based on the results of the biopsied cells and of the spare embryos it was concluded that 11 embryos were derived from an alternate (balanced) segregation pattern of gamete formation, 8 from adjacent-1, and one each from adjacent-2, 3:1 tertiary, and 3:1 interchange (all unbalanced) while for four embryos the segregation pattern was unknown.
3.4.2 Case 20-B: Reciprocal Translocation 46,XX,t(1;2)(q42.1;p23).

Couple B were referred for PGD as the female partner 32 years old, was the carrier of a reciprocal translocation 46,XX,t(1;2)(q42.1;p23). The male partner was found to be karyotypically normal. The couple had suffered years of secondary infertility having had a social termination of pregnancy in the past followed by two missed abortions.

A PGD protocol was developed for this case to exclude any imbalance of the chromosomes involved in the translocation. Two of the probes pUC 1.77, satellite III probe insert size 1.77kb-specific for the heterochromatic region of human chromosome 1 SO & SG (Cooke and Hindley 1979) and the subtelomeric probe Iqtel SO (Vysis, U.K.) flank the breakpoint on chromosome 1 and the third probe was the alpha-satellite biotinylated probe for chromosome 2 (Appligene Oncor). Using this FISH probe combination, 86% control lymphocyte nuclei (n=200) showed three clear signals per nucleus. Before clinical application these probes were also tested on blastomeres from day 3 post-insemination embryos, showing discrete FISH signals easily scored in all nuclei. Fig. 3.9 shows the PGD strategy employed for this case tested on patient lymphocytes. Fig. 3.10 shows the probes employed for the PGD case tested on the triploid fibroblast cell line and on blastomeres.

In the IVF cycle sixteen oocytes were collected and eleven fertilised (68.7%) resulting in all 11 embryos being suitable for biopsy. Two cells were biopsied on day three post-insemination from six embryos (embryos 5, 6, 7, 8, 9 and 10) at the 5-7 cell stage. A single cell biopsy only was possible from embryos 1, 2, 3, 4 and 11 which were all at the 4-cell stage except embryo four that was a 3-cell. FISH analysis of the biopsied blastomeres showed two embryos (4 and 5) that were balanced based on all biopsied cells. Blastomeres biopsied from embryos 2, 3, 7 and 8 failed to give results. Embryo one was trisomic for region 2pter—>2p23 and monosomic for region 1q42.1—>qter based on one cell biopsied. One blastomere from embryo six showed tertiary trisomy for regions 2p23—pter and 1pter—>1q42.1, while the other one was trisomic for region 1q42.1—>qter and monosomic for region 2p23—qter. Only one nucleus was analysed from the two biopsied from embryo 9, this was shown to be haploid. One blastomere from embryo ten was trisomic for region 2p23—qter and monosomic for region 1pter—>q42.1.
Fig. 3.9 PGD for case 20-B 46,XX,t(1;2)(q42.1;p23)

(a), (b): Strategy devised for PGD performed from case 20-B 46,XX,t(1;2)(q42.1;p23).
(c) FISH analysis of patient lymphocyte metaphase nucleus showing the normal and derivative chromosomes.
Fig. 3.10 FISH analysis on triploid fibroblasts and a blastomere from a spare embryo using the probe combination employed for PGD case 20-B 46,XX,t(1;2)(q42.1;p23) as preliminary work up.

(i), (ii): Triple colour FISH analysis on a metaphase (i) and and interphase (ii) nucleus of a triploid fibroblast cell line treated with hypotonic solution (i) or PBS (ii). Three copies of each probe are detected in both (i) and (ii). (iii): The same probe combination on a blastomere from a spare embryo, showing disomy for both chromosomes 1 and 2.
Finally, the cell biopsied from embryo eleven was trisomic for region 2p23→pter and monosomic for region 1q42.1→qter. The two embryos found to be balanced (embryos 4 and 5) were transferred on day four post-insemination but did not result in a pregnancy.

This diagnosis was confirmed by analysis of the remainder of the biopsied untransferred embryos (embryos 1, 2, 3, 6, 7, 8, 9, 10 and 11). Spare embryos 1, 6, 10 and 11 failed to provide results following spreading on day-4 mostly due to persistent granular cytoplasm therefore the embryo classification was based on the biopsied blastomeres. Classification for embryos 2 and 3 was not possible as no results were available at either stage. Embryos 1 and 11 were classified as aneuploid based on the one cell analysed. Embryo 6 was described as aneuploid/mosaic and embryo 10 was classified as chaotic based on the two biopsied blastomeres. Based on the four cells analysed from spare embryo 8 the embryo was classified as uniformly aneuploid. Finally, embryo 7 was classified as chaotic/balanced whilst embryo 9 was classed as chaotic/aneuploid.

Twenty per cent of the embryos analysed were found to be chromosomally balanced while the great majority (80%) were classified as abnormal. The embryos were of very poor quality; results obtained on three of the nine spare embryos showed one aneuploid and two chaotic mosaics. From the results of the biopsied cells and the spare embryos it was concluded that two embryos derived from an alternate segregation pattern of the gamete, three from adjacent-1, while for four embryos the segregation pattern was unknown. Table 3.23 summarises this cycle of treatment.
Table 3.23. Classification of embryos from the single PGD cycle of patient 20-B. Karyotype 46,XX,t(1;2)(q42.1;p23)

<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; NO OF CELLS</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>RESULTS FROM SPARE EMBRYO ON D4.</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION &amp; COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 2-4-cell</td>
<td>1 cell biopsied.</td>
<td>Embryo arrested with persistant granular cytoplasm</td>
<td>Adjacent-1 (2,der1)</td>
<td>Aneuploid (biopsied cell)</td>
</tr>
<tr>
<td></td>
<td>Trisomy 2p23→pter &amp; Monosomy for 1q42.1→qter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Aneuploid</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Grade 2 4-cell</td>
<td>1 cell biopsied</td>
<td>Morula stage. Spreading resulted in pieces of degenerated chromatin</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>No result</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Grade 2 4-cell</td>
<td>1 cell biopsied. Nuclei were lost during spreading</td>
<td>4-cell stage very fragmented. No nuclei were seen during spreading.</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>No result</td>
<td>No result</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Grade 2-3 3-cell</td>
<td>1 cell biopsied. Balanced for the translocation</td>
<td>Embryo transferred</td>
<td>Alternate</td>
<td>Balanced (Based on biopsied cell)</td>
</tr>
<tr>
<td></td>
<td>Balanced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>Cells</td>
<td>Biopsied</td>
<td>Embryo Transferred</td>
<td>Alternate</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>----------</td>
<td>--------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>5</td>
<td>7-cell</td>
<td>2 cells</td>
<td>Balanced</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>biopsied</td>
<td></td>
<td>Embryo transferred</td>
</tr>
<tr>
<td>6</td>
<td>6-cell</td>
<td>2 cells</td>
<td>4 cell stage arrested and very cytoplasmic. No nuclei were seen during spreading</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td>biopsied</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>7-cell</td>
<td>2 cells</td>
<td>5 cells. 5 Nuclei analysed</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td>biopsied</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>5-cell</td>
<td>2 cells</td>
<td>Adjacent-1 (2, der1)</td>
<td>Aneuploid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>biopsied</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>Cells</td>
<td>Nucleus 1</td>
<td>Nucleus 2</td>
<td>Analysis</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>-----------</td>
<td>-----------</td>
<td>----------</td>
</tr>
<tr>
<td>2</td>
<td>2-cell</td>
<td>lost during spreading</td>
<td>Haploid</td>
<td>9 nuclei analysed</td>
</tr>
<tr>
<td>2</td>
<td>2-cell</td>
<td>Trisomy for 2p23→qter and monosomy 1pter→q42.</td>
<td>Aneuploid mosaic</td>
<td>5 cell stage-no nuclei were seen during spreading</td>
</tr>
<tr>
<td>2-</td>
<td>4-cell</td>
<td>Partial trisomy 2p23→pter and monosomy 1q42.1→qter</td>
<td>Aneuploid</td>
<td>6cell stage-no nuclei were seen during spreading</td>
</tr>
</tbody>
</table>

11 oocytes were fertilised. 11 embryos were biopsied. All 11 were analysed.

***Embryos classified as abnormal with a normal cell line present
3.4.3 Case 13-C: Reciprocal Translocation

46,XX,t(16;17)(p13.3;p11.1).

Couple C were referred for PGD as the female partner, 33 years old, was the carrier of a reciprocal translocation 46,XX,t(16;17)(p13.3;p11.1). The male partner was found to be karyotypically normal. In the past they had suffered one termination of pregnancy for social reasons and three first trimester miscarriages. The patient underwent hysterosalpingography and presented with a unicornuate uterus and a normal patent left fallopian tube.

The strategy chosen for this case relies on three probes to detect all possible unbalanced translocation products in the embryo. Probes chosen were the satellite II DNA/D16Z3 16q11.2 probe (SO & SG), which maps to the centromeric region of chromosome 16 and the Miller-Dieker region probe detection set (LIS1 SO/RARA17q21.1 SG control probe), containing two probes which flank the breakpoint on chromosome 17, all from Vysis, U.K. Using this FISH probe combination, 92% of control lymphocyte nuclei (n=200) showed three clear signals per nucleus. Before clinical application these probes were also tested on blastomeres from day 3 post-insemination embryos, showing discrete FISH signals easily scored in all nuclei. Fig. 3.11 shows the PGD strategy employed for this case tested on patient lymphocytes. Fig. 3.12 shows the probes employed for the PGD case tested on the triploid fibroblast cell line and on blastomeres.

A single IVF/PGD treatment cycle was carried out for couple C. Eight oocytes were collected, all eight fertilised (100%) and seven were suitable to be biopsied. Two cells were biopsied on day three post-insemination from four embryos between 6 and 9 cell stage (embryos 2, 3, 4 and 7). A single cell biopsy only was possible from embryos 5 and 6 that were at 5 and 4-cell stage respectively, whilst three blastomeres were biopsied from the 7-cell stage embryo 1. FISH analysis of the biopsied blastomeres showed four embryos (3, 4, 5 and 7) that were balanced based on the biopsied cells. The diagnosis was based on FISH analysis on both biopsied blastomeres only for embryo 4 and 7. The two blastomeres biopsied from embryo 1 showed interchange monosomy 16, whilst the third biopsied cell was haploid.
(a), (b): Strategy devised for PGD performed from case 13-C 46,XX,t(16;17)(p13.3;p11.1).
(c) FISH analysis of patient lymphocyte metaphase nucleus showing the normal and derivative chromosomes.
Fig. 3.12 FISH analysis on a triploid fibroblast metaphase and two blastomeres from spare embryos using the probe combination employed for PGD case 13-C 46,XX,t(16;17)(p13.3;p11.1).

(a): Triple colour FISH analysis on a nucleus of a triploid fibroblast cell line treated with hypotonic solution. Three copies of each probe are detected. (b): The same probe combination on two blastomeres from spare embryos from this PGD case on day 4 post insemination showing (i): Tetrasomy for chromosome 17 and nullisomy for 16p13.3-pter, and (ii): Tetrasomy for both chromosomes 16 and 17.
Results

One of the blastomeres biopsied from embryo two was trisomic for region 17p11.1→pter and nullisomic for region 16p13.3→qter whilst the second one was disomic for chromosome 17 and nullisomic for region 16p13.3→qter. Finally, the single cell biopsied from embryo 6 revealed five micronuclei during spreading with only one providing results, being monosomic for region 16p13.3→qter and nullisomic for region 17p11.1→pter. The three embryos scored as balanced for the chromosomes examined (3, 4 and 7) were transferred on day four post-insemination and resulted in a biochemical pregnancy that failed to progress to a clinical one.

This diagnosis was confirmed by analysis of the remainder of the biopsied untransferred embryos (embryos 1, 2, 5, 6) and embryo 8 that was not biopsed. No result was available for the remainder of embryo 5, which was compacting at the time of spreading on day 4 as the preparation proved not to be analysable. Therefore embryo 5 was classified balanced based on the single biopsied blastomere. Embryos 2, 6 and 8 were classified as fully chaotic with no normal cells, while embryo 1 was classified as aneuploid/haploid. Based on the results of the biopsied cells and the spare embryos it was inferred that four embryos derived from an alternate segregation pattern at meiosis (embryos 3, 4, 5, and 7), one from 3:1 interchange, while for three embryos the segregation pattern was unknown. Table 3.24 summarises this cycle of treatment.
Table 3.24. Classification of embryos from the single PGD cycle for patient 13-C. Karyotype 46,XXt,(16;17)(p13.3;q11.1)

<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; NO OF CELLS</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>RESULTS FROM SPARE EMBRYO ON D4.</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION &amp; COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 1-7-cell</td>
<td>3 cells biopsied.</td>
<td>4 cell stage. Mosaic with a haploid cell line (2 cells) and another showing disomy for 17 and nullisomy for 16p13.3→pter (2 cells)</td>
<td>According to biopsied blastomeres 1 &amp; 2: Interchange 3:1 (17,-)</td>
<td>Aneuploid/haploid</td>
</tr>
<tr>
<td></td>
<td>Nuclei 1, 2: Interchange monosomy 16.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nucleus 3: Haploid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aneuploid/Haploid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Grade 1-7-cell</td>
<td>2 cells biopsied.</td>
<td>4 cell stage</td>
<td>Unknown</td>
<td>Fully chaotic (no normal cells)</td>
</tr>
<tr>
<td></td>
<td>Nuclei 1: Trisomy for 17p11.1→pter. &amp; nullisomy for 16p13.3→pter.</td>
<td>Chaotic (no normal cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nucleus 2: disomy for 17 and nullisomy for 16p13.3→pter.</td>
<td>Chaotic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aneuploid/Mosaic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Grade 2 8-cell</td>
<td>2 cells biopsied-Result from 1 cell</td>
<td>Embryo transferred</td>
<td>Alternate</td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td>Nucleus 1: Balanced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>Cells Biopsied</td>
<td>Details</td>
<td>Status</td>
<td>Notes</td>
</tr>
<tr>
<td>-------</td>
<td>---------------</td>
<td>---------</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td>4</td>
<td>2 cells</td>
<td>Embryo transferred</td>
<td>Alternate</td>
<td>Balanced</td>
</tr>
<tr>
<td>5</td>
<td>1 cell</td>
<td>Embryo was compacting, nuclei were faint and could not be analysed</td>
<td>Alternate</td>
<td>Based on one nucleus: balanced</td>
</tr>
<tr>
<td>6</td>
<td>1 cell</td>
<td>5 cell stage-2 nuclei analysed</td>
<td>Unknown-</td>
<td>Fully chaotic (no normal cells)</td>
</tr>
<tr>
<td>7</td>
<td>2 cells</td>
<td>Embryo transferred</td>
<td>Alternate</td>
<td>Balanced</td>
</tr>
<tr>
<td>Embryo</td>
<td>Grade</td>
<td>Stage</td>
<td>Nuclei</td>
<td>Unknown</td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>-------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2-3</td>
<td>3-6</td>
<td>5-6 cell stage and fragmented. 3 nuclei were analysed. <strong>Nucleus 1</strong>: Disomic for 17 and nullisomic for 16p13.1-16qter. <strong>Nucleus 2</strong>: Disomy 16 and monosomy 17. <strong>Nucleus 3</strong>: Tetrasomy for 17 and nullisomy for 16p13.1-16qter</td>
</tr>
</tbody>
</table>

8 oocytes were fertilised. 7 embryos biopsied. All 7 biopsied embryos were analysed plus 1 embryo not found suitable for biopsy. Tetrasomy 16 & 17 in embryos 8 and 6. Nullisomy 16 in embryos 1 and 8.
3.4.4 Case 6-D: Reciprocal Translocation 46,XY,t(5;19)(p12;p12).

Couple 6-D were referred for PGD due to a paternal balanced reciprocal translocation 46,XY,t(5;19)(p12;p12). The couple suffered primary infertility and the male partner was oligoasthenozoospermic. The maternal karyotype was normal and maternal age was 25 years.

A PGD protocol was developed for this case to exclude any imbalance of the chromosomes involved in the translocation. The two locus specific probes of the dual Cri Du Chat LSI EGR1 SO/SG (Vysis UK) were employed to flank the breakpoint on chromosome 5, with SO mapping to 5q31 and SG mapping to 5p15.2. The third probe was the laboratory prepared YAC locus specific probe for chromosome 19 mapping to position 19q13.2 (Frengen et al., 1999) labelled in SO and SG using the Vysis UK nick translation kit. Using this FISH probe combination, 96.75% of control lymphocyte nuclei (n=200) showed three clear signals per nucleus. Before clinical application these probes were also tested on blastomeres biopsied from day 3 post-insemination embryos, showing discrete FISH signals easily scored in all nuclei. Fig. 3.13 shows the PGD strategy employed for this case tested on patient lymphocytes and on blastomeres.

A single ICSI/PGD treatment cycle was carried out for couple D. Twenty oocytes were collected, 15 fertilised (75%) and 13 were suitable to be biopsied. Two cells were biopsied on day three post-insemination from all 13 embryos between the 6 and 8 cell stage. A single embryo was diagnosed as balanced and transferred, but no pregnancy ensued. All remaining untransferred embryos were spread to confirm the diagnosis. Unfortunately technical difficulties amplifying the YAC locus specific probe for chromosome 19 were experienced after PGD treatment, and consequently the spare embryos were analysed employing the Cri Du Chat dual probe only. The latter meant that information was obtained for chromosome five and the two derivatives but not for chromosome 19. Of the 12 non-transferred embryos, eight were found to be chaotic, and three aneuploid or aneuploid mosaic. The high number of chaotic embryos meant that segregation could be determined for two only; one alternate and the other adjacent 1. Analysis of sperm showed that 54% were the result of alternate segregation; 18% of adjacent 1, 11% of adjacent 2 and 13% of 3:1 disjunction. Due to the chaotic nature of the majority of these embryos it was
impossible to ascertain the exact chromosomal constitution of each blastomere. Therefore, a more generalised table presents the interpretation of the results. Table 3.25 summarises this cycle of treatment.
Fig. 3.13 PGD for case 6-D 46,XY,t(5;19)(p12;p12)

(a), (b): Strategy devised for PGD performed from case 6-D 6,XY,(5;19)(p12;p12).
(c) FISH analysis of patient lymphocyte metaphase nucleus showing the normal and derivative chromosomes.

(d) FISH analysis of blastomeres from biopsied (i) and spare embryos (ii), (iii) for PGD.
(i): The same probe combination applied to a blastomere biopsied from a day 3 post-insemination embryo during PGD for this couple, showing (i) disomy for both chromosomes 5 and 19. Application of the dual probe only for chromosome 5 on blastomeres from spare embryos of couple D showing (ii) trisomy 5p15.2->pter and tetrasomy 5q31.1->qter, (iii) trisomy for 5p12->qter.
Table 3.25. Classification of embryos from the single PGD cycle of patient 6-D. Karyotype 46,XY,t(5;19)(p12;p12)

<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; NO OF CELLS</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>RESULTS FROM SPARE EMBRYO ON D4*</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION &amp; COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 1-8cell</td>
<td>2 cells biopsied. Nucleus 1: Monosomy 5p12→pter &amp; trisomy 1p12→pter Nucleus 2: Could not be located. <em>Aneuploid</em></td>
<td>6 cell stage-4 nuclei analysed</td>
<td>Unknown- Chaotic</td>
<td>Chaotic</td>
</tr>
<tr>
<td>2 Grade 2 8cell</td>
<td>2 cells biopsied. Nucleus 1:Aneuploid- inconclusive possible failure of hybridisation of 19YAC Nucleus 2: Inconclusive <em>Aneuploid</em></td>
<td>6 cell stage-6 nuclei analysed</td>
<td>Unknown</td>
<td>Chaotic</td>
</tr>
<tr>
<td>3 Grade 2+6cell</td>
<td>2 cells lysed during spreading. <em>No result</em></td>
<td>6 cell stage-6 nuclei analysed</td>
<td>Unknown</td>
<td>Chaotic</td>
</tr>
<tr>
<td>4 Grade 1-7cell</td>
<td>2 cells biopsied. Nucleus 1:Balanced Nucleus 2: Inconclusive <em>Balanced</em></td>
<td>Embryo transferred</td>
<td>Alternate</td>
<td>Balanced (Based on one cell)</td>
</tr>
<tr>
<td>Grade</td>
<td>Cells</td>
<td>Stage</td>
<td>Nuclei</td>
<td>Results</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>7cell</td>
<td>5</td>
<td>Unknown</td>
</tr>
<tr>
<td>Grade 2-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>-</td>
<td>No results</td>
<td>Unknown-</td>
</tr>
<tr>
<td>Grade 2+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>8cell</td>
<td>5</td>
<td>Unknown-</td>
</tr>
<tr>
<td>Grade 2+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>8cell</td>
<td>8</td>
<td>Unknown</td>
</tr>
<tr>
<td>Grade 2+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>8cell</td>
<td>Embryo was compacting. Nuclei were faint 4 were analysed.</td>
<td>Unknown</td>
</tr>
<tr>
<td>Grade 2+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>8cell</td>
<td>8</td>
<td>Unknown-</td>
</tr>
<tr>
<td>Grade 1-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Aneuploid mosaic*
<table>
<thead>
<tr>
<th>Grade</th>
<th>8cell</th>
<th>2 cells biopsied—Both cells lysed during spreading</th>
<th>8 cell stage—6 nuclei analysed Uniformly abnormal.</th>
<th>Unknown-</th>
<th>Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td></td>
<td>2 cells biopsied—Both cells lysed during spreading</td>
<td>No results</td>
<td>Aneuploid</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>Single cell biopsied. Nucleus not located</td>
<td>Embryo was compacting. 6 nuclei analysed.</td>
<td>Adjacent-1</td>
<td>Aneuploid mosaic</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>2 cells biopsied.</td>
<td>6 cell stage—6 nuclei analysed.</td>
<td>Unknown</td>
<td>Chaotic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aneuploid mosaic</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

20 oocytes collected, 15 oocytes were fertilised—13 embryos biopsied.

*All spare D4 embryos were analysed only with the Cri Du Chat probe as the yeast strain carrying the YAC insert failed to grow.*
Results

3.4.5 Case 23-G: Reciprocal Translocation 46,XX,t(8;12)(q11.2;q12).

Couple G were referred for PGD due to a maternal balanced reciprocal translocation 46,XX,t(8;12)(q11.2;q12). The paternal karyotype was normal 46,XY. Chromosomal analysis of two previous spontaneous abortions revealed that both resulted from an adjacent-2 segregation pattern during maternal meiosis. The maternal age was 32 years.

A PGD protocol was developed for this case to exclude any imbalance of the chromosomes involved in the translocation. Two of the probes α-satellite DNA/D8Z2 8p11.1-q11.1 in spectrum green (SG), and the subtelomeric probe 8qtel SO (Vysis, U.K.) flank the breakpoint on chromosome 8 and the third probe was the chromosome 12 α-satellite labelled in SO and SG (Baldini et al., 1990). Using this FISH probe combination, 87.5% of control lymphocyte nuclei (n=200) showed three clear signals per nucleus. Before clinical application these probes were also tested on blastomeres biopsied from day 3 post-insemination embryos, showing discrete FISH signals easily scored in all nuclei. Fig. 3.14 shows the PGD strategy employed for this case tested on patient lymphocytes and on blastomeres.

In the PGD cycle twenty-one oocytes were collected and seventeen fertilised (80.9%) resulting in 13 embryos, being suitable for biopsy on day 3. Two cells were biopsied on day three post-insemination from 7 embryos (embryos 1, 4, 6, 7, 8, 9 and 13) between the 7-8-cell stage. A single cell biopsy only was possible for six embryos (embryos 2, 3, 5, 10, 11 and 12) all being at 4-6-cell stage.

In the single cycle carried out for this couple biopsy results were obtained for seven embryos of which a single embryo was balanced. This embryo (1) was transferred on day four and resulted in a normal live birth. The majority of embryos for which no result was obtained were less than six cells and the single biopsied cell was lost either during spreading or during FISH. Of the 12 untransferred embryos four were either balanced or balanced/aneuploid or balanced/chaotic mosaics, three were aneuploid or aneuploid mosaics and four were fully chaotic, with one tetraploid. Reprobing confirmed the chaotic nature of five embryos but another five were diploid for chromosomes uninvolved in the translocation.
Fig. 3.14 PGD for case 32-G 46,XX,t(8;12)(q11.2;q12).

(a), (b): Strategy devised for PGD performed from case 23-G 46,XX,t(8;12)(q11.2;q12).
(c) FISH analysis of patient lymphocyte metaphase nucleus showing the normal and derivative chromosomes.

(d) FISH analysis of blastomeres for PGD
(d): The same probe combination applied to blastomeres from biopsied (i) and spare embryos (ii, iii) during PGD for this couple, showing (i) disomy for both chromosomes 8 and 12, (ii) tetrasomy for chromosome 12 and pentasomy for 8q11.2->qter, (iii) monosomy for chromosome 12 and partial trisomy for 8pter->q11.2
Of note was embryo 4 (embryo g Table 3.39) which was diagnosed as balanced/aneuploid mosaic at the time of biopsy; further analysis (including CGH) of the embryo, confirmed the nature of the mosaicism; including the biopsied cells, eight of 12 cells were balanced, three had lost a derivative chromosome 8 and one had a duplicated chromosome 12. Table 3.39 lists the other two embryos, 2 and 7 (embryos f and h respectively), with both chromosomally balanced and abnormal cells. Segregation patterns were deduced to be alternate in six cases and in three cases to be adjacent 2, similar to two of the products of conception (POCs) investigated for this couple. Table 3.26 summarises this cycle of treatment.
Table 3.26. Classification of embryos from the single PGD cycle of patient 23-G. Karyotype 46,XX,t(8;12)(q11.2;q12)

<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; NO OF CELLS</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>RESULTS FROM SPARE EMBRYO ON D4 ACCORDING TO FISH PERFORMED WITH PROBES SELECTED FOR THE CASE</th>
<th>RESULTS FROM SPARE EMBRYO ON D4 ACCORDING TO SECOND ROUND OF FISH PERFORMED WITH THE VYSIS ANEUVYSION PROBES (X, Y, 18)</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION &amp; COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 1- 8-cell</td>
<td>Both cells balanced</td>
<td>Embryo transferred</td>
<td>N/A</td>
<td>Alternate</td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td>**</td>
<td></td>
<td>**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Grade 2+ 6-cell</td>
<td>1 cell biopsied. Nucleus 1: Monosomy 8pter→q11.2 &amp; 12q12→qter. Aneuploid</td>
<td>6cell stage. 5 nuclei analysed.</td>
<td>5 nuclei analysed.</td>
<td>Alternate</td>
<td>Chaotic/Balanced</td>
</tr>
<tr>
<td></td>
<td>***</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Grade 1- 5-cell</td>
<td>1 cell biopsied-No results were obtained</td>
<td>6cell stage. 6 nuclei analysed.</td>
<td>6 nuclei analysed.</td>
<td>Unknown</td>
<td>Chaotic</td>
</tr>
<tr>
<td></td>
<td>**</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

No Result

Balanced

Alternate

Balanced

Chaotic

Chaotic

Unknown

Chaotic
<table>
<thead>
<tr>
<th>Grade</th>
<th>Cell Stage</th>
<th>Embryo</th>
<th>Cells Biopsied</th>
<th>Nucleus 1</th>
<th>Nucleus 2</th>
<th>Nuclei analysed</th>
<th>Aneuploid</th>
<th>Chaotic</th>
<th>Balanced</th>
<th>Diploid</th>
<th>Adjacent</th>
<th>Aneuploid/Chaotic</th>
<th>Balanced/Chaotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>7-cell</td>
<td>g</td>
<td>2</td>
<td>Balanced</td>
<td>Trisomy 12</td>
<td>Balanced/Aneuploid</td>
<td>Balanced</td>
<td></td>
<td>Aneuploid</td>
<td></td>
<td>Adjacent-2 (12, der12)</td>
<td>Balanced/Chaotic</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6-cell</td>
<td>**</td>
<td>1</td>
<td>Nullisomy 12</td>
<td></td>
<td>Aneuploid</td>
<td>Aneuploid</td>
<td></td>
<td>Chaotic</td>
<td></td>
<td></td>
<td>Aneuploid/Chaotic</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8-cell</td>
<td>**</td>
<td>2</td>
<td>Monosomy 8</td>
<td>Monosomy 12 qpter-q11.2 &amp; 12q12-&gt;qter</td>
<td>Aneuploid mosaic</td>
<td>Aneuploid mosaic</td>
<td></td>
<td>Diploid</td>
<td></td>
<td>Adjacent-2 (12, der12)</td>
<td>Aneuploid/mosaic</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>7-cell</td>
<td>**</td>
<td>1</td>
<td>No result obtained</td>
<td>Monosomy 8pter-q11.2</td>
<td>Balanced(2)/Chaotic(4)</td>
<td></td>
<td></td>
<td>Diploid(1)/Chaotic</td>
<td></td>
<td></td>
<td>Chaotic/ Balanced</td>
<td></td>
</tr>
</tbody>
</table>

No Result
<table>
<thead>
<tr>
<th>Grade</th>
<th>Stage</th>
<th>Cells Biopsied</th>
<th>Chromosomal Abnormalities</th>
<th>Nuclei Analyzed</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>7-cell</td>
<td>2 cells biopsied.</td>
<td>Nucleus 1: Trisomy 12, monosomy 8q11.2→qter, nullisomy 8pter→q11.2. Nucleus 2: Monosomy 8q11.2→qter, nullisomy 8pter→q11.2.</td>
<td>Aneuploid mosaic</td>
<td>Chaotic</td>
</tr>
<tr>
<td>9</td>
<td>1-8-cell</td>
<td>Both cells: Trisomy 12pter→12q12, monosomy 8q11.2→qter.</td>
<td></td>
<td>Chaotic</td>
<td>Diploid/Chaotic</td>
</tr>
<tr>
<td>10</td>
<td>4-cell</td>
<td>1 cell biopsied. No results were obtained</td>
<td></td>
<td>No Result</td>
<td>Balanced</td>
</tr>
<tr>
<td>11</td>
<td>5-cell</td>
<td>1 cell biopsied. No results were obtained</td>
<td></td>
<td>No Result</td>
<td>Chaotic</td>
</tr>
</tbody>
</table>

222
<table>
<thead>
<tr>
<th>Grade</th>
<th>Stage</th>
<th>Cell Biopsied</th>
<th>Morphology</th>
<th>Nuclei Analysed</th>
<th>Tetra/Treploid</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2+</td>
<td>1-cell</td>
<td>1 cell</td>
<td>No signals</td>
<td>4 nuclei</td>
<td>Tetraploid</td>
<td>No Result</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abnormal</td>
<td>morphology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2+</td>
<td>8-cell</td>
<td>2 cells</td>
<td>Nucleus 1</td>
<td>9 nuclei</td>
<td>Aneuploid</td>
<td>Adjacent-2 (12, der12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>biopsied</td>
<td>No signals</td>
<td>Diploid</td>
<td></td>
<td>Aneuploid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cytoplasm</td>
<td>Nucleus 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Could not be</td>
<td>located</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

21 oocytes were collected, 17 were fertilised. 13 embryos were biopsied.
**Spare embryos that CGH analysis was performed on single cells biopsied on D3.
*** Balanced and abnormal cells in one embryo
† 1PN: A single pronucleus instead of two was visible at time of fertilisation assessment indicating possible abnormal fertilisation.
3.4.6 Case 18-H: Reciprocal Translocation 46,XY,t(1;18)(p23;q23)

Couple H were referred for PGD due to a paternal balanced reciprocal translocation 46,XY,t(1;18)(p23;q23). The couple had experienced six years of subfertility and two spontaneous abortions early in gestation. Unfortunately, no cytogenetic data was obtained from the conceptuses from these miscarriages. The sperm analysis for the husband was normal. Maternal age was 36 years.

A PGD protocol was developed for this case to exclude any imbalance of the chromosomes involved in the translocation. Two of the probes pUC 1.77, satellite III probe (insert size 1.77kb) specific for the heterochromatic region of human chromosome 1 in SO and SG (Cooke and Hindley 1979) and the subtelomeric probe 1ptel SG (Vysis, U.K.) flank the breakpoint on chromosome 1 and the third probe was the alpha-satellite 18p11.1-q11.1 in spectrum A (SA). Using this FISH probe combination, 91% control lymphocyte nuclei (n=200) showed three clear signals per nucleus. Before clinical application these probes were also tested on blastomeres biopsied from day 3 post-insemination embryos, showing discrete FISH signals easily scored in all nuclei. Fig. 3.15 shows the PGD strategy employed for this case tested on patient lymphocytes and on blastomeres.

In the single PGD cycle sixteen oocytes were collected and fourteen fertilised (87.5%) resulting in 12 embryos, being suitable for biopsy on day 3. Two cells were biopsied on day three post-insemination from 10 embryos (embryos 1, 2, 3, 5, 6, 7, 9, 10, 11 and 12) between the 6-8-cell stage. A single cell biopsy only was possible for embryos 4 and 8, which were at the 5-cell stage. As both biopsied cells from embryo 5 were lysed during spreading, a single cell was re-biopsied from the embryo on day four. Re-biopsy on day four was also performed for embryo 9, as the FISH results of the two biopsied blastomeres on day three were inconclusive.

FISH analysis of the biopsied blastomeres revealed two embryos diagnosed as having a balanced complement (embryos 1 and 10) but for one (embryo 10) the single cell analysed was a binucleate with a haploid set in each nucleus. Of the remainder, eight were aneuploid on diagnosis (embryos 2, 3, 4, 5, 6, 8, 11 and 12) and two were balanced/aneuploid mosaics (embryos 7 and 9). Unfortunately, the patient became hyperstimulated and the cycle had to be discontinued. The single normal embryo (embryo 1) along with three others (embryos 5, 9 and 12) for which there had been some ambiguity was frozen on day 5.
Fig.3.15 PGD for case 18-H 46,XY,(1;18)(p32;q23)

(a), (b): Strategy devised for PGD performed from case 18-H 46,XY,(1;18)(p32;q23).
(c) FISH analysis of patient lymphocyte metaphase nucleus showing the normal and derivative chromosomes.

(d) FISH analysis of biopsied blastomeres for PGD
(d): The same probe combination applied to blastomeres from day 4 post-insemination embryos following PGD for this couple, shows (i) and (ii) trisomy for 1p32->pter, (iii) monosomy for 1p32.1->pter.
The normal embryo (embryo 1-a blastocyst) was later thawed and transferred but no pregnancy ensued. Analysis of the available (eight) spare embryos on day 4 with the diagnostic probe set showed two balanced/chaotic mosaics (embryos 8 and 10), four aneuploids (embryos 2, 4, 6 and 11) and one each of aneuploid/balanced (embryo 7) and aneuploid/chaotic mosaic (embryo 3). Of the two embryos diagnosed as balanced/aneuploid mosaics on biopsy (embryos 7 and 9), embryo 9 was frozen, while full analysis (including one CGH result) of the other (embryo 7) showed six cells to be the product of adjacent-1 segregation and two cells to be apparently balanced. The difference was the loss of one signal for the sub-telomere probe for chromosome 1p in the two balanced cells. Re-probing of these spare embryos performed with 16 β-satellite (SO) and 12 α-satellite (SG) showed three diploid embryos, one aneuploid and four diploid/aneuploid or chaotic mosaics.

Of the three abnormal embryos that were frozen, embryo 9 fragmented on thawing, FISH analysis with the diagnostic probe set provided interesting results for the other two. Of the 44 nuclei comprising one embryo, (number 5), 20 were consistent with being the product of adjacent 1 segregation; 17 were similar except that there was a only one signal for the heterochromatic region probe on derivative chromosome 1 (instead of two) while three nuclei had three such signals, suggesting reciprocal loss and gain. The remaining four nuclei seemed chaotic. The embryo was classified as aneuploid mosaic. The second embryo (embryo number 12) had 11 nuclei; five chaotic, three balanced and three lacking a derivative chromosome 1 (heterochromatic region signal) but otherwise balanced. Three embryos in this cycle embryos 7, 8, and 10, were found to have both balanced and abnormal cells, and are listed in Table 3.42 as embryos i, j, and k respectively.

Where this was possible, the segregation pattern deduced from the embryos was alternate in three, adjacent 1 in six and adjacent 2 in one. This compares with that from sperm analysis of 64% alternate, 17% adjacent 1, 5% adjacent 2 and 12% 3:1. Table 3.27 summarises this cycle of treatment.
Table 3.27. Classification of embryos from the single PGD cycle of patient 18-H. Karyotype 46,XY,t(1;18)(p23;q23)

<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; NO OF CELLS</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>RESULTS FROM SPARE EMBRYO ON D4 ACCORDING TO FISH PERFORMED WITH PROBES SELECTED FOR THE CASE</th>
<th>RESULTS FROM SPARE EMBRYO ON D4 OR BIOPSIED CELLS ACCORDING TO SECOND ROUND OF FISH PERFORMED WITH 16 β-SAT (SO) &amp; 12 α-SAT (SG)</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION &amp; COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 2+ 6-cell</td>
<td>Both cells balanced <em>Balanced</em></td>
<td>Embryo frozen</td>
<td>Both biopsied cells diploid.</td>
<td>Alternate</td>
<td>Balanced</td>
</tr>
<tr>
<td>2 Grade 2+ 7-cell **</td>
<td>2 cells biopsied. Nucleus 1: Monosomy 18q23–qter &amp; Trisomy 1p23–pter. Nucleus 2: Could not be located following FISH. <em>Aneuploid</em></td>
<td>5cell stage. 2 nuclei analysed. Both showed Monosomy 18q23–qter &amp; Trisomy 1p23–pter. <em>Aneuploid</em></td>
<td>2 nuclei analysed. Both showed trisomy 16.</td>
<td>Adjacent-1 (1,der18)</td>
<td>Aneuploid</td>
</tr>
<tr>
<td>3 Grade 2+ 7-cell **</td>
<td>2 cells biopsied. Nuclei: 1 &amp; 2: Monosomy 18q23–qter &amp; Trisomy 1p23–pter. <em>Aneuploid</em></td>
<td>8cell stage. 7 nuclei analysed. <em>Aneuploid(4)/Chaotic (3)</em></td>
<td>6 nuclei analysed. 4 Diploid /1 Aneuploid (tetrasomy 16)</td>
<td>Adjacent-1 (1,der18)</td>
<td>Aneuploid/Chaotic for the translocation chromosomes. Diploid /Aneuploid for chromosomes 12 &amp;16.</td>
</tr>
<tr>
<td>Grade</td>
<td>Stage</td>
<td>Cells Biopsied</td>
<td>Details</td>
<td>Analysis</td>
<td>Additional Notes</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>----------------</td>
<td>---------</td>
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<td>------------------</td>
</tr>
<tr>
<td>5</td>
<td>2+</td>
<td>7-cell</td>
<td>2 cells biopsied on D3 were lost during spreading. 1 cell (binucleate) biopsied on D4. Nucleus 1: Balanced. Nucleus 2: Monosomy 18q23→qter &amp; Trisomy 1p23→pter.</td>
<td>Aneuploid</td>
<td>Alternate</td>
</tr>
<tr>
<td>Grade</td>
<td>Stage</td>
<td>Grade</td>
<td>Stage</td>
<td>Grade</td>
<td>Stage</td>
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<tr>
<td>-------</td>
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<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>7</td>
<td>1-</td>
<td>9-cell</td>
<td>2 cells biopsied. Nucleus 1: Monosomy 18q23→qter &amp; Trisomy 1p23→pter. Nucleus 2: Balanced.</td>
<td>Balanced/Aneuploid</td>
<td>8 cell stage. 8 nuclei analysed. 2 Balanced/6 Aneuploid. Monosomy 18q23→qter &amp; Trisomy 1p23→pter.</td>
</tr>
<tr>
<td>8</td>
<td>1-</td>
<td>5-cell</td>
<td>1 cell biopsied. Nucleus 1: Monosomy 1 and nullisomy 18.</td>
<td>Balanced/Aneuploid</td>
<td>5 nuclei analysed.</td>
</tr>
<tr>
<td>9</td>
<td>2+</td>
<td>7-cell</td>
<td>2 cells biopsied on D3 Nucleus 1: Balanced. Nucleus 2: Aneuploid (Monosomy for 1p23→qter). 1 cell biopsied on D4: Nucleus not located after FISH.</td>
<td>Balanced/Aneuploid</td>
<td>Embryo frozen</td>
</tr>
<tr>
<td>Grade</td>
<td>Cells Biopsied</td>
<td>Nuclei Details</td>
<td>Number of Nuclei Analysed</td>
<td>Adjacent</td>
<td>Ploidy</td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
<td>----------------</td>
<td>---------------------------</td>
<td>----------</td>
<td>--------</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-</td>
<td>2</td>
<td>Trisomy 18p&lt;sup&gt;ter&lt;/sup&gt; → 18q&lt;sup&gt;23&lt;/sup&gt; &amp; Monosomy 1p&lt;sup&gt;23&lt;/sup&gt; → q&lt;sup&gt;ter&lt;/sup&gt;.</td>
<td>4</td>
<td>Adjacent-1 (18, der-1)</td>
<td>Aneuploid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

16 oocytes were collected, 14 were fertilised. 12 embryos were biopsied.

**Spare embryos that CGH analysis was performed on single cells biopsied on D3.

***Balanced and abnormal cells in one embryo.
Results

3.5 PGD for Two Couples with Robertsonian Translocations.

PGD was attempted for two couples with balanced Robertsonian translocations; one case of 45,XY,der(13;21)(q10;q10) and one case of 45,XY,der(13;14)(q10;q10). Both paternal translocation carriers showed oligoasthenozoospermia necessitating ICSI. Four cycles of PGD were performed for both couples (two cycles for each). A dual colour FISH strategy was employed for the case involving chromosomes 13 and 14, while for the case involving chromosomes 13 and 21 a triple colour FISH strategy was developed. Both cases employed commercially obtained probes.

3.5.1 Case 32-I: Robertsonian Translocation 45,XY,der(13;14)(q10;q10).

Couple I was the single satellite PGD case carried out in collaboration with the Nottingham IVF centre "Care". The couple were referred for PGD after several years of primary infertility. The male partner carrying the translocation was assessed as oligoasthenozoospermic accounting for their fertility status. Maternal age was 35 years.

A dual colour FISH protocol was developed for PGD in this case using a locus specific probe binding at the retinoblastoma 1 region RB1 LSI 13 directly labelled in spectrum green combined with a subtelomeric probe for chromosome 14 in spectrum orange TelVysion 14q. Preliminary testing of this dual probe combination showed strong discrete signals in both lymphocyte control and blastomere nuclei showing the expected number of signals in 92% of lymphocyte interphase nuclei. Before clinical application these probes were also tested on blastomeres biopsied from day 3 post-insemination embryos, showing discrete FISH signals easily scored in all nuclei. Fig. 3.16 shows the PGD strategy employed for this case tested on patient lymphocytes and on blastomeres.

After an initial cancelled cycle due to poor ovarian stimulation, two treatment cycles were carried out for PGD to exclude imbalance of chromosomes 13 and 14. For the first treatment cycle 7 oocytes were collected, five fertilised (71.4%) and all were biopsied.
Results

Fig. 3.16 PGD for case 32-I 45, XY, der(13;14)(q10;q10)

(a), (b): Strategy devised for PGD performed from case 32-I 45, XY, t(13;14)(q10;q10)
(c) FISH analysis of patient lymphocyte metaphase nucleus showing the normal and derivative chromosomes.

(d) FISH analysis of biopsied blastomeres for PGD

(d): The same probe combination applied to blastomeres from biopsied embryos during the first PGD cycle for this couple, shows (i) trisomy for chromosome 13, (ii) disomy for both chromosome 13 and 14, (iii) Tetrasomy 13 and trisomy 14.
Results

Three cells were biopsied on day three post-insemination from embryos 4 and 5 at the 6 and 8-cell stage respectively. Although the intention was to biopsy two cells from each embryo, due to difficulties encountered regarding the embryonic cell organisation, biopsying three cells could not be avoided. A single cell biopsy only was possible from embryos 1, 2 and 3 that were at 4 and 5 cell stages.

FISH analysis of the biopsied blastomeres showed only embryo 3 to be balanced based on the single biopsied cell, and an embryo transfer was performed but no pregnancy was achieved. Embryo one showed tetrasomy 14 and trisomy 13 based on the single biopsied blastomere, while the cell biopsied from embryo 2 lysed during spreading failing to provide any results. Based on the FISH analysis of the biopsied cells from embryos 4 and 5 they were classified as monosomic for chromosome 13 and trisomic for chromosome 13 respectively. Unfortunately, the slides of the spare embryos were damaged during transportation from the distant IVF unit. Therefore, no information was available. The embryo classification was based on the results provided by the biopsied cells alone. Embryos 4 and 5 were classified as aneuploid being a product of adjacent-1 segregation in the gamete, and embryo one was also classified as aneuploid although the segregation pattern could not be deduced, while no classification was applicable for embryo 2. Table 3.28 summarises this cycle of treatment.
Table 3.28. Classification of embryos from the 1st PGD cycle of patient 32-I. Karyotype 45,XY,der(13;14)(q10;q10)

<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; CELL NO</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>EMBRYO CLASSIFICATION BASED ON THE BIOPSY RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 1 4-cell</td>
<td>1 cell biopsied. Nucleus 1: Tetrasomy 13, trisomy 14</td>
<td>Unknown-</td>
<td>Aneuploid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Grade 2 4-cell</td>
<td>1 cell biopsied. Cell lysed during spreading</td>
<td>Unknown--</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Grade 1 5-cell</td>
<td>1 cell biopsied. Nucleus 1: Balanced</td>
<td>Alternate</td>
<td>Balanced-Embryo transferred</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Grade 2 6-cell</td>
<td>3 cells biopsied- 1 cell lysed Nuclei 1 &amp; 2: Monosomy 13</td>
<td>Adjacent (nullisomy 13)</td>
<td>Aneuploid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Grade 1 8-cell</td>
<td>3 cells biopsied 1 cell lysed, 1 failed to provide results Nucleus 1: Trisomy 13</td>
<td>Adjacent (disomy 13)</td>
<td>Aneuploid</td>
</tr>
</tbody>
</table>

7 oocytes were collected, 5 fertilised and 5 biopsied. All 5 biopsied embryos were analysed.
The slides of the spare embryos were damaged during transportation to the centre, so no information is available.
In the second ICSI cycle, 6 oocytes were collected, 3 were fertilised (50%) and frozen on day three of development as the patient hyperstimulated and an embryo transfer was not advisable. Later, the patient had a PGD cycle with these frozen embryos. All three were thawed and biopsied. For embryos 1 and 2 at the 4- and 6-cell stage a single cell biopsy was performed, however no results were provided for embryo 1 due to nuclear degeneration. Embryo 2 showed monosomy for chromosome 13 based on the single biopsied cell. Four cells were biopsied from embryo 3 at the 8-cell stage. The intention was for two cells to be removed from the embryo. However, due to difficulties encountered regarding the embryonic cell organisation, biopsying four cells could not be avoided. All four biopsied blastomeres lysed during spreading and unfortunately no information was available for this embryo. No transfer took place for this cycle.

This diagnosis was confirmed by analysis of the remainder of the biopsied untransferred embryos. Embryo 1 was classified as aneuploid/mosaic based on the two nuclei analysed. Embryo 2 being at 5-cell stage on day four revealed three nuclei and five micronuclei and was classified as chaotic. Finally, embryo 3 that reached blastocyst stage on day 4 revealed 74 nuclei during spreading all of which were found to be balanced indicating the embryo originated from an alternate segregation pattern. The segregation pattern for embryos 1 and 2 was unknown. Table 3.29 summarises this cycle of treatment.
Table 3.29. Classification of embryos from the 2nd PGD cycle of patient 32-1. Karyotype 45,XY,der(13;14)(q10;q10)

<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; CELL NO</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>RESULTS FROM SPARE EMBRYO ON D4.</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 1 4-cell</td>
<td>1 cell biopsied. Nucleus was degenerated. No results were provided</td>
<td>3-cell stage. 2 nuclei analysed. 1: Tetrasomy 13, Trisomy 14 1: Trisomy 13</td>
<td>Unknown-</td>
<td>Aneuploid/Mosaic</td>
</tr>
<tr>
<td>2 Grade 1 6-cell</td>
<td>1 cell biopsied. Nucleus 1: Monosomy 13</td>
<td>5-cell stage. Spreading revealed 3 nuclei and 5 micronuclei. 2: Tetrasomy 14 1: Pentasomy 14, trisomy 13 4 micronuclei: monosomy 13 1 micronucleus: monosomy 14</td>
<td>Unknown-</td>
<td>Chaotic</td>
</tr>
<tr>
<td>3 Grade 1 8-cell</td>
<td>4 cells biopsied. All blastomeres lysed during spreading. None could be located under DAPI</td>
<td>Blastocyst 74 nuclei: Balanced</td>
<td>Alternate</td>
<td>Balanced</td>
</tr>
</tbody>
</table>

6 oocytes were collected, 3 fertilised and frozen on day 3 of development. All 3 embryos were thawed and biopsied. No embryos were transferred.
3.5.2 Case 35-E: Robertsonian Translocation 45,XY,der(13;21) (q10;q10).

Couple E were referred for PGD after several years of primary infertility. The male partner carrying the translocation was assessed as oligoasthenoteratozoospermic accounting for their fertility status. Maternal karyotype was normal and age was 34 years.

A triple colour FISH protocol was developed for PGD in this case using the Vysis locus specific probe cocktail for chromosomes 13 (SG) mapping to 13q14 spanning the retinoblastoma gene (RB1), and 21(SO) mapping to 21q22.13-q22.2. The third probe was a combination of two directly labelled subtelomeric probes for chromosome 21 in red and green from Oncor, UK providing extra information on chromosome 21. Preliminary testing of this dual probe combination showed strong discrete signals in both lymphocyte control and blastomere nuclei showing the expected number of signals in 94% lymphocyte interphase nuclei. Fig. 3.17 shows the PGD strategy employed for this case tested on patient lymphocytes and on blastomeres.

Two treatment cycles, were carried out for PGD to exclude imbalance of chromosomes 13 and 21. The fertilisation rate for the first ICSI cycle was 77%, resulting in six embryos suitable for biopsy. The second ICSI cycle (76.9% fertilisation) resulted in nine embryos for biopsy. In total, 22 oocytes were collected, seventeen (77.3%) fertilised resulting in sixteen embryos suitable for biopsy.

In the first PGD cycle six embryos were biopsied, with two cells obtained from five embryos (1, 3, 4, 5, & 6) all being at the 6-8 cell stage, and a single cell from embryo 2 consisting of 4 cells. FISH analysis showed embryos 2, 4, and 5 to be carrying two copies of the chromosomes involved in the translocation and all were subsequently transferred on day four post-insemination, but no pregnancy resulted. Only the diagnosis for embryo 4 was based on FISH analysis of both biopsied cells. On the day of the diagnosis, embryos 1 and 3 were found to be aneuploid while no results were available for embryo 6. The untransferred embryos were subjected to confirmation of the diagnosis using the original probe set, and further analysis employing the AneuVysion kit from Vysis, UK for chromosomes X, Y and 18. Moreover, embryo 6 was subjected to further biopsy for the purpose of obtaining single blastomeres for CGH analysis.
Fig. 3.17 PGD for case 35-E 45,XY,der(13;21)(q10;q10)

(a), (b): Strategy devised for PGD performed from case 35-E 45,XY,t(13;21)(q10;q10)
(c) FISH analysis of patient lymphocyte metaphase nucleus showing the normal and derivative chromosomes.

(d) FISH analysis of blastomeres for PGD
(d): The same probe combination excluding the LSI probe on chromosome 21 applied to blastomeres from biopsied (ii, iii) and spare embryos (i) during the second PGD cycle performed for this couple, shows (i) pentasomy for chromosome 13, (ii) Trisomy for chromosome 13, (iii) Trisomy for chromosome 13 and monosomy for chromosome 21.
Results

Based on the available results from this investigation embryo 3 was classified as chaotic and embryo 6 as aneuploid based on results from a single cell.

In the second PGD cycle ten embryos were biopsied, with two cells obtained from four embryos (embryos 1, 4, 5, & 9) all at the 5-8 cell stage, and a single cell from six embryos (embryos 2, 3, 6, 7, 8 and 10) all at the 4-5 cell stage. FISH analysis showed that embryos 1, 5, and 6 carried two copies of the chromosomes involved in the translocation and all were subsequently transferred on day four post insemination, but no pregnancy resulted. Only the diagnosis for embryo 1 was based on FISH analysis on both biopsied cells. Based on the diagnoses, embryos 2, 3, 4, 7, 8, 9 and 10 were all found to be aneuploid. The untransferred embryos were subjected to confirmation of the diagnosis as well as further analysis employing the AneuVysion kit from Vysis, UK for chromosomes X, Y and 18. Based on the available results from this investigation of the embryos, all were classified as chaotic.

In total, 8 of the 10 spare embryos were chaotic, and a single one (embryo 6 from the first PGD cycle) was found to be aneuploid based a single cell. However, additional CGH analysis performed on three cells from embryo 6 classified it as balanced. Prior analysis revealed that 89% of sperm were the result of alternate segregation; similarly, where it was possible to determine the segregation from the embryonic outcome, seven of the eight were the result of alternate segregation. Table 3.30 and 3.31 summarise the treatment.
<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; NO OF CELLS</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>RESULTS FROM SPARE EMBRYO ON D4 ACCORDING TO FISH PERFORMED WITH PROBES SELECTED FOR THE DIAGNOSIS</th>
<th>RESULTS FROM SPARE EMBRYO ON D4 ACCORDING TO SECOND ROUND OF FISH PERFORMED WITH THE VYSIS ANEUVYSIS PROBES (X, Y, 18)</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION &amp; COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 2+ 8-cell</td>
<td>2 cells biopsied. Nucleus 1: Lost during spreading Nucleus 2: Trisomy 13</td>
<td>Embryo lysed no results available.</td>
<td>Adjacent (disomy13)</td>
<td>Based on one nucleus: Trisomy 13</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Aneuploid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Grade 1-4-cell</td>
<td>1 cell biopsied. Nucleus 1: balanced</td>
<td>Embryo transferred)</td>
<td>Alternate</td>
<td>Based on one nucleus: Balanced</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Balanced</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Grade 2+ 6-cell</td>
<td>2 cells biopsied-Result from 1 cell Nucleus 1: Trisomy 21</td>
<td>5-6 cell. 4 nuclei analysed. 2 showed trisomy 21 and 2 nuclei showed: monosomy 13 &amp; 21</td>
<td>Unknown</td>
<td>Chaotic</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Aneuploid</strong></td>
<td><strong>Chaotic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Grade 2+ 8-cell</td>
<td>2 cells biopsied-Both balanced</td>
<td>Embryo transferred</td>
<td>Alternate</td>
<td>Balanced</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Balanced</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.30. Classification of embryos from the 1st PGD cycle of patient 35-E. Karyotype 45,XY,der(13;21)(q10;q10)
<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; NO OF CELLS</th>
<th>RESULTS FROM BIOPSIED BLASTOMERES</th>
<th>RESULTS FROM SPARE EMBRYO ON D4 ACCORDING TO FISH PERFORMED WITH PROBES SELECTED FOR THE DIAGNOSIS</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION</th>
</tr>
</thead>
</table>
| 5 Grade 2 6-cell              | 2 cells biopsied  
Nucleus 1: balanced  
Nucleus 2: Lost during spreading  
Balanced | Embryo transferred | Alternate | Based on one nucleus: Balanced |
| 6 Grade 2+ 8-cell **          | 2 cells biopsied. None provided results | 6-7 cell compacting. Results available from one cell suggesting nullisomy 13. | Alternate | **Aneuploid** |

9 oocytes retrieved. 7 oocytes were fertilised. 6 embryos biopsied.

**Embryo that CGH analysis was performed on single cells biopsied on D3

Table 3.31. Classification of embryos from the 2nd PGD cycle for patient 35-E. Karyotype 45,XY,der(13;21)(q10;q10)
<table>
<thead>
<tr>
<th>Grade</th>
<th>Cells</th>
<th>Biopsy Details</th>
<th>Cell Details</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5-cell</td>
<td>One cell biopsied. <strong>Nucleus 1</strong>: Trisomy 13</td>
<td>7 cell. 4 nuclei analysed. 1 nucleus: Monosomy 21. 1 nucleus: Monosomy 13 &amp; 21. 2 nuclei: No signals</td>
<td>Unknown</td>
</tr>
<tr>
<td>3</td>
<td>4-cell</td>
<td>One cell biopsied. <strong>Nucleus 1</strong>: Monosomy 21</td>
<td>5-6 cell. 3 nuclei analysed. 2 nuclei: trisomy 13 1 nucleus: Monosomy 21 &amp; trisomy 13</td>
<td>Unknown</td>
</tr>
<tr>
<td>4</td>
<td>8-cell</td>
<td>2 cells biopsied. <strong>Nucleus 1</strong>: Trisomy 13, monosomy 21. <strong>Nucleus 2</strong>: Lost during spreading</td>
<td>6 cell. 4 nuclei analysed. 2 nuclei: monosomy 13, nullisomy 21. 1 nucleus: Monosomy 21 1 nucleus: Monosomy 21 &amp; trisomy 13</td>
<td>Unknown</td>
</tr>
<tr>
<td>5</td>
<td>7-cell</td>
<td>2 cells biopsied. <strong>Nucleus 1</strong>: Balanced. <strong>Nucleus 2</strong>: Could not be located. <strong>Balanced</strong></td>
<td>Embryo transferred</td>
<td>Alternate</td>
</tr>
<tr>
<td>6</td>
<td>Grade 2+ 4-cell</td>
<td>One cell biopsied. <em>Balanced</em></td>
<td>Embryo transferred</td>
<td>Alternate</td>
</tr>
<tr>
<td>---</td>
<td>----------------</td>
<td>-------------------------------</td>
<td>-------------------</td>
<td>----------</td>
</tr>
<tr>
<td>7</td>
<td>Grade 2+ 5-cell</td>
<td>One cell biopsied: Incocclusive results <em>Aneuploid</em></td>
<td>6 cell. 4 nuclei analysed.</td>
<td>Unknown</td>
</tr>
<tr>
<td>8</td>
<td>Grade 2+ 5-cell</td>
<td>One cell biopsied: <em>Nucleus 1</em>: Trisomy 21 <em>Aneuploid</em></td>
<td>6-7 cell. 6 nuclei analysed.</td>
<td>Unknown</td>
</tr>
<tr>
<td>9</td>
<td>Grade 2+ 5-cell</td>
<td>2 cells biopsied. <em>Nucleus 1</em>: Inconclusive <em>Nucleus 2</em>: Trisomy 21 &amp; tetrasomy 13. <em>Aneuploid</em></td>
<td>5 cell. 4 nuclei analysed.</td>
<td>Unknown</td>
</tr>
<tr>
<td>10</td>
<td>Grade 2+ 4-cell</td>
<td>One cell biopsied: Trisomy 21 monosomy 13 <em>Aneuploid</em></td>
<td>4 cell. 4 nuclei analysed</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

13 oocytes retrieved 10 oocytes were fertilised-9 embryos biopsied.
3.6 PGD for a Couple with an Intrachromosomal Insertion.

3.6.1 Case 40-F: Intrachromosomal Insertion
46,XX,ins(7)(p22q32q31.1)

This couple were referred for PGD in preference to prenatal diagnosis having experienced pregnancy termination because of chromosome imbalance. The mother aged 33, carried a rearrangement for chromosome 7 and her karyotype was 46,XX,ins(7)(p22q32q31.1). The couple's first pregnancy was a miscarriage at 11 weeks of gestation. The patient had a further pregnancy, and the results of transabdominal amniocentesis revealed a normal female fetus with a karyotype the same as the mother's. The pregnancy resulted in a normal livebirth. Finally, the third pregnancy was terminated as the analysis of cultured amniocytes revealed that the fetus had an abnormal karyotype with a duplication of the inserted fragment.

The complexity of any approach for PGD for these cases was increased by the problem that it was impossible to determine the orientation of the inverted segment. To cope with this eventuality an approach was developed including a sub-telomeric probe incorporated along with a locus-specific probe for the inserted segment. Possible recombinant chromosomes resulting from both direct and inverted insertions could then be detected. The Williams microdeletion probe from Vysis, U.K. (Elastin gene probe SO [D7S486], and a control probe [D7S522] SG mapping to the inserted segment 7q31) was employed for the diagnosis to detect duplication or deletion of the inserted fragment, which causes partial trisomy and monosomy respectively. A chromosome 7 sub-telomeric probe (7p) was also selected as the orientation of this inversion was unclear from the G-banded karyotype. Although the selected probes could be distinguished on patient chromosomes, the FISH efficiency in interphase nuclei proved difficult to optimise due to the small signal size from the 7p probe labelled 50:50 FITC:TRITC (orange). Following counselling and consultation with the patient, it was decided to use the dual probe alone, detecting all recombinant chromosome outcomes for an inverted insertion and all but two possibilities for a direct insertion. Preliminary testing of this dual probe combination showed strong discrete signals in both lymphocyte control and blastomere nuclei showing the expected number of signals in 78% lymphocyte interphase nuclei. Before clinical application these probes were also tested on blastomeres biopsied from day 3 post-
Results

insemination embryos, showing discrete FISH signals easily scored in all nuclei. Fig. 3.18 shows the PGD strategy employed for this case tested on patient lymphocytes. Fig. 3.19 shows the probes employed for the PGD case tested on the triploid fibroblast cell line and on blastomeres.

The biopsied cells and the spare embryos were re-FISH-ed with subtelomeric probes for 7q [Labelled with red fluorochrome (TRITC)] and 7p [Labelled with green fluorochrome (FITC)] (Appligene Oncor, UK) to refine the diagnostic results and, in case the insertion was direct, to distinguish certain recombinant chromosomes that would not have been detected otherwise.

A single PGD cycle was performed for couple 40-F. Sixteen oocytes were collected 16 fertilised (100%). Eleven embryos were suitable to be biopsied. Two cells were biopsied on day three post insemination from 4 embryo at the 8-cell stage (embryos 6, 8, 9 and 11), whilst a single cell was obtained from 2 embryos at the 7-cell stage (embryos 1 and 10), 3 embryos at the 6-cell stage (embryos 2, 5 and 7), embryo 3 that was at the 5-cell stage and embryo 4 that consisted of 4 cells. During spreading it was noted that embryos 7, 8, 9, 10 and 11 showed vacuoles and inclusion bodies. FISH analysis of the biopsied blastomeres showed 7 embryos to be balanced (embryos 2, 4, 5, 7, 8, 9 and 11). The single biopsied cell from embryo 3 failed to provide any results. Embryo 1 showed duplication of region 7q31.1→q32 and nullisomy for the centromeric region 7pter→7q31.1 based on the single blastomere that was analysed. Embryo 6 similarly showed duplication of region 7q31.1→q32 based on the single blastomere that was analysed. Finally, embryo 10 showed deletion of region 7q31.1→q32 supported by both the blastomeres analysed. Embryos 2 and 9 scored as balanced were transferred on day four post-insemination resulting in a healthy baby. Embryos 4, 5, 7, 8 and 11 also scored as balanced, were cryopreserved for future possible transfer.

This diagnosis was confirmed by analysis of the remainder of the embryos 1, 3, 6 and 10. These embryos were biopsied on day 4 to obtain 1-2 blastomeres for CGH analysis. Furthermore, these embryos were later re-probed with sub-telomeric probes for both the long and the short arm of chromosome 7, allowing the most likely mode of segregation of the maternal chromosome 7 to be predicted. Unfortunately no results were obtained after re-probing for embryos 6 and 10.
Fig. 3.18 PGD for case 40-F 46,XX,ins(7)(p22q31.1q32) (direct or inverted)

(a), (b): Strategy devised for PGD performed from case 40-F 46,XX,ins(7)(p22q31.1q32) (direct or inverted).

(c) FISH analysis of patient lymphocyte metaphase nucleus showing the normal and derivative chromosomes.

(d) FISH analysis of blastomeres from biopsied (i) and spare embryos (ii), (iii) for PGD.

(i), (ii): The same probe combination applied to blastomeres biopsied from a day 3 post-insemination embryo during PGD for this couple, shows (i): Monosomy for 7q31.1->q32 and (ii): Monosomy 7pter->q31.1 (iii): Application of the dual probe on blastomeres from spare embryos of couple F showing (iii) trisomy for chromosome 7.
Results

Fig. 3.19 FISH analysis on triploid fibroblasts using the probe combination employed for PGD for case 40-F 46,XX,ins(7)(p22 q31.1q32) as preliminary work up.

(i), (ii): Dual colour FISH analysis on metaphase (i), (ii) and interphase (iii) nuclei of a triploid fibroblast cell line treated with hypotonic solution (i), (ii) or PBS (iii). Three copies of each probe are detected in all (i), (ii) and (iii).
Results

Based on the results available from the FISH and re-FISH embryo 1 was classified as fully chaotic originating for an unknown segregation pattern, whilst all nine blastomeres from embryo 3 were balanced (no result on biopsy). Finally, embryos 6 and 10 were uniformly abnormal carrying duplication and deletion of the inserted segment respectively.

Based on the FISH analysis the possible segregation patterns of the oocytes were deduced. The majority of the embryos derived from an oocyte carrying a balanced copy of chromosome 7 (embryos 2, 3, 4, 5, 7, 8, 9 and 11). Embryo 6 derived from an oocyte with a partially duplicated copy for chromosome 7. While, embryo 10 derived from an oocyte involving a copy for chromosome 7 that was carrying a deletion for the inserted segment. It was not possible to determine the segregation pattern giving rise to embryo 1. Table 3.32 summarises this cycle of treatment.
<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; NO OF CELLS</th>
<th>RESULT FROM BIOPSIED BLASTOMERE</th>
<th>RESULT FROM SPARE EMBRYO ON D4 ACCORDING TO FISH PERFORMED WITH PROBES SELECTED FOR THE CASE</th>
<th>RESULT FROM SPARE EMBRYO ON D4 ACCORDING TO SECOND ROUND OF FISH PERFORMED WITH SUBTELOMERIC PROBES FOR 7P &amp; 7Q</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>FINAL EMBRYO CLASSIFICATION &amp; COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 2 7-cell **</td>
<td>2 cells biopsied. Results obtained from nucleus 1 only: Duplication of the inserted segment. Nullisomy for the centromeric segment</td>
<td>5 cells-Chaotic showing monosomies &amp; trisomies for both the inserted and the non-inserted segments (no normal cells) A: 2 cells carrying the deletion of the insertion, B: 2 cells missing one copy of the control. C: 1 cell carrying 3 copies of 7</td>
<td>A: balanced (2 copies each cell) B: Balanced (2 copies each cell C: 3 copies indicating trisomy 7</td>
<td>Unknown.</td>
<td>Chaotic</td>
</tr>
<tr>
<td>2 Grade 1 6-cell</td>
<td>1 cell biopsied.</td>
<td>Embryo cavitating transferred</td>
<td></td>
<td>Oocyte was carrying a balanced copy for chromosome 7</td>
<td>Embryo was classified as balanced</td>
</tr>
<tr>
<td>3 Grade 1 5-cell **</td>
<td>1 cell biopsied-no results were obtained</td>
<td>9 cells- all balanced</td>
<td>9 cells- all balanced</td>
<td>Oocyte was carrying a balanced copy for chromosome 7</td>
<td>Balanced</td>
</tr>
</tbody>
</table>

Table 3.32. Classification of embryos from the single PGD cycle of patient 40-F karyotype 46,XX,ins(7)(p22q31.1q32)
<table>
<thead>
<tr>
<th>Grade</th>
<th>Stage</th>
<th>Cells Biopsied</th>
<th>Biopsied from</th>
<th>Results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>4-cell</td>
<td>1 cell</td>
<td>Embryo frozen</td>
<td></td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Balanced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6-cell</td>
<td>1 cell</td>
<td>Embryo frozen</td>
<td></td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Balanced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8-cell</td>
<td>2 cells</td>
<td>6 cells</td>
<td>No results</td>
<td>Uniformly abnormal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Duplication</td>
<td>all carrying</td>
<td></td>
<td>carrying duplication for the inserted segment</td>
</tr>
<tr>
<td>7</td>
<td>6-cell</td>
<td>1 cell</td>
<td>Embryo frozen</td>
<td></td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Balanced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>8-cell</td>
<td>2 cells</td>
<td>Embryo frozen</td>
<td></td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Balanced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>Grade 2</td>
<td>Grade 1-</td>
<td>8-cell</td>
<td>Oocyte was carrying a balanced copy for chromosome 7</td>
<td>Balanced</td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
<td>--------</td>
<td>--------</td>
<td>--------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>8-cell</td>
<td>2 cells biopsied-Results obtained from nucleus 1 only: Balanced</td>
<td>Embryo cavitating transferred</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2 cells biopsied: Deletion of the inserted segment. Deletion</td>
<td>10 cells- all carrying deletion of the inserted segment. Deletion</td>
<td>No results were obtained</td>
<td>Oocyte was carrying a copy for chromosome 7 carrying the deleted insertion segment</td>
<td>Uniformly abnormal carrying deletion for the inserted segment</td>
</tr>
<tr>
<td>11</td>
<td>2 cells biopsied-Results obtained from nucleus 1 only: Balanced</td>
<td>Embryo frozen</td>
<td></td>
<td>Oocyte was carrying a balanced copy for chromosome 7</td>
<td>Balanced</td>
</tr>
</tbody>
</table>

* Embryos were cultured to day five (all developed to morulas) and frozen
** Embryos were biopsied on D4 to obtain single blastomere for CGH analysis

16 oocytes were fertilised. 11 embryos were biopsied. All were analysed
3.7 Cancelled PGD cycles.

3.7.1 Case 12-K: Reciprocal Translocation 46,XY,t(8;9)(q24.3;q21.2).

Couple K were referred for PGD after several years of primary infertility. The male partner carrying the translocation was assessed as oligoasthenozoospermic accounting for their fertility status and necessitating ICSI. Maternal age was 33 years.

A PGD protocol was developed for this case to exclude any imbalance of the chromosomes involved in the translocation. Two of the probes subtelomeric probe 8qtel SO (Vysis, U.K.) and the alpha satellite centromeric 8 CEP probe SG (Vysis U.K.) flank the breakpoint on chromosome 8 and the third probe was the alpha-satellite centromeric 9 CEP probe SG (Vysis U.K.). Using this FISH probe combination, 87% of control lymphocyte nuclei (n=200) showed three clear signals per nucleus. Before clinical application these probes were also tested on blastomeres biopsied from day 3 post-insemination embryos, showing discrete FISH signals easily scored in all nuclei. Fig. 3.20 shows the PGD strategy employed for this case tested on patient lymphocytes. Fig. 3.21 shows the probes employed for the PGD case tested on the triploid fibroblast cell line and on blastomeres.

In the ICSI/PGD cycle sixteen oocytes were collected and only four fertilised (25%) resulting in 4 embryos between the 2- and 5- cell stage, none suitable for biopsy. The PGD cycle was therefore cancelled. The patient opted to have three embryos transferred on day 3 post insemination, without diagnosis, which resulted in an early spontaneous termination of pregnancy. The remaining untransferred embryo was subjected to FISH analysis revealing a fully chaotic embryo. The three nuclei that provided results showed monosomy for chromosome 8 and nullisomy 9, haploidy and disomy for the chromosomes examined.
Fig. 3.20 Strategy for case 12-K 46,XY,t(8;9)(q24.3;q21.2).

(a), (b): Strategy devised for PGD performed for case 12-K 46,XY,t(8;9)(q24.3;q21.2)
(c) FISH analysis of patient lymphocyte metaphase nucleus showing the normal and derivative chromosomes.
Fig. 3.21 FISH analysis on triploid fibroblasts and spare embryos, using the probe combination employed for cancelled PGD case 12-K 46,XY,t(8;9)(q24.3;q21.2).

(i), (ii): Triple colour FISH analysis on metaphase (i) and interphase (ii) nuclei of a triploid fibroblast cell line treated with hypotonic solution (i) or PBS a (ii). Three copies of each probe are detected in both (i) and (ii). (iii), (iv): The same probe combination on blastomeres from the spare embryo from this couple on day-4 post insemination, showing (iii): Monosomy 8 and 9, (iv): monosomy 8 and nullisomy 9, and (v): disomy for both chromosomes 8 and 9.
3.7.2 Case 9-J: Reciprocal Translocation 46,XX,t(12;22)(q24.1;q13.3).

The female partner 43 years old, was the carrier of a reciprocal translocation 46,XX,t(12;22)(q24.1;q13.3). Her husband was found to be karyotypically normal. The patient has one affected son carrying an unbalanced translocation 47,XY,der22. The child shows evidence of retarded mental development.

The strategy chosen for this case relied on three probes to detect all possible unbalanced translocation products in the embryo. Probes chosen were the α-satellite DNA/D12Z3 12q11.11q11 probe (SO & SG), which maps to the centromeric region of chromosome 12 and the Di George region probe detection set (LIS Di George/VCFS region probe LSI TUPLE 1/ARSA control), that contains two probes which flank the breakpoint on chromosome 22, all from Vysis, U.K. Using this FISH probe combination, 91% control lymphocyte nuclei (n=200) showed three clear signals per nucleus. Before clinical application these probes were also tested on blastomeres biopsied from day 3 post-insemination embryos, showing discrete FISH signals easily scored in all nuclei. Fig. 3.22 shows the PGD strategy employed for this case tested on patient lymphocytes. Fig. 3.23 shows the probes employed for the PGD case tested on the triploid fibroblast cell line and on blastomeres.

The patient came through for a cycle with 9 embryos that had previously been cryopreserved on day two post-insemination. All embryos were thawed and 7 survived the thawing procedure, whilst the other two (embryos 5 and 6) were degenerate. Following 24 hours of culture two embryos were at the 5-cell stage, one was at the 6-cell stage, while the rest consisted of 1 to 4 cells. PGD was not performed, as the embryos had not divided sufficiently to be subjected to biopsy. The patient had the three embryos that were between 5-6 cells transferred but no pregnancy was evident. The remaining four embryos plus the two degenerate ones were analysed by FISH employing the selected probes for this case. Embryos 2, 3 and 4 failed to provide results. Embryo 1 revealed three nuclei and was classified as chaotic. The single nucleus analysed from degenerate embryo 5 was trisomic for regions 22pter→22q11.2 and 12q24.1→qter and classified as aneuploid. Finally, degenerate embryo 6 provided two nuclei both of which were monosomic for region 12q24.1→qter and trisomic for region 22q11.2→qter, and was also classified as aneuploid. Based on the FISH analysis embryo 5 could have been derived from a 3:1
tertiary segregation pattern and embryo 6 could be the result of the adjacent-1 segregation mode. Table 3.33 summarises this cycle of treatment.
Fig. 3.22 PGD work up for case 9-J 46,XX,t(12;22)(q24.1;q11.2)

(a), (b): Strategy devised for PGD for case 9-J 46,XX,t(12;22)(q24.1;q11.2).
(c) FISH analysis of patient lymphocyte metaphase nucleus showing the normal and derivative chromosomes.
Fig. 3.23 FISH analysis on triploid fibroblasts and blastomeres from a spare embryo using the probe combination employed for PGD case J 46,XX,t(12;22)(q24.1;q11.2) as preliminary work up.

(i): Triple colour FISH analysis on (i) a metaphase nucleus of a triploid fibroblast cell line treated with hypotonic solution. Three copies of each probe are detected. (ii), (iii): The same probe combination on blastomeres from spare embryos used as preliminary work-up in this case, showing (ii): monosomy 22 and (iii): disomy for both chromosomes 12 and 22.
Table 3.33. Classification of embryos from the cancelled PGD cycle of patient 9-J. Karyotype 46,XX,t(12;22)(q24.1;q11.2)

<table>
<thead>
<tr>
<th>EMBRYO NO, GRADE &amp; NO OF CELLS</th>
<th>RESULTS FROM SPARE EMBRYO ON DAY 4.</th>
<th>SEGREGATION PATTERN OF THE GAMETE</th>
<th>EMBRYO CLASSIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Grade 2 2-cell</td>
<td>2 cell stage. 3 nuclei were analysed. Nucleus1: Monosomy 22q11.2→qter, 12pter→q24.1 Nucleus2: Trisomy 12, nullisomy 22 Nucleus3: Tertiary trisomy 22pter→22q11.2, 12q24.1→qter</td>
<td>Unknown</td>
<td>Chaotic</td>
</tr>
<tr>
<td>2 Grade 2 2-cell</td>
<td>2 cell stage- No nuclei seen while spreading.</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>3 Grade 3 1-cell</td>
<td>1 cell stage- Nuclei lost during spreading.</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>4 Grade 3 4-cell</td>
<td>4 cell stage Embryo failed to lyse</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>5 (degenerated)</td>
<td>1 nucleus: Tertiary trisomy 22pter→22q11.2, 12q24.1→qter</td>
<td>3:1 Tertiary</td>
<td>Aneuploid</td>
</tr>
<tr>
<td>6 (degenerated)</td>
<td>6 nuclei fragments revealed degenerated chromatin. Only two nuclei were scored Nucleus1 &amp; 2: Monosomy 12q24.1→qter, trisomy 22q11.2-qter.</td>
<td>Adjacent-1-</td>
<td>Aneuploid</td>
</tr>
</tbody>
</table>

9 embryos (frozen on day 2 of development) were thawed. Seven survived, 2 were degenerated (embryos 5 and 6). Following 24 hours 2 embryos were at a 5 cells stage, 1 was a 6 cell, 1 was a 4 cell, 1 was a 2 cell, and 2 were arrested at 1 cell stage. PGD was cancelled as embryos were not suitable for biopsy. The patient had a 3 embryos transferred and the remaining 4 were analysed.
3.8 Summary of Outcome of PGD Cycles.

Of sixteen PGD cycles initiated for eleven couples referred for PGD of chromosomal abnormalities; one was cancelled due to poor stimulation, whilst fifteen cycles reached the oocyte retrieval stage. Two cycles resulted in embryos showing retarded or arrested development on day three post-insemination and PGD was cancelled. The thirteen remaining cycles (five IVF, eight ICSI) proceeded to PGD for nine couples; six couples with balanced reciprocal translocations (17-A, 20-B, 13-C, 6-D, 23-G, 18-H) two with balanced Robertsonian translocations (32-I, 35-E), and one for an intrachromosomal insertion (40-F). Maternal age ranged from 25 to 36 years in these nine couples (mean 32.7 years). The patient’s reproductive histories are summarised in Table 3.34 Details of probe combinations for diagnosis and for re-probing are given in Table 3.35 together with the efficiencies determined on control lymphocytes.

Table 3.36 summarises the outcome of the 13 PGD cycles performed for 9 couples carrying a chromosomal rearrangement. In total, 166 oocytes were collected, 135 (83.7%) fertilised and 113 of the resulting embryos were suitable for biopsy. Only 21.2% (24/113) of embryos available were suitable for transfer, representing 14.4% of the oocytes retrieved. Twelve cycles resulted in embryo transfer (four triple embryo transfers, four double embryo transfers and four single embryo transfers) that resulted in three normal livebirths and one biochemical pregnancy (Table 3.36).
<table>
<thead>
<tr>
<th>Case</th>
<th>Fertility Status</th>
<th>Female age</th>
<th>Parental Karyotype</th>
<th>Normal Livebirths</th>
<th>Chromosomally Abnormal Livebirths</th>
<th>Previous PND &amp; TOP or miscarriages</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-A</td>
<td>Secondary Infertility</td>
<td>35</td>
<td>Balanced reciprocal translocation 46,XX,t(5;11)(q34;q25)</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>20-B</td>
<td>Secondary Infertility</td>
<td>32</td>
<td>Balanced reciprocal translocation 46,XX,t(1;2)(q42.1;p23)</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>13-C</td>
<td>Primary Infertility</td>
<td>33</td>
<td>Balanced reciprocal translocation 46,XX,t(16;17)(p13.3:p11.1)</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>6-D</td>
<td>Primary Infertility</td>
<td>25</td>
<td>Balanced Reciprocal Translocation 46,XY,t(5;19)(p12;p12)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>35-E</td>
<td>Primary Infertility</td>
<td>34</td>
<td>Balanced Robertsonian Translocation 45,XY,der(13;21)(q10;q10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>40-F</td>
<td>Fertile</td>
<td>33</td>
<td>Intrachromosomal Between arm insertion 46,XX,ins(7)(p22q32q31.1)</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>23-G</td>
<td>Multiple miscarriages</td>
<td>32</td>
<td>Balanced reciprocal translocation 46,XX,t(8;12)(q11.2;q12)</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>18-H</td>
<td>Sub-fertile (2 miscarriages)</td>
<td>36</td>
<td>Balanced Reciprocal Translocation 46,XY,t(1;18)(p32;q23)</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>32-I</td>
<td>Primary Infertility</td>
<td>35</td>
<td>Balanced Robertsonian Translocation 45,XY,der(13;14)(q10;q10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Patients</td>
<td>Probes Used for Diagnosis</td>
<td>Probes used for re-FISH</td>
<td>Diagnostic Probe Combination Efficiency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------</td>
<td>------------------------</td>
<td>-----------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17-A</td>
<td>CEP11 α-satelliteDNA/D11Z1 11p11.1-q11.1: SO &amp; SG</td>
<td>Vysis UroVysion Multicolour Probe (4-colour DNA probes for chr 3 SR, chr7 SG, chr17 SA, chr 9 Sg)</td>
<td>84%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TelVysion 11q SO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LSI D5S23/D5S721 L SG maps to chromosome 5p15.2. All probes were from Vysis U.K.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-B</td>
<td>Laboratory prepared satellite III 1q12 – pUC 1.77 insert size 1.77kb. (Cooke and Hindley 1979). Labelled SO &amp; SG using Vysis nick translation kit.</td>
<td></td>
<td>86%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TelVysion 1q SO-Vysis UK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-satellite biotinylated 2p11.1-q11.1. Indirectly labelled. Detection kit biotin-FITC. Oncor-Appligene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13-C</td>
<td>Satellite II DNA/D16Z3 16q11.2 probe SO &amp; SG</td>
<td></td>
<td>92%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Miller-Dieker region probe detection set (LSI SO/RARA17q21.1 SG control probe)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>All probes were from Vysis U.K.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-D</td>
<td>LSI EGR1 SO/ SG D5S721, D5S23. SO maps to chromosome 5q31, SG maps to chromosome 5p15.2</td>
<td></td>
<td>96.75%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Laboratory prepared YAC locus specific probe for chromosome 19, mapping to position 19q13.2 (Frengen et al., 1999). Labelled SO and SG using Vysis UK nick translation kit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| 25-E | -LSI 21 SO, D21S529, D21S341, D21S342. SO maps to chromosome 21q22.13-q22.2  
-LSI 13 SG, spans the Retinoblastoma gene (RB1). SG maps to chromosome 13q14  
Both from AneuVysion Kit, probe mixture 2, Vysis UK  
-TEL 21q, labelled in green, Oncor, UK  
-TEL 21q, labelled in red, Oncor, UK |
| 40-F | -LSI Williams Syndrome Region, Elastin Probe LSI Elastin 7q11.23 SO/D7S486,D7S522 control 7q31 SG- 
- α-satellite DNA/D8Z2 8p11.1-q11.1, SG Vysis UK  
-TelVysion 8q SO- Vysis UK |
| 23-G | -Laboratory prepared centromeric 12 α-satellite (Baldini et al., 1990). Labelled SO and SG using Vysis UK nick translation kit.  
- α-satellite DNA/D8Z2 8p11.1-q11.1, SG Vysis UK  
-TelVysion 8q SO- Vysis UK |
-TelVysion 1p SG- Vysis UK  
- centromeric alpha satellite DNA/D18Z1, 8p11.1-q11.1, SA, Vysis UK |

-CEP 18, centromeric α-satellite D18Z1, 18p11.1-q11.1, SA  
-CEP X, centromeric satellite DXZ1, Xp11.1-q11.1, SG  
-CEP Y, α-satellite DYZ3, Yp11.1-q11.1, SO  
-AneuVysion Kit from Vysis UK  
- α-satellite DNA/D8Z2 8p11.1-q11.1, SG Vysis UK  
-TelVysion 7q SO  
-TelVysion 7p SG  
All probes were from Vysis UK  
-CEP 18, centromeric α-satellite D18Z1, 18p11.1-q11.1, SA  
-CEP X, centromeric satellite DXZ1, Xp11.1-q11.1, SG  
-CEP Y, α-satellite DYZ3, Yp11.1-q11.1, SO  
-AneuVysion Kit from Vysis UK  
- α-satellite DNA/D8Z2 8p11.1-q11.1, SG Vysis UK  
-TelVysion 7q SO  
-TelVysion 7p SG  
87.5%  
-AneuVysion Kit from Vysis UK  
-Laboratory prepared satellite III 1q12 – pUC 1.77 insert size 1.77kb.(Cooke and Hindley 1979). Labelled SO & SG using Vysis nick translation kit.  
-TelVysion 1p SG- Vysis UK  
- centromeric alpha satellite DNA/D18Z1, 8p11.1-q11.1, SA, Vysis UK  
-Laboratory prepared centromeric 12 α-satellite (Baldini et al., 1990). Labelled SG using Vysis UK nick translation kit.  
91%  
264
Table 3.36- Summary of the outcome of 13 cycles of PGD for 9 couples carrying a chromosomal rearrangement.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Cycles</th>
<th>Oocytes Retrieved</th>
<th>Oocytes Fertilised</th>
<th>Embryos Biopsied</th>
<th>Normal/ Balanced</th>
<th>Abnormal*</th>
<th>Embryos Transferred</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-A</td>
<td>3</td>
<td>34</td>
<td>29</td>
<td>22</td>
<td>8 (30.8%)</td>
<td>18 (69.2%)</td>
<td>7</td>
<td>Normal livebirth</td>
</tr>
<tr>
<td>20-B</td>
<td>1</td>
<td>16</td>
<td>11</td>
<td>11</td>
<td>2 (20%)</td>
<td>8 (80%)</td>
<td>2</td>
<td>No pregnancy</td>
</tr>
<tr>
<td>13-C</td>
<td>1</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>4 (50%)</td>
<td>4 (50%)</td>
<td>3</td>
<td>Biochemical pregnancy</td>
</tr>
<tr>
<td>6-D</td>
<td>1</td>
<td>20</td>
<td>15</td>
<td>13</td>
<td>1 (8.3%)</td>
<td>12 (91.7%)</td>
<td>1</td>
<td>No pregnancy</td>
</tr>
<tr>
<td>35-E</td>
<td>2</td>
<td>22</td>
<td>17</td>
<td>15</td>
<td>6 (40%)</td>
<td>9 (60%)</td>
<td>6</td>
<td>No pregnancy</td>
</tr>
<tr>
<td>40-F</td>
<td>1</td>
<td>16</td>
<td>16</td>
<td>11</td>
<td>7 (70%)</td>
<td>3 (30%)</td>
<td>2</td>
<td>Normal livebirth</td>
</tr>
<tr>
<td>23-G</td>
<td>1</td>
<td>21</td>
<td>17</td>
<td>14</td>
<td>2 (14.3%)</td>
<td>11 (85%)</td>
<td>1</td>
<td>Normal livebirth</td>
</tr>
<tr>
<td>18-H</td>
<td>1</td>
<td>16</td>
<td>14</td>
<td>12</td>
<td>1 (8.3%)</td>
<td>11 (91.7%)</td>
<td>1</td>
<td>Balanced embryo frozen as</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>frozen/thawed</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>blastocyst</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>no pregnancy</td>
<td></td>
</tr>
</tbody>
</table>

32-I: LSI 13 SG, spans the Retinoblastoma gene (RB1). SG maps to chromosome 13q14- Vysis UK - TelVysion 14q SR- Vysis UK
<table>
<thead>
<tr>
<th>32-I</th>
<th>2</th>
<th>13</th>
<th>8</th>
<th>8</th>
<th>2 (28%)</th>
<th>5 (72%)</th>
<th>1</th>
<th>No pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>13</td>
<td>166</td>
<td>135</td>
<td>113</td>
<td>33 (29%)</td>
<td>81 (71%)</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

*Abnormal includes embryos shown to have an unbalanced chromosome complement either due to abnormal meiotic segregation or to post-zygotic errors.

The % of normal/abnormal embryos is based on the embryos that have been analysed (including biopsied embryos but also embryos not suitable for biopsy spread and FISH-ed on day 4 or 5) and provided results for embryo classification.
Results

Two blastomeres were removed from 60% of the embryos biopsied, while a single cell biopsy was taken for the remainder. For 30% of the biopsied embryos, results were obtained from both biopsied cells. For the majority (60%) of these embryos the results of the two blastomeres were in concordance (Table 3.37). For approximately 30% of embryos from which a single cell was taken, no result was available from the biopsy, as cells were lost during spreading or FISH. For 40% of the embryos recommended for transfers/cryopreservation, two biopsied blastomeres had given a normal result, whilst for the majority (60%) of the embryos, selection was based on the results obtained from one blastomere (Table 3.37).

Analysis of the remaining cells in untransferred embryos was performed to confirm the biopsy results. Table 3.38 shows the detailed outcome of PGD for 9 couples carrying chromosomal rearrangements. Detailed analysis provided evidence for the co-existence of chromosomally balanced and abnormal cells in embryos a, b, c, d, e, f, g, h, i, j, and k. Table 3.39 lists the embryos that belong to this category for which a possible mechanism of origin could be suggested. Embryos f, h and k are classified as balanced/chaotic and therefore no possible mechanism could be suggested for them.
Table 3.37- Summary of embryos biopsied and results obtained.

<table>
<thead>
<tr>
<th>Case</th>
<th>No of Embryos from which ≥2 cells were biopsied, but No result was available</th>
<th>No of Embryos from which ≥2 cells were biopsied &amp; result was obtained from 1</th>
<th>No of Embryos from which ≥2 cells were biopsied but results disagree</th>
<th>No of Embryos from which 1 cell was biopsied and results from both agree</th>
<th>No of Embryos in which &gt;1 nucleus was in biopsied cell</th>
<th>No of Embryos subjected to ET/freezing based on results from 2 cells</th>
<th>No of Embryos subjected to ET/freezing based on results from 1 cells</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-A</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>9</td>
<td>1</td>
<td>3</td>
</tr>
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<td></td>
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<td>Normal Livebirth</td>
</tr>
<tr>
<td>20-B</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>No pregnancy</td>
</tr>
<tr>
<td>13-C</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
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<td>Biochemical pregnancy</td>
</tr>
<tr>
<td>6-D</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>No pregnancy</td>
</tr>
<tr>
<td>35-E</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>No pregnancy</td>
</tr>
<tr>
<td>40-F</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Normal Livebirth</td>
</tr>
<tr>
<td>23-G</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>0</td>
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<td></td>
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<td>Normal Livebirth</td>
</tr>
<tr>
<td>18-H</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>No pregnancy</td>
</tr>
<tr>
<td>32-I</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No pregnancy</td>
</tr>
<tr>
<td>Total</td>
<td>10 (8.8%)</td>
<td>24 (21.2%)</td>
<td>20 (17.7%)</td>
<td>13 (11.5%)</td>
<td>14 (12.5%)</td>
<td>13 (11.5%)</td>
<td>6 (5.3%)</td>
<td>20 (17.7%)</td>
</tr>
</tbody>
</table>
Table 3.38- Detailed outcome of 11 cycles of PGD for 8 couples carrying chromosomal rearrangements

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cycle</th>
<th>Biopsy Result</th>
<th>Follow up: Diagnostic probes</th>
<th>Follow up: Re-probe</th>
<th>Segregation</th>
<th>Embryos Transferred</th>
<th>Comment</th>
</tr>
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<tbody>
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<td>1</td>
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<td>-</td>
<td>Alternate: 3</td>
<td>2</td>
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<tr>
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<td></td>
<td>3:1 tertiary: 1</td>
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<td>Aneuploid: 1</td>
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<td>3</td>
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<td>Aneuploid/bal: 2</td>
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<td>Adjacent 1: 3</td>
<td></td>
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<tr>
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<td>Chaotic/aneup.: 1</td>
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<td>Adjacent 2: 1</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Aneuploid/mos.: 1</td>
<td>Aneup. Mos: 1</td>
<td></td>
<td>3:1 interch.: 1</td>
<td>2 embryos aneuploid with balanced cells detected</td>
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<td>No result: 2</td>
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<td>Dip/chaotic: 2</td>
<td>Alternate: 5</td>
<td>2 live birth</td>
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<td>2</td>
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<td>Adjacent 1: 3</td>
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<td>Aneuploid mos: 1</td>
<td>Chaotic/aneup: 1</td>
<td></td>
<td>Unknown: 3</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>No result: 6</td>
<td></td>
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</tr>
</tbody>
</table>

269
| 13-C | 1 | Balanced: 4  
Aneuploid: 1  
Aneuploid mos: 1  
Aneup/haploid: 1 | Aneup/haploid: 1  
Chaotic: 3  
No result: 1 | - | Alternate: 4  
3:1 interch.: 1  
Unknown: 3 | 3 biochem.  
preg. |
| 6-D | 1 | Balanced: 1  
Aneuploid: 4  
Aneuploid mos: 4  
No result: 4 | Aneuploid: 1  
Aneup.mos: 2  
Chaotic: 8  
No result: 1 | - | Alternate: 1  
Adjacent 1: 1  
Unknown: 11 | 1 |
| 35-E | 1 | Balanced: 3  
Aneuploid: 2  
No result: 1 | Bal/aneuploid: 1  
Chaotic: 1  
No result: 1 | Chaotic: 1  
No result: 2 | Alternate: 4  
Adjacent 1: 1  
Unknown: 1 | 3  
bal/aneu  
embryo  
(includes  
CGH  
analysis) |
| 2 | Balanced: 3  
Aneuploid: 7 | Chaotic: 7 | Chaotic: 4  
No result: 3 | Alternate: 3  
Unknown: 7 | 3 |
| 40-F | 1 | Balanced: 7  
Duplication: 2  
Deletion: 1  
No result: 1 | Balanced: 1  
Duplication: 1  
Deletion: 1  
Chaotic: 1  
Frozen: 5 | - | Balanced: 8  
Duplicated: 1  
Deleted: 1  
Unknown: 1 | 2  
live birth |
| 23-G | 1 | Balanced: 1  
Bal/aneuploid: 1  
Aneuploid: 3  
Aneuploid mos: 2  
No result: 6 | Balanced:1  
Bal/aneuploid: 1  
Bal/chaotic: 2  
Aneuploid: 1  
Aneuploid mos: 1  
Aneup/chaotic: 1  
Chaotic: 4  
Tetraploid: 1 | Diploid: 5  
Diploid/chaotic: 2  
Chaotic: 3  
Tetraploid +  
Aneuploid: 1  
No result: 1 | Alternate: 6  
Adjacent 2: 3  
Unknown: 4 | 1  
live birth  
3 embryos  
contained  
balanced  
and  
aneuploid  
cells |
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<td></td>
<td>Bal/chaotic: 1</td>
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<td>Aneuploid mos: 1*</td>
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<tr>
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<td>Bal/chaotic: 2</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Alternate: 1</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adjacent-1:2</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Unknown: 2</td>
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<tr>
<td>Total</td>
<td>Balanced: 32 (27%)</td>
<td>Balanced: 5</td>
<td>Aneuploid: 11 (13%)</td>
<td>Bal/aneuploid: 7 (8%)</td>
<td>Altern:** Balanced: 8</td>
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<tr>
<td></td>
<td>Aneuploid: 40 (34%)</td>
<td>Aneuploid: 11 (13%)</td>
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<td>Adjacent: 2:1 (21%)</td>
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<tr>
<td></td>
<td>Bal/aneup: 4 (3.4%)</td>
<td>Bal/aneuploid: 7 (8%)</td>
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<td></td>
<td>Adjacent: 2:5 (5%)</td>
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<tr>
<td></td>
<td>Aneup. mos: 18 (15%)</td>
<td>Aneup. mos: 7 (8%)</td>
<td></td>
<td></td>
<td>3:1 Inter: 2 (1%)</td>
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<td></td>
<td>Haploid: 1 (0.8%)</td>
<td>Aneup. mos: 7 (8%)</td>
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<td></td>
<td>3:1 Tertiary: 1 (1%)</td>
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<td></td>
<td>Aneu/haploid: 1(0.8%)</td>
<td>Chaotic: 30 (36%)</td>
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<td>Duplication: 2</td>
<td>Aneup/chaotic: 4 (5%)</td>
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<td>**Balanced: 8</td>
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<tr>
<td></td>
<td>Deletion: 1</td>
<td>Bal/chaotic: 6 (8%)</td>
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<tr>
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<td>No result: 23 (19%)</td>
<td>Chaotic/ bal/aneup: 1 (1.3%)</td>
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<tr>
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<td>Not biopsied: 6</td>
<td>Tetraploid: 1 (1.3%)</td>
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<td>Unknown: 1</td>
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<tr>
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<td></td>
<td>Aneu/haploid: 1 (1.3%)</td>
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<td>**CaseF</td>
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</tr>
<tr>
<td></td>
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<td>Duplication: 1</td>
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<td>Frozen: 5</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Deletion: 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>No result: 11 (13%)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**KEY- / indicates mosaicism; Mos: mosaic; Aneup: aneuploid; Bal: chromosomally balanced; Dip: diploid for the chromosomes investigated; Chaotic: randomly varying chromosome constitution; Biochem preg: biochemical pregnancy.**

*Embryos frozen on day 5, thawed prior to analysis*
<table>
<thead>
<tr>
<th>Patient</th>
<th>Cycle</th>
<th>Embryo</th>
<th>Biopsy Result</th>
<th>Number of cells on day 4/5</th>
<th>Chromosome Constitution</th>
<th>Mechanism</th>
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</thead>
<tbody>
<tr>
<td>17-A</td>
<td>2</td>
<td>a</td>
<td>1 binucleate aneuploid</td>
<td>23</td>
<td>16 adjacent 1 balanced; loss 11qtel.</td>
<td>Chromosome breakage and loss of segment</td>
</tr>
<tr>
<td>17-A</td>
<td>2</td>
<td>b</td>
<td>1 balanced</td>
<td>13</td>
<td>6 adjacent 1 balanced; loss 11q tel.</td>
<td>Chromosome breakage and loss of segment or hybridisation failure</td>
</tr>
<tr>
<td>17-A</td>
<td>3</td>
<td>c</td>
<td>1 aneuploid</td>
<td>4</td>
<td>2 balanced 2 -5; -11; -11</td>
<td>Chromosome loss</td>
</tr>
<tr>
<td>17-A</td>
<td>3</td>
<td>d</td>
<td>No result</td>
<td>7</td>
<td>4 adjacent-1 balanced; loss 11qtel.</td>
<td>Chromosome breakage and loss of segment or hybridisation failure</td>
</tr>
<tr>
<td>17-A</td>
<td>3</td>
<td>e</td>
<td>No result</td>
<td>5</td>
<td>3 balanced 2 -der 5; -11</td>
<td>Chromosome loss</td>
</tr>
<tr>
<td>23-G</td>
<td>1</td>
<td>g</td>
<td>1 balanced 1 +12</td>
<td>10</td>
<td>7 balanced 3 -der8</td>
<td>Chromosome loss; Chromosome gain</td>
</tr>
<tr>
<td>18-H</td>
<td>1</td>
<td>i</td>
<td>1 balanced 1 adjacent 1</td>
<td>8</td>
<td>6 adjacent 1 balanced; loss 1p tel.</td>
<td>Chromosome breakage and loss of segment or hybridisation failure</td>
</tr>
<tr>
<td>18-H</td>
<td>1</td>
<td>j</td>
<td>1 balanced 1 -derl</td>
<td>11</td>
<td>3 balanced 3 -der1 5 chaotic</td>
<td>Chromosome loss</td>
</tr>
</tbody>
</table>


Combined results of embryo analysis show that 29% of embryos were normal for the chromosomes tested and that 71% were chromosomally abnormal (Table 3.36). Based on the biopsy results 27% of the embryos subjected to biopsy were balanced for the chromosomes examined, while 4 more embryos presented with one cell balanced and one aneuploid at the time of the diagnosis. An impressive 49% of the embryos biopsied were categorised at the time of biopsy as aneuploid or aneuploid mosaic. These were categorised as 34% aneuploid or aneuploid mosaic and 15% chaotic. Results obtained from follow-up analysis showed 23% of the embryos carrying a balanced cell line with 7.5% of them being uniformly balanced. Aneuploid and aneuploid/mosaics accounted for 20 %, while fully chaotic were 36% (Table 3.38). On the basis of the total results it was deduced that 36% of the embryos were a result of alternate segregation however, for the same percentage of embryos the segregation pattern was unknown. Adjacent-1 segregation gave rise to 21% of the embryos analysed, adjacent-2 gave rise to 5%, while 3:1 interchange and tertiary accounted for 2% of the embryos analysed (Table 3.38).
3.9 Wolf Hirschhorn Syndrome Project Results

3.9.1 Background Information.

Karyotyping initially revealed an unbalanced translocation involving chromosome 4p. The origin of the extra material translocated on to 4p was unknown and both parental karyotypes were normal. Certain phenotypic characteristics (see section 2.1.3.1) such as the “Greek helmet” appearance implied the involvement of the WHSCR. The deletion of the whole WHCR was excluded by application of a cosmid probe for D4S96, a marker situated within the critical region. However, the phenotype suggested that there might be partial deletion of the WHSCR or, alternatively disruption of the expression of one or more genes due to the translocation. According to the above information given by the preliminary work carried out by cytogeneticists at UCLH and in the Royal Free Haematology Department, the project was undertaken to determine if there was evidence for deletion within the WHSCR at the molecular level. The availability of material from this interesting patient and of cloned DNA (cosmids) from the WHSCR provided an ideal opportunity for further delineation of the region. We proposed to use molecular cytogenetic techniques (CGH and PCR) to determine the origin of the additional chromosome material and to determine the extent, if any, of the deletion of the WHSCR in this case. We employed polymorphic DNA markers in this study to examine in a more detailed molecular fashion the possibility of the involvement of the WHSCR in this case and the parental origin of the abnormality. It was hoped that this information might then provide the basis on which to proceed to molecular studies towards gene isolation.

While the molecular studies were in progress, further work was performed by cytogeneticists at UCLH and in the Royal Free Haematology Department. Cytogenetic analysis, G-banding, application of whole chromosome paints, and FISH with probes: centromeric, locus specific and pantelomeric, revealed a case of pure trisomy 20p arising from de novo isochromosome formation associated with non-reciprocal translocation involving 4p without any concomitant loss of material from 4p. The karyotype was interpreted as 46,XY,der(4)t(4;20)(p16.3;q11.1)i(20)(q11.1). Fig. 3.24 summarises the extensive FISH investigation performed on this patient. Der(4)t(4;20)(p16;q11.1)i(20)(q11.1) shown by (a) G banding. (b) whole
Results

chromosome paints for chromosome 4 (cy3) and 20 (FITC). (c) Subtelomeric probes for 20p (FITC) and 20q (Cy3) showing 20q signal on der(4)t(4;20). (d) Subtelomeric probes for 4p (FITC) and 4q (Cy3), showing interstitial 4p signal near the breakpoint junction on der(4)t(4;20). (e) All human telomeres probe (FITC) showing interstitial signal on der(4)t(4;20). (f) Probe for D20Z1 (rhodamine) showing similar signals on the normal and isochromosome 20, but lack of signal at the breakpoint junction on der(4)t(4;20). Chromosome 4 was identified by the centromeric signal of D4Z1 (rhodamine). (g) All human centromeres probe (FITC) showing signal at the breakpoint junction of der(4)t(4;20). Chromosome 4 was identified by previous hybridisation with D4Z1 (rhodamine) resulting in a yellow signal. Fig. 3.24 is courtesy of The Royal Free Hematology Department and the cytogenics unit that performed all the investigations.
Fig. 3.24 Extensive FISH investigation performed for the WHS project.
(Courtesy of the Royal Free Cytogenetics Department)
3.9.2 DNA extraction.

DNA extraction was performed for the patient and his family (father, mother, sister). The efficiency of the original DNA extraction was assessed by performing PCR amplification of an unrelated sequence. The PCR employed a standard protocol and primers for the thalassaemia gene. The PCR products were run on an agarose gel and the results of the gel electrophoresis, based on the brightness and the thickness of the bands, showed that the DNA obtained from the father (F), the mother (M), and the sister (S) was in fact very dilute while the DNA of the patient (P) was at an optimal concentration. DNA precipitation was performed in order to increase DNA concentration of the samples as required for further investigation to take place. The DNA precipitation was successful however the optimal concentration failed to be achieved for the F, M, and S DNA samples.

As the project progressed it was essential to perform another DNA extraction from the family which provided DNA samples of optimal concentrations for all the members of the family F1, M1, S1, P1.

3.9.3 PCR protocols and DNA analysis

The PCR protocols that have been devised to ensure optimal conditions for the markers and primers involved have been altered numerous times to achieve improvements. Those alterations will be discussed and explained in the discussion (section 4.3). Several random DNA samples (DA, BW, JE, SF52α, WH54α, M257α) other than those obtained from the patient's family were employed in the project, acting as internal controls, to ensure the authenticity of the results obtained.

The markers employed, their sequence and source are listed in Table 2.4.

**PCR protocols and DNA analysis employing polymorphic marker D4S127.**

<table>
<thead>
<tr>
<th>Protocol 1.</th>
<th>PCR MIX</th>
<th>PCR CONDITIONS (Gradient )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5μl 10 X Buffer</td>
<td>Hot Start</td>
<td>Denaturation 95°C, 4'30'', 1 Cycle</td>
</tr>
<tr>
<td>2.5μl dNTPs</td>
<td></td>
<td>Denaturation 95°C, 30'', 25 Cycles</td>
</tr>
<tr>
<td>1μl D4S127(F) 9.3pmol</td>
<td></td>
<td>Annealing temperature: 48.7°C, 50.6°C, 52.8°C, 55.1°C, 57.4°C, 59.4°C, 62.1°C, 45'', 25 Cycles</td>
</tr>
<tr>
<td>1μl D4S127® 9.7pmol</td>
<td></td>
<td>Extention: 72°C, 45'', 25 Cycles</td>
</tr>
<tr>
<td>0.1 Taq</td>
<td></td>
<td>Final extention: 72°C, 10', 1 Cycle</td>
</tr>
<tr>
<td>16.9μl water</td>
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</tr>
</tbody>
</table>
The PCR products were loaded on the ABI 310 PRISM™ for analysis. BW proved to be homozygous for an allele of 159bp size while JE was heterozygous for alleles 152/154 bp.

According to the peak size representing the intensity of a band if the samples were analyzed with gel electrophoresis, the optimal annealing temperature for this set of primers was 52.8°C.

Protocol 2.

**PCR MIX**

- 2.5μl 10 X Buffer
- 2.5μl dNTPs
- 0.7μl D4S127(F) 6.5pmol
- 0.65μl D4S127® 6.3pmol
- 0.1 Taq
- 17.55μl water

**PCR CONDITIONS**

- DNA: BW, JE
- Hot Start
- Denaturation 95°C, 4'30" , 1 Cycle
- Denaturation 95°C, 30" , 25 Cycles
- Annealing temperature: 52.8°C
- Extension: 72°C, 45" , 25 Cycles
- Final extension: 72°C, 10', 1 Cycle.

The PCR products were analysed on the ABI 310 PRISM™. Samples F, M, and S: Showed no peaks, while P proved to be heterozygous for alleles of 152bp and 159 bp.

Protocol 3

**PCR MIX**

- 2.5μl 10 X Buffer
- 2.5μl dNTPs
- 0.7μl D4S127(F) 6.5pmol
- 0.65μl D4S127® 6.3pmol
- 0.1 Taq
- 13.75μl water

**PCR CONDITIONS**

- DNA: F, M, S, P
- Hot Start
- Denaturation 95°C, 4'30" , 1 Cycle
- Denaturation 95°C, 30", 25 Cycles
- Annealing temperature: 52.8°C
- Extension: 72°C, 45", 25 Cycles
- Final extension: 72°C, 10', 1 Cycle.

The results obtained from the patient and his family after analysis on the ABI 310 PRISM™ indicated that the father proved heterozygous for alleles 152bp and 159 bp.
The mother was homozygous for allele 159bp, while both the patient and his sister were heterozygous for the same alleles as the father (152/159).

**Protocol 4**

<table>
<thead>
<tr>
<th>PCR MIX</th>
<th>PCR CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5μl 10 X Buffer</td>
<td>Hot Start</td>
</tr>
<tr>
<td>2.5μl dNTPs</td>
<td>Denaturation 95°C, 4'30'', 1 Cycle</td>
</tr>
<tr>
<td>0.7μl D4S127(F) 6.5pmol</td>
<td>Denaturation 95°C, 30'', 25 Cycles</td>
</tr>
<tr>
<td>0.65μl D4S127® 6.3pmol</td>
<td>Annealing temperature:52.8°C</td>
</tr>
<tr>
<td>0.1 Taq</td>
<td>Extention: 72°C, 45'', 25 Cycles</td>
</tr>
<tr>
<td>13.755μl water</td>
<td>Final extention: 72°C, 10'', 1 Cycle.</td>
</tr>
<tr>
<td>1μl DNA</td>
<td></td>
</tr>
<tr>
<td>DNA: F1, M1, S1, Pl(New DNA Extraction)</td>
<td></td>
</tr>
</tbody>
</table>

The results were the same as in protocol 3 analysis.

**PCR protocols and DNA analysis employing polymorphic marker D4S43.**

**Protocol 1**

<table>
<thead>
<tr>
<th>PCR MIX</th>
<th>PCR CONDITIONS GRADIENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5μl 10 X Buffer</td>
<td>Hot Start</td>
</tr>
<tr>
<td>2.5μl dNTPs</td>
<td>Denaturation 95°C, 4'30'', 1 Cycle</td>
</tr>
<tr>
<td>0.6μl D4S43(F) 6.96pmol</td>
<td>Denaturation 95°C, 30'', 25 Cycles</td>
</tr>
<tr>
<td>0.65μl D4S43® 6.48pmol</td>
<td>Annealing temperature:48.4°C, 50.6°C, 52.8°C, 55.1°C, 57.4°C, 59.4°C, 45'', 25 Cycles</td>
</tr>
<tr>
<td>0.1 Taq</td>
<td>Extention: 72°C, 45'', 25 Cycles</td>
</tr>
<tr>
<td>17.74μl water</td>
<td>Final extention: 72°C, 10'', 1 Cycle.</td>
</tr>
<tr>
<td>1μl DNA</td>
<td></td>
</tr>
<tr>
<td>DNA: BW, JE</td>
<td></td>
</tr>
</tbody>
</table>

The PCR products were loaded on the PRISM for analysis. BW proved to be heterozygous for alleles of 194 and 207 bp size, JE was heterozygous for the same alleles.

According to the peak size representing the intensity of a band if the samples were analyzed with gel electrophoresis, the optimal annealing temperature for this set of primers was 55.1°C.
### Results

**Protocol 2**

<table>
<thead>
<tr>
<th>PCR MIX</th>
<th>PCR CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5μl 10 X Buffer</td>
<td>Hot Start</td>
</tr>
<tr>
<td>2.5μl dNTPs</td>
<td>Denaturation 95°C, 4'30'', 1 Cycle</td>
</tr>
<tr>
<td>0.6μl D4S43(F) 6.96pmol</td>
<td>Denaturation 95°C, 30'', 28 Cycles (DNA1μl)/ 35 Cycles (DNA4μl)</td>
</tr>
<tr>
<td>0.65μl D4S43® 6.48pmol</td>
<td>Annealling temperature: 55.1°C, 45''28 Cycles (DNA1μl)/ 35 Cycles (DNA4μl)</td>
</tr>
<tr>
<td>0.1 Taq</td>
<td>Extention: 72°C, 45'', 28 Cycles (DNA1μl)/ 35 Cycles (DNA4μl)</td>
</tr>
<tr>
<td>17.65μl water/14.65μl water</td>
<td>Final extention: 72°C, 10', 1 Cycle.</td>
</tr>
<tr>
<td>1μl DNA(P)/4μl DNA(F, M, S)</td>
<td>DNA: F, M, S, P</td>
</tr>
</tbody>
</table>

All the members of the family were shown to be heterozygous for the same alleles as BW and JE, which would be unlikely as D4S43 is considered to be a highly polymorphic marker. Based on that, contamination or non-specific amplification of primers was presumed.

**Protocol 3:** All the reagents used were new in order to avoid contamination

<table>
<thead>
<tr>
<th>PCR MIX</th>
<th>PCR CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5μl 10 X Buffer</td>
<td>Cold Start(to avoid contamination)</td>
</tr>
<tr>
<td>2.5μl dNTPs</td>
<td>Denaturation 95°C, 4'30'', 1 Cycle</td>
</tr>
<tr>
<td>0.6μl D4S43(F) 6.96pmol</td>
<td>Denaturation 95°C, 30'', 28 Cycles</td>
</tr>
<tr>
<td>0.65μl D4S43® 6.48pmol</td>
<td>Annealling temperature: 55.1°C, 45''28 Cycles</td>
</tr>
<tr>
<td>0.1 Taq</td>
<td>Extention: 72°C, 45'', 28 Cycles</td>
</tr>
<tr>
<td>17.65μl water</td>
<td>Final extention: 72°C, 10', 1 Cycle.</td>
</tr>
<tr>
<td>1μl DNA</td>
<td>DNA: BW, JE, DA.</td>
</tr>
</tbody>
</table>

BW, JE, and DA were shown to be heterozygous for 194/207bp alleles. Those peaks were not a contamination effect but most likely to be resulting from the primers amplifying a non-variable region.
### Protocol 4

<table>
<thead>
<tr>
<th>PCR MIX</th>
<th>PCR CONDITIONS GRADIENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5μl 10 X Buffer</td>
<td>Cold Start</td>
</tr>
<tr>
<td>2.5μl dNTPs</td>
<td>Denaturation 95°C, 4'30'', 1 Cycle</td>
</tr>
<tr>
<td>2.1μl D4S43(F) 24.36pmol</td>
<td>Denaturation 95°C, 30'', 35 Cycles</td>
</tr>
<tr>
<td>3μl D4S43® 24.3pmol</td>
<td>Annealing temperature: 54.2°C, 55.8°C, 57.2°C, 58.9°C, 60.6°C, 62.3°C, 63.8°C, 65°C, 65.7°C, 45'', 35Cycles</td>
</tr>
<tr>
<td>0.1 Taq</td>
<td>Extention: 72°C, 45'', 35Cycles</td>
</tr>
<tr>
<td>13.8μl water</td>
<td>Final extension: 72°C, 10'', 1 Cycle</td>
</tr>
<tr>
<td>1μl DNA</td>
<td>DNA: SF52α</td>
</tr>
</tbody>
</table>

The PCR products were subjected to DNA gel electrophoresis for analysis but the visualisation under UV light showed no amplification.

### Protocol 5

<table>
<thead>
<tr>
<th>PCR MIX</th>
<th>PCR CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5μl 10 X Buffer</td>
<td>Cold Start</td>
</tr>
<tr>
<td>2.5μl dNTPs</td>
<td>Denaturation 95°C, 4'30'', 1 Cycle</td>
</tr>
<tr>
<td>2.1μl D4S43(F) 24.36pmol</td>
<td>Denaturation 95°C, 30'', 35 Cycles</td>
</tr>
<tr>
<td>3μl D4S43® 24.3pmol</td>
<td>Annealing temperature: 55°C, 45'', 35Cycles</td>
</tr>
<tr>
<td>0.1 Taq</td>
<td>Extention: 72°C, 45'', 35Cycles</td>
</tr>
<tr>
<td>13.8μl water</td>
<td>Final extension: 72°C, 10'', 1 Cycle</td>
</tr>
<tr>
<td>1μl DNA</td>
<td>DNA: SF52α, WH54α, MZ57α</td>
</tr>
</tbody>
</table>

The PCR products were analysed by DNA gel electrophoresis but again there were no bands.

### Protocol 6

Protocol 6 was the same as 5 but the annealing temperatures were 58°C, and 60°C. The products were analysed by DNA gel electrophoresis again but there were no bands to prove any amplification.
**Protocol 7**

<table>
<thead>
<tr>
<th>PCR MIX</th>
<th>PCR CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5μl 10 X Buffer</td>
<td>Cold Start</td>
</tr>
<tr>
<td>2.5μl dNTPs(Special mix including 60% Deaza dGTP)</td>
<td>Denaturation 94°C, 4'30'', 1 Cycle</td>
</tr>
<tr>
<td>0.6μl D4S43(F) 6.96pmol</td>
<td>Denaturation 94°C, 20'', 28 Cycles</td>
</tr>
<tr>
<td>0.8μl D4S43® 6.48pmol</td>
<td>Annealling temperature:55°C, 20'', 28Cycles</td>
</tr>
<tr>
<td>0.2 Taq</td>
<td>Extention: 74°C, 20'', 28Cycles</td>
</tr>
<tr>
<td>17.4μl water</td>
<td>Final extention: 74°C, 5', 1 Cycle.</td>
</tr>
<tr>
<td>1μl DNA</td>
<td></td>
</tr>
<tr>
<td>DNA: SF52α, WH54α, MZ57α</td>
<td></td>
</tr>
</tbody>
</table>

The results were analysed on the ABI 310 PRISM™ as follows:

- SF52α was shown to be heterozygous for alleles of this size 326bp, and 378 bp.
- WH54α was shown to be heterozygous for alleles of these sizes 160bp and 184bp.
- MZ57α was shown to be heterozygous for alleles of these sizes 312 bp and 340bp.

**Protocol 8** was the same as 7 but PCR was performed on DNA from the patient and his family(F1, M1, S1, P1). The results were analysed on the ABI 310 PRISM™ and indicating:

- Father (F1) was heterozygous for alleles 230bp and 266 bp.
- Mother (M1) was homozygous for allele 184bp.
- Sister (S1) was heterozygous for alleles 184bp and 266bp.
- Patient (P1) was heterozygous for the same alleles as the sister 184bp and 266 bp.

**PCR protocols and DNA analysis employing polymorphic marker D4S169.**

**Protocol 1**

<table>
<thead>
<tr>
<th>PCR MIX</th>
<th>PCR CONDITIONS GRADIENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5μl 10 X Buffer</td>
<td>Hot Start</td>
</tr>
<tr>
<td>2.5μl dNTPs</td>
<td>Denaturation 95°C, 4'30'', 1 Cycle</td>
</tr>
<tr>
<td>1.25μl D4S169(F) 5.5pmol</td>
<td>Denaturation 95°C, 30'', 24 Cycles</td>
</tr>
<tr>
<td>0.85μl D4S169® 5.52pmol</td>
<td>Annealling temperature:56.3°C, 60.8°C, 63.1°C, 65.1°C, 67.6°C, 45'', 24Cycles</td>
</tr>
<tr>
<td>0.1 Taq</td>
<td>Extention: 72°C, 45'', 24Cycles</td>
</tr>
<tr>
<td>16.8μl water</td>
<td>Final extention: 72°C, 10', 1 Cycle.</td>
</tr>
<tr>
<td>1μl DNA</td>
<td></td>
</tr>
<tr>
<td>DNA: BW, JE</td>
<td></td>
</tr>
</tbody>
</table>

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Results

The PCR products were run on the ABI 310 PRISM™ failing to obtain results as no peaks were observed. Later, it was realised that the size of the particular PCR product was such that it prevented its detection (too large). In fact the product that we were interested in was the result of Rsal digestion. Therefore, the PCR product was digested and the digested product was run on the PRISM. However, as there were no peaks amplification failure seemed likely.

Protocol 2

<table>
<thead>
<tr>
<th>PCR MIX</th>
<th>PCR CONDITIONS GRADIENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5µl 10 X Buffer</td>
<td>Hot Start</td>
</tr>
<tr>
<td>2.5µl dNTPs</td>
<td>Denaturation 95°C, 4'30&quot;, 1 Cycle</td>
</tr>
<tr>
<td>1.5µl D4S169(F) 6.6pmol</td>
<td>Denaturation 95°C, 30&quot;, 35 Cycles</td>
</tr>
<tr>
<td>1µl D4S169® 6.5pmol</td>
<td>Annealing temperature:55°C, 56.4°C, 57.6°C, 60.3°C, 61.7°C, 63°C, 64°C, 64.8°C, 45&quot;, 35Cycles</td>
</tr>
<tr>
<td>0.1 Taq</td>
<td>Extention: 72°C, 45&quot;, 35Cycles</td>
</tr>
<tr>
<td>16.4µl water</td>
<td>Final extention: 72°C, 10&quot;, 1 Cycle.</td>
</tr>
<tr>
<td>1µl DNA</td>
<td></td>
</tr>
<tr>
<td>DNA: BW, JE</td>
<td></td>
</tr>
</tbody>
</table>

The PCR products were digested and both the digested and undigested products were analysed with DNA gel electrophoresis. However, no bands were visualised.

Protocol 3

<table>
<thead>
<tr>
<th>PCR MIX</th>
<th>PCR CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5µl 10 X Buffer</td>
<td>Hot Start</td>
</tr>
<tr>
<td>2.5µl dNTPs</td>
<td>Denaturation 95°C, 4'30&quot;, 1 Cycle</td>
</tr>
<tr>
<td>1.5µl D4S169(F) 6.6pmol</td>
<td>Denaturation 95°C, 30&quot;, 35 Cycles</td>
</tr>
<tr>
<td>1µl D4S169® 6.5pmol</td>
<td>Annealing temperature:65°C, 45&quot;, 35Cycles</td>
</tr>
<tr>
<td>0.1 Taq</td>
<td>Extention: 72°C, 45&quot;, 35Cycles</td>
</tr>
<tr>
<td>16.4µl water</td>
<td>Final extention: 72°C, 10&quot;, 1 Cycle.</td>
</tr>
<tr>
<td>1µl DNA</td>
<td></td>
</tr>
<tr>
<td>DNA: BW, JE</td>
<td></td>
</tr>
</tbody>
</table>

The products were digested with Rsal and both digested and undigested samples were analysed using the PRISM as well as DNA gel electrophoresis. However, there were no peaks or bands identified.
Table 3.40 summarises the results obtained from analysis of the DNA from the patient and his family.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Marker D4S43 allele size(bp)</th>
<th>Marker D4S127 allele size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>230, 266. Heterozygous</td>
<td>152, 159. Heterozygous</td>
</tr>
<tr>
<td>Mother</td>
<td>184 Homozygous</td>
<td>159. Homozygous</td>
</tr>
<tr>
<td>Sister</td>
<td>184, 266. Heterozygous</td>
<td>152, 159. Heterozygous</td>
</tr>
<tr>
<td>Patient</td>
<td>184, 266. Heterozygous</td>
<td>152, 159. Heterozygous</td>
</tr>
</tbody>
</table>

Fig. 3.25 Showing the family pedigree based on the analysis performed on the patient and his family.

Fig. 3.25 showing pedigree of the patient and his family following DNA analysis on the ABI PRISM with primers D4S43 and D4S127.

Analysis revealed that there was no deletion for D4S127 polymorphic marker, minimizing the possibility of the WH involvement as their results supported normal patterns of inheritance. The same was concluded from the analysis for the polymorphic marker D4S43, as the patient and his sister were both heterozygous inheriting one maternal and one paternal allele. No results were obtained from analysis of the polymorphic marker D4S169.
Results

Polymorphic marker D4S169

The PCR product of the primers for this marker was very large (1.6Kb) and we were interested in a small part of it that would result after digestion with Rsal (214bp). PCR was performed according to published suggestions and both the digested and the undigested products were analysed both by agarose gel electrophoresis and the™ 310 PRISM, however, no results, no bands or peaks were obtained. The activity of Rsal was tested on other PCR products and the enzyme was working satisfactorily. The fact that there were no bands when the original products were run on agarose gel could be due to the lack of sensitivity of the detection technique and the fact that the original PCR product was too big to be detected by electrophoresis. Similarly, the ABI 310 PRISM™ could not detect a product of this size (1.6Kb) and it was speculated that the reason why the 214bp Rsal fragment was not detected by the ABI 310 PRISM™ was possibly the fact that the fluorescently tangled fragment of the 1.6Kb product would be not the 214 bp but the other Rsal fragment. The results obtained by this molecular refinement study, support the cytogenetic data showing there is no WHS involvement in this case.

3.9.4 CGH

CGH was employed as a molecular cytogenetic technique in order to eliminate the possible involvement of other chromosomal regions for the patient. The CGH investigation as illustrated in Fig. 3.26 confirmed the cytogenetic finding that the patient was carrying pure trisomy of the short arm of chromosome 20. The karyotype deduced from the CGH results is rev ish XY,enh(20p13q11.1).
Fig. 3.26 Showing CGH analysis and interpretation on the patient.

(a) captured metaphase showing efficient hybridisation of the fluorescently labelled DNA of the patient against normal control reference DNA.

(b) identification of chromosome 20 for the analysis shows the short arm of chromosome 20 to be green, indicating its duplication and confirming the isochromosome formation as shown by FISH in Fig. 3.24.

(c) CGH interpretation. The software detects successfully duplication of the short arm of chromosome 20 in a male DNA sample.
Chapter 3

Results – Part IV Molecular Cytogenetic Analysis of Fetal DNA (ICH Project)
3.10 Molecular Cytogenetic analysis of Fetal DNA 
(ICH Project)

3.10.1 Touch preparations

Fetal tissues from different organs were directly dabbed on a clean slide using the cut surface. A total of 58 slides were provided accounting for 58 aborted fetuses and all were subjected to FISH employing the Vysis UK AneuVysion set screening for chromosomes X(SG), Y(SO), and 18(SA) in order to assess the ploidy status of the samples. The efficiency of the same probe cocktail had been tested on normal male lymphocyte nuclei and was shown to be 96% based on scoring 200 nuclei. Results of ploidy status were successfully obtained for 50 of the samples and 50 interphase nuclei were scored per sample. For the remaining 8 samples no results were obtained due to the fact that the DNA was degenerate and therefore not suitable for FISH. Throughout all the experiments and to ensure probe efficiency a slide of normal male lymphocytes was used along with the touch preps. Table 3.41 summarises the results.

Table 3.41. Summary of results obtained by FISH employing probes for chromosomes X, Y, and 18 in order to assess the ploidy status of 50 fetal samples

<table>
<thead>
<tr>
<th>20 Samples</th>
<th>Male, disomic for chromosome18 (XY, 18, 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26 Samples</td>
<td>Female, disomic for chromosome18 (XY, 18, 18)</td>
</tr>
<tr>
<td>2 Samples</td>
<td>Mosaics carrying both male and female cells, both disomic for chromosome 18. (XX, 18, 18/XY, 18, 18)</td>
</tr>
<tr>
<td>1 Sample</td>
<td>Complex aneuploid mosaic carrying 3 cell types</td>
</tr>
<tr>
<td>(546)</td>
<td>- 42 cells: Female, disomic for chromosome18 (XX, 18, 18)</td>
</tr>
<tr>
<td></td>
<td>- 5 cells: Male, disomic for chromosome18 (XY, 18, 18)</td>
</tr>
<tr>
<td></td>
<td>- 3 cells: Male monosomic for chromosome 18 (XY, 18)</td>
</tr>
<tr>
<td>1 Sample</td>
<td>Complex aneuploid mosaic carrying 4 different cell types</td>
</tr>
<tr>
<td>(542)</td>
<td>- 18 cells: Male, disomic for chromosome18 (XY, 18, 18)</td>
</tr>
<tr>
<td></td>
<td>- 22 cells: Female, disomic for chromosome18 (XY, 18, 18)</td>
</tr>
<tr>
<td></td>
<td>- 4 cells: Male monosomic for chromosome 18 (XY, 18)</td>
</tr>
<tr>
<td></td>
<td>- 6 cells: Disomic for chromosome 18, trisomic for chromosome X (XXX, 18, 18)</td>
</tr>
</tbody>
</table>
3.10.2 DNA Analysis

A total of 46 DNA samples of known concentration were made available (from the Institute of Child Health) and subjected to CGH in order to obtain copy number information on all the chromosomes of the respective fetal tissues. Eighteen out of the 46 sample provided analysable results. All 18 samples were classified as disomic for all 22 sets of chromosomes. Eleven were female and 7 were male. Comparing the CGH results against those of the touch-preps 16 samples showed consistency of results between FISH and CGH, and 2 samples were inconsistent with regard to the sex, suggesting either maternal contamination or a twin pregnancy initially. Unfortunately no DNA was provided from the two cases shown to be complex mosaics by FISH (542, 546). Table 3.42-compares the results obtained from FISH performed on the touch preps against the results obtained from CGH performed on the respective extracted DNA.

Table 3.42- Comparison of FISH and CGH results.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>CGH Classification of extracted DNA</th>
<th>FISH classification of Touch preps</th>
</tr>
</thead>
<tbody>
<tr>
<td>490*</td>
<td>Rev ish XY</td>
<td>XY, 18, 18</td>
</tr>
<tr>
<td>491*</td>
<td>Rev ish XY</td>
<td>XY, 18, 18</td>
</tr>
<tr>
<td>492*</td>
<td>Rev ish XX</td>
<td>XX, 18, 18</td>
</tr>
<tr>
<td>493*</td>
<td>Rev ish XX</td>
<td>XX, 18, 18</td>
</tr>
<tr>
<td>494*</td>
<td>Rev ish XY</td>
<td>Degenerate DNA not suitable for FISH analysis.</td>
</tr>
<tr>
<td>498**</td>
<td>Rev ish XY</td>
<td>Mosaic XY,18,18/XX,18,18</td>
</tr>
<tr>
<td>500*</td>
<td>Rev ish XY</td>
<td>XY, 18, 18</td>
</tr>
<tr>
<td>502*</td>
<td>Rev ish XY</td>
<td>XY, 18, 18</td>
</tr>
<tr>
<td>504*</td>
<td>Rev ish XX</td>
<td>XX, 18, 18</td>
</tr>
<tr>
<td>508*</td>
<td>Rev ish XX</td>
<td>XX, 18, 18</td>
</tr>
<tr>
<td>509*</td>
<td>Rev ish XX</td>
<td>XX, 18, 18</td>
</tr>
<tr>
<td>525**</td>
<td>Rev ish XX</td>
<td>XY, 18, 18</td>
</tr>
</tbody>
</table>
**Results**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Karyotype</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>532*</td>
<td>Rev ish XX</td>
<td>XX, 18, 18</td>
</tr>
<tr>
<td>533*</td>
<td>Rev ish XX</td>
<td>No material was provided.</td>
</tr>
<tr>
<td>534*</td>
<td>Rev ish XX</td>
<td>XX, 18, 18</td>
</tr>
<tr>
<td>536*</td>
<td>Rev ish XX</td>
<td>Degenerate DNA not suitable for FISH analysis.</td>
</tr>
<tr>
<td>547*</td>
<td>Rev ish XY</td>
<td>XY, 18, 18</td>
</tr>
<tr>
<td>548*</td>
<td>Rev ish XX</td>
<td>No material was provided.</td>
</tr>
</tbody>
</table>

*CGH analysis of all samples showed efficient hybridisation, and where classification from FISH on the respective touch-prep was available the FISH and CGH results were consistent.

**Comparison of CGH results to the touch-prep result implies the possibility of contamination of the samples provided, or twin conception.

Following CGH analysis on all the samples provided, the remaining DNA samples that failed to give a result were classified into two categories, A and B.

Category A includes the following samples: 495, 497, 499, 506, 516, 517, 518, 526, 529, 535, 536, 537, 538, 546, 550, 552, 553, 69. The CGH results for these samples were not considered to be analyzable. The hybridisation was uneven, the fluorescence weak, the signals produced speckling, and the chromosome morphology was poor.

Category B includes the following samples: 52, 59d, 60d, 69d, 489, 523, 535, 539, 540, 546. Analysis of CGH performed on these samples proved that the control DNA (spectrum red) had hybridised uniformly on the chromosomes, and evenly on the metaphases producing clear distinct signals, while the test DNA (spectrum green) failed to hybridise on the chromosomes providing only background fluorescence. The same observations were noted on both the commercial and our own indicator slides (same slide pre-treatment), for the same DNA samples, indicating that the test DNA provided could be degenerate.

Further investigation was carried out on the samples from both categories. Agarose gels (1%, 1000volts, 30 minutes) were run for all the DNA samples in question, in order to obtain information as to whether they were suitable for labelling (nick
translation) and further CGH analysis. As a control for this investigation, the control DNA (46,XY that had showed efficient hybridisation) that co-hybridised with the test DNA was included in running the gels. Furthermore, other DNA samples proved to have amplified successfully in previous PCR experiments were included as extra controls for this investigation.

The results obtained from the control DNA samples, showed a clear distinct band indicating DNA of high molecular weight that could be used successfully for CGH experiments to provide efficient hybridisation. The DNA samples from the category A group that fell in that classification, indicating that the DNA was not degraded, include: 69, 495, 497, 499, 526, 529, 536, 537, 538, 550, 552, 553. Therefore, CGH was repeated for these samples. However, analysable results failed to be obtained. This was later attributed to contamination of the test DNA causing failure of hybridisation.

All the remaining samples from the group A (506, 516, 517, 518, 546) and all the samples from group B (where results suggested that the poor DNA quality lead to unsuccessful CGH experiments) (52, 59d, 60d, 69d, 489, 535, 539, 540, 543, 546), gave smears at the gel, indicating degraded or low molecular weight DNA. In the cases where DNA is degraded the failure of CGH experiments is explained. For low molecular weight DNA, the nick translation reaction while labelling would “cut” the DNA into even shorter fragments not suitable for hybridisation.
Chapter 3

Results – Part V Assessing CGH as a Diagnostic Tool on Human Preimplantation Embryos.
3.11 Preliminary Work

Quality control was performed for every step of this study. Prior to commencing this study a preliminary study was performed to ensure the optimal method for analysing single blastomeres by CGH. Single cell isolation of 100 buccal cells was carried out. Subsequent tubing of the buccal cells was followed by DOP-I-PCR (See section 2.2.7.1.1). The final amplified product was run on a 2% agarose gel electrophoresis carried out at 50V for 30 minutes after which gels were viewed via ultra-violet trans-illumination to assess the efficiency of the tubing technique which proved to be 97%. Efficient tubing was confirmed by successful amplification, which was in turn revealed by a smear on the gel. Bands indicating amplification were observed corresponding to a range of fragment sizes of DNA of approximately 1550, 1200, 600 and 450 bp in length, while the average fragment size was approximately 600 bp in length. Positive and negative controls were always included in the tubing process, to ensure reliability of results. The frequency of amplification in negative controls was <5%, while amplification in positive controls was successful with a frequency of 98%.

Five different trisomic fibroblast cell cultures (trisomy 13, 14, 18, 21, and 22 respectively) were made available for this study acting as positive controls in the identification of aneuploidy in single cells. Fig. 3.27 illustrates CGH analysis of some of the trisomic samples. Aneuploidy was correctly identified in single cells with a success rate of 96%, while no false positives were recorded. Repeated CGH analysis of both control lymphocytes and trisomic cells was carried out to optimise the protocol.

Prior to performing CGH on the embryos of the selected couples undergoing PGD, three day 3 spare embryos were donated from an infertile couple undergoing a frozen embryo transfer at the Assisted Conception Unit. These embryos were biopsied and their blastomeres were tubed and subjected to CGH to provide familiarity and understanding of single blastomere CGH. Six blastomeres were tubed from embryo W1, 4 blastomeres from embryo W2, and 7 blastomeres from embryo W3. All 17 blastomeres were subjected to CGH and nine provided analysable results. One sample did not show any hybridisation possibly because the blastomere lysed during the isolation procedure.
Fig. 3.27 showing CGH analysis and interpretation of single cell DNA isolated from four cell cultures trisomic for chromosomes 14, 18, 21, and 22 respectively.

(a) Trisomy 14 analysis and interpretation.

(b) Trisomy 18 analysis and interpretation.

(c) Trisomy 21 analysis and interpretation.

(d) Trisomy 22 analysis and interpretation.
Three blastomeres showed uneven granular hybridisation and for four samples the metaphase target slide was not of optimal quality as it presented with poor chromosome morphology and excess cytoplasm. Therefore for eight blastomeres the results did not provide any reliable information as to their chromosomal constitution. Three blastomeres from embryo W1 provided adequate hybridisation for CGH analysis and were all revealed to be normal male [rev ish XY]. Both blastomeres that provided CGH results from embryo W2 indicated monosomy for chromosomes 16 and X [rev ish X,dim(16),dim(Y)] as shown in Fig. 3.28. Finally, all 4 blastomeres with CGH results from embryo W3 were normal male [rev ish XY]. Table 3.43 summarises the results from the 3 spare embryos.

Table 3.43- CGH results of blastomeres biopsied from three day 3 spare embryos.

<table>
<thead>
<tr>
<th>Embryo no</th>
<th>Cells retrieved</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1</td>
<td>6</td>
<td>3 blastomeres: rev ish XY</td>
</tr>
<tr>
<td>W2</td>
<td>4</td>
<td>2 blastomeres: rev ish X, dim(16),dim(Y)</td>
</tr>
<tr>
<td>W3</td>
<td>7</td>
<td>4 blastomeres: rev ish XY</td>
</tr>
</tbody>
</table>
Results

Fig. 3.28 Showing analysis and interpretation of CGH on biopsied blastomeres from embryo W2.

(a) Captured metaphase showing efficient hybridisation of the fluorescently labelled DNA of a blastomere from embryo W2 against normal control reference DNA.

(b) Identification of chromosomes 16, X, and Y for the analysis shows chromosome 16 to be red, indicating its deletion, and chromosome X showing an equal ratio of red:green indicating that the test DNA (W2) carries a single copy of chromosome X as does the reference DNA (normal male). Finally, chromosome Y appears to be red indicating its exclusion from the test DNA’s chromosomal constitution, and confirming the karyotype of rev ish X, dim(16), dim(Y).

(c) CGH interpretation. The software detects successfully deletion of one copy of chromosomes 16 and X as well as lack of chromosome Y.
3.12 Assessing CGH as a diagnostic tool on untransferred embryos from translocation cases.

The surplus embryos chosen for this study were donated by five couples carrying a chromosomal rearrangement undergoing PGD (described in sections 3.6, 3.7 and 3.8). Patients 17-A, 40-F, 23-G and 18-H had experienced multiple miscarriages and/or termination of pregnancies for reasons of chromosome imbalance, with the exception of patients D and E with primary infertility. Table 3.44 shows their reproductive histories, karyotypes, fertility status and outcome of the cycles performed at the Assisted Conception Unit of University College Hospital London. Following PGD and embryo transfer the spare embryos were again biopsied based on morphological criteria and 1-2 blastomeres of the best quality were retrieved.

During this study 50 blastomeres were investigated from 30 embryos. Table 3.45 lists the embryos involved in the study, including the number of cells biopsied from each embryo, the number of blastomeres for which information was gained regarding their chromosomal status and the number of blastomeres that failed to provide analysable results. Overall, CGH efficiency of single blastomeres in this series was shown to be high, with 82% of the blastomeres providing analysable CGH results. Failure to obtain results involved nine blastomeres and was restricted to patients G and H.
Table 3.44- Reproductive histories of patients included in CGH study

<table>
<thead>
<tr>
<th>CASE</th>
<th>FERTILITY STATUS</th>
<th>FEMALE AGE</th>
<th>PREVIOUS NORMAL LIVE BIRTHS</th>
<th>PREVIOUS MISCARRIAGES, TOP OR ABNORMAL BIRTHS</th>
<th>OUTCOME</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 46,XX,t(5;11)(q34;q25)</td>
<td>Secondary infertility - Low sperm count</td>
<td>35</td>
<td>1</td>
<td>3</td>
<td>1 healthy live birth</td>
</tr>
<tr>
<td>E 45,XY,der(13;21)(q10;q10)</td>
<td>Primary infertility- Oligasthenoteratozoospermia</td>
<td>34</td>
<td>0</td>
<td>0</td>
<td>No pregnancy</td>
</tr>
<tr>
<td>F 46,XX,ins(7)(p22q31.1q32)</td>
<td>Fertile</td>
<td>33</td>
<td>1</td>
<td>2</td>
<td>1 healthy live birth</td>
</tr>
<tr>
<td>G 46,XX,t(8;12)(q11.2;q12)</td>
<td>Secondary infertility</td>
<td>32</td>
<td>0</td>
<td>5</td>
<td>1 healthy live birth</td>
</tr>
<tr>
<td>H 46,XY,t(1;18)(p32;q23)</td>
<td>Secondary infertility</td>
<td>36</td>
<td>0</td>
<td>2</td>
<td>No pregnancy</td>
</tr>
</tbody>
</table>
Table 3.45- List of the embryos involved in the study, how many cells were retrieved and the blastomeres from which results were obtained.

<table>
<thead>
<tr>
<th>Case &amp; Karyotype of carrier parent</th>
<th>Embryos</th>
<th>Blastomeres biopsied</th>
<th>No of blastomeres from which results were obtained</th>
<th>No of blastomeres from which no results were obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 46,XX,t(5;11)(q34;q25)</td>
<td>6 (A1,A2,A6,A8,A10,A11)</td>
<td>6 (one from each embryo)</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>E 45,XY,der(13;21)(q10;q10)</td>
<td>1 (E6)</td>
<td>3 (all from E6)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>F 46,XX,ins(7)(p22q31.1q32)</td>
<td>4 (F1,F3,F6,F10)</td>
<td>5 (one from F1,F6,F10) (two from F3)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>G 46,XX,t(8;12)(q11.2;q12)</td>
<td>11 (G2,G3,G4,G5,G6,G7,G8,G9,G10,G15,G13)</td>
<td>21 (two from G2,G3,G4,G6,G7,G8,G9,G10,G15,G13) (one from G5)</td>
<td>16</td>
<td>5 (G2.2,G8.1,G8.2, G9.2,G13.2)</td>
</tr>
<tr>
<td>H 46,XY,t(1;18)(p32;q23)</td>
<td>8 (H2,H3,H4,H6,H7,H8,H10,H11)</td>
<td>15 (two from H2,H3,H4,H6,H7,H8,H11) (one from H10)</td>
<td>11</td>
<td>4 (H4.1,H6.1,H6.2, H8.2)</td>
</tr>
<tr>
<td>OVERALL</td>
<td>30 Embryos.</td>
<td>50</td>
<td>41 (82%)</td>
<td>9</td>
</tr>
</tbody>
</table>
Results

Tables 3.46-3.50 shows the CGH and FISH results of the embryos and blastomeres involved in this study. Based on these results, the meiotic segregation patterns of the rearranged chromosomes were deduced. According to the comparison between the CGH and FISH analysis the results were classified into three categories. In the first category, "in agreement", embryo chromosomal constitution was confirmed by both CGH and FISH results. Embryos that belong in this group include those that the chromosomal status of the blastomere analysed by CGH is the same as of another blastomere of the embryo analysed by FISH. The largest group of the embryos studied 43% (12) belong in this category.

In the case where the CGH result was not confirmed by the FISH analysis but results are apparently not discordant, then they were classified as "not discordant". Taking into consideration difficulties regarding single blastomere CGH efficiency, and differences in the individual chromosomal rearrangements involved, and in the blastomeres sampled, feasible explanations could be offered for the respective FISH and CGH results (discussed in chapter 4). The "not discordant" category includes embryos where CGH and FISH results identify different cell lines (i.e. the presence of mosaicism), but both cell lines are compatible with the overall embryo classification according to both the FISH and CGH results. In this way the CGH and FISH results could complement rather than contradict one another, since both cell lines can be mutually inclusive for the embryo classification. Thirty two per cent (9) of embryos belong to this category with the majority of these being generated by cases F, G and H.

Finally in the case where the CGH result contradicts the FISH data, then the results "disagree". Possible factors that could explain these contradictory results are discussed in more detail in chapter four. Twenty five per cent (7) of the embryos are classified in this category.

Nine of the 50 blastomeres subjected to CGH failed to give analysable results. There is a variety of factors which may account for this. G2.2 and H6.1 failed to give hybridisation from the test DNA, while the control DNA hybridised successfully. That could indicate either an anucleate blastomere (as visibility of the nucleus was not possible for all the blastomeres biopsied), or that the cell was mistaken for an anuclear fragment which is a common finding in day three embryos (Vouillaire et al., 2000). Another explanation accounting for failure of results could be premature cell lysis or loss of cell during transfer to PCR tube.
Results

A cytoplasmic metaphase preparation of poor chromosome morphology would present a major obstacle to the successful hybridisation of both control and test DNA. Apart from the non-specific fluorescence and the granular hybridisation effect (meaning that although hybridisation is successful it presents with a granular effect due to poor chromosome quality, failing to provide a smooth, analysable fluorescence), the strength of the counterstain banding, essential for the identification of chromosomes, would also be negatively affected by this factor. These problems accounted for lack of analysable results for blastomeres G8.1, G8.2, G 9.2, G 13.2, H4.2, H6.2 and H8.2.

Eighty two per cent (41) of the blastomeres obtained for this study provided good analysable results. Hybridisation presented with no granulation, dynamic fluorescence (strength, evenness and consistency of fluorescent signals) was good, and good chromosome morphology allowed a strong counterstain banding essential for the analysis. Sex chromosomes could be determined confidently providing an internal positive control regarding the efficiency and reliability of the technique, as all cells from each embryo were consistent for the determination of the sex chromosome CGH pattern as confirmed by FISH analysis. With regard to the autosomes, the literature suggests the exclusion of certain regions of the karyotype as they have been proven to show variation in the profile, (as previously mentioned) and that was also taken into consideration during this study. Moreover, deletions or amplifications concerning small subtelomeric regions were found extremely difficult to detect and interpret, as reported by other groups (Kallionemi et al., 1994). Consequently abnormalities involving translocations with distal breakpoints could not be confidently detected.

3.12.1 Case A 46,XX,t(5;11)(q34;q25)- 3rd PGD cycle

One blastomere was biopsied from each of the 6 spare embryos of case A.

According to FISH analysis two of the embryos (A1 and A6) were classified as fully chaotic, two as aneuploid/balanced (A8 and A11), embryo A2 was balanced/chaotic and embryo A10 uniformly aneuploid. Blastomeres A2.1, A6.1, A8.1, A10.1 and A11.1 were all found to be normal according to CGH while A1.1 presented with extensive aneuploidy. It is important to note that embryos A2, A8, and A11 presented with a balanced selection of cells (≥50%) in apparent agreement with their CGH results. However, in the case of embryo A8, if the segregation pattern was adjacent-1,
it is difficult to accept that a truly normal cell can be present. It is most likely that the apparently balanced cells detected by FISH are due to post zygotic events, and CGH was failing to detect abnormalities in terminal regions, as for embryo A10. Embryo A1 was fully chaotic and according to FISH analysis with both probe sets, certain cells exhibited trisomy 3, partial trisomy 5, and trisomy 17, supported by the CGH results as well. In the case of embryo A10 where the CGH and FISH results are not in agreement, it is important to consider that the breakpoints at both chromosomes are distal, particularly the one on chromosome 11. Therefore it was anticipated that any duplications or deletions involving the translocated segments might not be detected by CGH of single cells. Sex detection agreed for all CGH and FISH results. Results are summarised in Table 3.46. Fig. 3.29 shows CGH analysis and interpretation of blastomere A1.1.
Results

Fig. 3.29 CGH analysis of blastomere A1.1. The chromosomes showing deletions or duplications according to the interpretation, are pictured next to the relevant ideogram.

Fig. 3.30 CGH analysis of blastomere F1.1. Chromosome 7 showing partial duplication as shown by the interpretation and pictured by the relevant ideogram.
Table 3.46 - Comparison of CGH & FISH results on day-4 untransferred embryos from Case A 46,XX,t(5;11)(q34;q25)-3/' PGD cycle.

<table>
<thead>
<tr>
<th>BLASTOMERE</th>
<th>A1.1</th>
<th>A2.1</th>
<th>A6.1</th>
<th>A8.1</th>
<th>A10.1</th>
<th>A11.1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CGH RESULTS</strong></td>
<td>rev ish XX,enh(3.5p21q35, 6p12p25,6q24q26, 12p12q11,12q12q24, 3, 16p13.3p13.2,16q23 q24,17p11.2p13), dim(18)</td>
<td>rev ish XX</td>
<td>rev ish XX</td>
<td>rev ish XY</td>
<td>rev ish XY</td>
<td>rev ish XX</td>
</tr>
<tr>
<td><strong>EMBRYOS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>A2</td>
<td>A6</td>
<td>A8</td>
<td>10</td>
<td>A11</td>
<td></td>
</tr>
<tr>
<td><strong>FISH RESULTS</strong></td>
<td>Fully chaotic. Certain cells presented with trisomy 3 &amp; 17 as well as partial trisomy for the chromosomes involved in the translocation</td>
<td>Balanced/Chaotic Biopsied cell: Trisomy 11/Monosomy 5. Spare embryo: 2 cells balanced for the translocation, 2 cells: nullisomy 11, monosomy 5. All cells abnormal for chromosomes 3, 7, 9, and 19.</td>
<td>Fully chaotic, there were no balanced cells.</td>
<td>Aneuploid/Balanced 3 cells balanced and 4 cells product of adjacent-1 segregation; monosomy 5q34→qter, trisomy 11q25→qter.) 3 cells were normal for chromosomes 3, 7, 9, and 19, while others showed mitotic non-disjunction.</td>
<td>Uniformly aneuploid Adjacent-1 segregation product. Trisomy 5q34→qter, monosomy 11q25→qter). Mosaicism (trisomy and monosomy 17) due to non-disjunction</td>
<td>Balanced/Aneuploid with one cell line balanced for the translocation (3 cells) and the other line having lost der5 and 11 (2 cells).</td>
</tr>
<tr>
<td><strong>INTERPRETATION</strong></td>
<td>CGH &amp; FISH results agree. The meiotic segregation pattern of the translocated chromosomes cannot be deduced.</td>
<td>CGH &amp; FISH results agree. The embryo originated from an alternate segregation.</td>
<td>CGH &amp; FISH results disagree. The embryo originated from an unknown segregation.</td>
<td>CGH and FISH results are not discordant. The embryo originated from an adjacent-1 segregation.</td>
<td>CGH and FISH results disagree. The embryo originated from an adjacent-1 segregation.</td>
<td>CGH &amp; FISH results agree. The embryo originated from an alternate segregation.</td>
</tr>
</tbody>
</table>
3.12.2 Case E-45,XY,der(13;21)(q10;q10)

Three blastomeres were biopsied from embryo E9 and all three were classified as normal female by CGH. It is important to consider that nullisomy 13 was detected in only one biopsied cell and based on the CGH results the possibility of mosaicism should be considered. Results are summarised in Table 3.47.

Table 3.47 -Comparison of CGH & FISH results on day 4 untransferred embryos from Case E 45,XY,der(13;21)(q10;q10)-1st PGD cycle.

<table>
<thead>
<tr>
<th>BLASTOMERE</th>
<th>E9.1</th>
<th>E9.2</th>
<th>E9.3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CGH RESULTS</strong></td>
<td>rev ish XX</td>
<td>rev ish XX</td>
<td>rev ish XX</td>
</tr>
<tr>
<td><strong>EMBRYO</strong></td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FISH RESULTS</strong></td>
<td>Biopsied cell: Two copies for 21, nullisomy 13 Spare embryo failed to give results</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>INTERPETATION</strong></td>
<td>CGH &amp; FISH results on the biopsied cell disagree. The embryo probably originated from an alternate segregation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.12.3 Case F-46,XX,ins(7)(p22q31.1q32)

Four spare embryos were involved in this study. Embryo F3 was found to be balanced with all 11 blastomeres analysed found to be balanced based on both CGH and FISH results. Embryo F6 was classified uniformly aneuploid by FISH carrying duplication of the inserted chromosomal region. The CGH analysis showed extensive aneuploidy involving chromosomes 18 and 17 as well as duplication of the short arm of chromosome 7 and duplication of the inserted segment of chromosome 7 also supported by the FISH results. As chromosomes 17 and 18 were not assessed by FISH, no overall conclusion as to the embryo’s chromosome constitution can be made, however, the FISH result was confirmed by CGH and therefore the results between FISH and CGH are in agreement. For embryos F1 and F10 CGH showed duplication of the non-inserted distal segment covering most of the short arm. Interestingly, a selection of cells from embryo F1 and all blastomeres from embryo F10 showed partial monosomy of the inserted segment according to the FISH results. As the insertion segment is small the possibility of CGH not detecting partial monosomies involving this region should be considered. Therefore, in the case that the cells carry both the deletion of the insertion and trisomy 7p21→q11.1 and assuming that the insertion is inverted rather than direct (see Fig. 3.6), a possible explanation would be duplication of the recombinant chromosome carrying the deletion of the inserted segment within the same cell. Results are summarised in Table 3.48. Fig. 3.30 shows CGH analysis and interpretation of blastomere F1.1.
Table 3.48 - Comparison of CGH & FISH results on day-4 untransferred embryos from Case F 46,XX,ins(7)(p22q31.1q32)

<table>
<thead>
<tr>
<th>BLASTOMERE</th>
<th>F1.1</th>
<th>F3.1</th>
<th>F3.2</th>
<th>F6.1</th>
<th>F10.1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CGH RESULTS</strong></td>
<td>rev ish XY, enh(7p21q11.1)</td>
<td>rev ish XY</td>
<td>rev ish XY</td>
<td>rev ish XY, enh(7p11.2p21,7q31q34,18p11.1p11.3,18q12q21),dim(17p12p13,17p11.2q11.2)</td>
<td>rev ish XY, enh(7p21q11.1)</td>
</tr>
<tr>
<td><strong>EMBRYOS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FISH RESULTS</strong></td>
<td>Chaotic with 3 cell types showing A: 2 cells carrying the deletion of the insertion, B: 2 cells missing one copy of the control probe. C: 1 cell carrying 3 copies of 7</td>
<td>Balanced (9 cells)</td>
<td>Uniformly aneuploid. Trisomy 7q31.1→q32 (7 cells)</td>
<td>Uniformly aneuploid. Monosomy 7q31.1→q32 (12 cells)</td>
<td></td>
</tr>
<tr>
<td><strong>INTERPRETATION</strong></td>
<td>FISH &amp; CGH results are not discordant. CGH shows partial duplication of chromosome 7 confirmed by FISH. Unknown segregation pattern</td>
<td>FISH &amp; CGH results agree. Oocyte was carrying a balanced copy for chromosome 7</td>
<td>FISH and CGH results agree as CGH shows partial duplication of chromosome 7 confirmed by FISH. Oocyte had chromosome 7 carrying the duplicated insertion segment</td>
<td>CGH and FISH results are not discordant. Oocyte was carrying a copy for chromosome 7 carrying the deleted insertion segment</td>
<td></td>
</tr>
</tbody>
</table>

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3.12.4 Case G-46,XX,t(8;12)(q11.2;q12)

Ten spare embryos were involved from this case. CGH detected duplication of the non-translocated segments of chromosome 12 for embryos G3, G7, G9, G13 and duplication of the non-translocated segment of 8 for embryo G5. However, CGH failed to detect any deletions involving any segments of the chromosomes 8 and 12. The failure of CGH to detect monosomies was clearly illustrated with the example of embryo 6 in which FISH detected only monosomies and the CGH results classified both cells G6.1, G6.2 as normal. For embryos G2, G4, G7, and G10 that presented with a balanced selection of cells (≥50%) according to FISH, the CGH results indicated balanced blastomeres (in the case of embryo G7 only one cell was found to be normal). For embryo G5 the duplication of the non-translocated segment of chromosome 8 detected by CGH was confirmed by the FISH results. Therefore, the results between the two techniques were in agreement for 50% of the embryos. For embryos G9 and G13 CGH picked up the same partial trisomy that was partly confirmed by FISH results. Therefore allowing for the fact that CGH failed to detect any monosomies, the results do not disagree. Embryo G15 was a 3PN embryo that was excluded from PGD analysis. FISH provided results only for 3 cells from embryo 15 all abnormal and both cells analysed by CGH were classified as normal. As the embryo fertilisation was abnormal, the chromosomal constitution is expected to be abnormal. Based on that and the fact that CGH fails to detect certain abnormalities, interpreting the result can prove particularly difficult. Sex detection agreed for all CGH and FISH results. For embryos G3 and G7 from which two blastomeres were analysed, the CGH results disagreed which was not expected as both embryos were classified as fully chaotic or balanced/chaotic by FISH. Results are summarised in Table 3.49. Fig. 3.31 shows CGH analysis and interpretation of blastomere G5.1.
Table 3.49 - Comparison of CGH & FISH results on day-4 untransferred embryos from Case G 46, XX, t(8;12)(q11.2;q12)

<table>
<thead>
<tr>
<th>BLASTOMERE</th>
<th>G2.1</th>
<th>G3.1</th>
<th>G3.2</th>
<th>G4.1</th>
<th>G4.2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CGH RESULTS</strong></td>
<td>rev ish XY</td>
<td>rev ish XY</td>
<td>rev ish XY, enh(12p11.1p13)</td>
<td>rev ish XY</td>
<td>rev ish XY</td>
</tr>
<tr>
<td><strong>EMBRYOS</strong></td>
<td>G2</td>
<td>G3</td>
<td>G4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FISH RESULTS</strong></td>
<td>Balanced/Chaotic (2 out of 5 cells were balanced for the translocation). Embryo found to be male</td>
<td>Fully chaotic – Unbalanced chromosome complement for both the sex chromosomes and the ones involved in the translocation (6 cells) FISH indicated the embryo to be male</td>
<td>Balanced/chaotic-3 cell lines (Balanced 8 out of 12 cells- Monosomy 8pter→q11.2 – Trisomy 12q12→qter). Embryo found to be male</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>INTERPETATION</strong></td>
<td>CGH &amp; FISH agree. Embryo originated from an alternate segregation pattern, and developed to be a chaotic mosaic</td>
<td>The CGH results between the cells examined disagree. The CGH &amp; FISH results disagree as embryo’s chromosomes are chaotic. Originated from unknown segregation pattern</td>
<td>CGH &amp; FISH results agree. Embryo originated from an alternate segregation pattern.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLASTOMERE</td>
<td>G5.1</td>
<td>G6.1</td>
<td>G6.2</td>
<td>G7.1</td>
<td>G7.2</td>
</tr>
<tr>
<td>------------</td>
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<td>------</td>
</tr>
<tr>
<td><strong>CGH RESULTS</strong></td>
<td>rev ish XY, enh(8p21q11.2)</td>
<td>rev ish XX</td>
<td>rev ish X</td>
<td>rev ish XY</td>
<td>rev ish XY, enh(12p13p11.1)</td>
</tr>
<tr>
<td><strong>EMBRYOS</strong></td>
<td>G5</td>
<td>G6</td>
<td></td>
<td>G7</td>
<td></td>
</tr>
<tr>
<td><strong>FISH RESULTS</strong></td>
<td>Aneuploid/Chaotic. Trisomy 8pter→q12 was seen in 6/9 cells. X, Y, 18: 7 out of 9 cells were normal male</td>
<td>Aneuploid/Mosaic (monosomy 8/monosomy 12q12→qter, monosomy 8pter→q11.2). Embryo was uniformly female &amp; diploid for 18.</td>
<td>Balanced/Chaotic (2 out of 6 cells balanced for the translocation) Embryo was found to be male</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>INTERPETATION</strong></td>
<td>CGH &amp; FISH results agree. Embryo originated from unknown segregation pattern</td>
<td>CGH &amp; FISH results disagree. Embryo originated from an adjacent-2 segregation (chrom 12 and der 12) with absent chromosome 8. Mosaicism developed later.</td>
<td></td>
<td>The CGH results between the cells examined are discordant. CGH &amp; FISH results agree. Embryo originated from an alternate segregation pattern.</td>
<td></td>
</tr>
</tbody>
</table>
Case G 46,XX,t(8;12)(q11.2;q12)

<table>
<thead>
<tr>
<th>BLASTOMERE</th>
<th>G9.1</th>
<th>G10.1</th>
<th>G10.2</th>
<th>G13.1</th>
<th>G15.1</th>
<th>G15.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGH RESULTS</td>
<td>rev ish XY, enh(12p11.1p13)</td>
<td>rev ish XY</td>
<td>rev ish XY</td>
<td>Rev ish XY, enh(12p13p11.1)</td>
<td>rev ish XX</td>
<td>rev ish XX</td>
</tr>
<tr>
<td>EMBRYOS</td>
<td>G9</td>
<td>G10</td>
<td>G13</td>
<td>G15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FISH RESULTS</td>
<td>Biopsied cell: Adjacent-2 segregation products: Trisomy 12pter→q12-, monosomy 8q11.2→qter. Remainder of the embryo chaotic (6 cells indicated partial trisomy for 12). All cells were male diploid for 18.</td>
<td>Balanced for the translocation. Male diploid for 18</td>
<td>Uniformly aneuploid. Male 8 cells (trisomy 12pter→12q12, monosomy 8q11.1→qter.</td>
<td>Embryo (3PN). Was not biopsied and not included in PGD analysis. Female. Chaotic both for the translocation and X, Y, 18. (3 cells analysed)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>INTERPRETATION</td>
<td>CGH &amp; FISH results are not discordant as embryo is chaotic mosaic. The embryo originated from an adjacent-2 segregation (based on the biopsied cell).</td>
<td>CGH &amp; FISH results agree. Embryo originated from an alternate segregation pattern.</td>
<td>CGH &amp; FISH results are not discordant. Embryo originated from an adjacent-2 segregation pattern based on the FISH results.</td>
<td>CGH &amp; FISH results disagree. Embryo originated from an alternate segregation pattern based on the CGH results.</td>
<td></td>
<td></td>
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</tbody>
</table>

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Results

Fig. 3.31 CGH analysis and interpretation of blastomere G5.1. Chromosome 8 showing partial duplication according to the interpretation, is pictured next to the relevant ideogram.

Fig. 3.32 CGH analysis and interpretation of blastomere H11.1. The chromosomes showing deletions or duplications according to the interpretation are pictured next to the relevant ideogram.
3.12.5 Case H-46,XY,t(1;18)(p32;q23)

Failure of CGH to detect specific partial monosomies as detected uniformly in all cells analysed by FISH, is shown in blastomeres H2.1, H2.2, and H4.1. The only blastomere where monosomies are detected is H11.1 including detection of monosomy for 1p32.1→qter. In blastomeres H2.1 and H2.2 it is partial monosomy 18 that is not picked up. In blastomere H4.1 the embryo is classified uniformly abnormal based on FISH results but CGH fails to pick up either of the partial abnormalities. Failure of CGH to detect partial monosomies/trisomies of the translocated segment of chromosome 18 could be due to the fact that the breakpoint is extremely distal and so beyond the limit of CGH resolution. In all cases CGH detects partial trisomy of the non-translocated segment of chromosome 1, agreeing with FISH performed on spare embryos H2, H3, and H7. Embryos H8 and H10, both classified as balanced/chaotic by FISH, the presence of balanced cells was confirmed by the CGH results indicating both blastomeres H8.1 and H10.1 to be normal male. Sex detection agreed for all CGH and FISH results. Results are summarised in Table 3.50. Fig. 3.32 shows CGH analysis and interpretation of blastomere H11.1, showing artefactual enhancement of 1pter→31.2.
<table>
<thead>
<tr>
<th>BLASTOMERE</th>
<th>H2.1</th>
<th>H2.2</th>
<th>H3.1</th>
<th>H3.2</th>
<th>H4.1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CGH RESULTS</strong></td>
<td>rev ish XY,enh(1p36.3p31)</td>
<td>rev ish XY,enh(1p36.3p31)</td>
<td>rev ish XY,enh(1p36.3p31)</td>
<td>rev ish XY,enh(1p36.3p31)</td>
<td>rev ish XX</td>
</tr>
<tr>
<td><strong>EMBRYOS</strong></td>
<td>H2</td>
<td>H3</td>
<td>H4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FISH RESULTS</strong></td>
<td>Uniformly aneuploid (trisomy 1p32→pter, monosomy 18q23→qter. All cells showed trisomy 16</td>
<td>Biopsied cells (2): Adjacent-1 (trisomy 1p32→pter, monosomy 18q23→qter. Remainder of the embryo (7 cells) Aneuploid/Chaotic (3/7 adjacent-1). Chromosomes 12 &amp; 16 showed imbalance in one cell only</td>
<td>Uniformly aneuploid-adjacent-1. 5 cells (monosomy 1p32→pter, trisomy 18q23-qter).</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>INTERPRETATION</strong></td>
<td>CGH &amp; FISH results are not discordant. The embryo originated from an adjacent-1 segregation.</td>
<td>CGH &amp; FISH results are not discordant. The embryo originated from an adjacent-1 segregation</td>
<td>CGH &amp; FISH results disagree. The embryo originated from an adjacent-1 segregation</td>
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</tbody>
</table>
### Case H 46,XY,t(1;18)(p32;q23)

<table>
<thead>
<tr>
<th>BLASTOMERE</th>
<th>H7.1</th>
<th>H7.2</th>
<th>H8.1</th>
<th>H10.1</th>
<th>H11.1</th>
<th>H11.2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CGH RESULTS</strong></td>
<td>rev ish XX,enh(1p31p36.3)</td>
<td>rev ish XX,enh(1p31p36.3)</td>
<td>rev ish XY</td>
<td>rev ish XY</td>
<td>rev ish XY,enh((1p31p36.3,5,12,18,22),dim(1p31.2q44,10,13,16,20))</td>
<td>rev ish XY.</td>
</tr>
<tr>
<td>EMBRYOS</td>
<td>H7</td>
<td>H8</td>
<td>H10</td>
<td>H11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Chromosomes examined: 1, 18. 2nd round: 12, 16)</td>
<td>3 cells balanced/7 cells aneuploid (trisomy 1p32→pter/monosomy 18q23→qter). Chromosomes 12 &amp; 16 showed monosomy in one cell</td>
<td>Balanced/Aneuploid.</td>
<td>Balanced(1)/Chaotic (4) for both the chromosomes involved in the translocation and 12, &amp; 16.(1 cell out of 4 normal). Biopsied cell binucleate-2 haploids</td>
<td>Balanced(1)/Chaotic (4) for both the chromosomes involved in the translocation and 12, &amp; 16.(1 cell out of 4 normal). Biopsied cell binucleate-2 haploids</td>
<td>Uniformly aneuploid(4)-adjacent-2. Monosomy 1p32→qter, trisomy 18pter→18q23. Diploid for both 12 &amp;16.</td>
<td></td>
</tr>
<tr>
<td>INTERPETATION</td>
<td>CGH &amp; FISH results are not discordant. The embryo originated from an adjacent-1 segregation</td>
<td>CGH &amp; FISH results agree. Embryo originated from an alternate segregation pattern.</td>
<td>CGH &amp; FISH results agree. Embryo originated from an alternate segregation pattern.</td>
<td>CGH &amp; FISH results agree. Embryo originated from an alternate segregation pattern.</td>
<td>CGH &amp; FISH results not discordant. Embryo originated from an adjacent-2 segregation pattern according to FISH results</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 4

DISCUSSION
4.1 PGD Preliminary Work
4.1.1 Selection and Evaluation of DNA Probes and Blastomere Fixation Techniques.

Early work to develop interphase cytogenetics using FISH was hampered in part by the lack of access to a reliable panel of locus-specific DNA probes and as such was limited in scope. Methods of screening DNA clones for use as FISH probes can be labour intensive and time-consuming as many will be chimaeric (large BAC/YAC clones etc), map to another location (cross-hybridize) or produce weak FISH signals and so need to be discarded. Most of the plasmid clones for centromeric probes used in this study were found to be suitable, producing discrete, easily scored FISH signals in lymphocyte interphase nuclei. However, that was not the case for centromeric probes for chromosomes 3 and 15. The probe for chromosome 3 showed cross hybridisation, while the probe for chromosome 15 revealed a very low hybridisation efficiency of 35% due to complete or partial absence of one of the two signals in the majority of the cells scored. This is explained by the nature of α-satellite DNA located at the centromere, being made up of varying numbers of monomeric repeat units. If the number of repeated units is below the level of detection with a particular probe, a missing signal will lead to misdiagnosis (Verma and Luke, 1992). In the case of the selection of YAC and cosmid clones, unfortunately only one successfully amplified, the clone for chromosome 19 (b484C16). Fortunately, the increasing availability of a wide range of commercial probes has made the method of producing ‘laboratory prepared’ diagnostic probes largely redundant. These commercial probes have revolutionised the use of interphase cytogenetics particularly in clinical laboratories where FISH is now used routinely to complement standard karyotyping for a wide range of pre- and postnatal applications (Blennow et al., 1995; Knight and Flint, 2000; Quilter et al., 2001). In addition the introduction of multi-probe cocktails specifically designed for polar bodies and blastomere analysis have contributed to the increase in the number of groups carrying out PGD and related research. Finally, the introduction and subsequent general accessibility to subtelomeric probes for all chromosomes has greatly simplified the strategies for PGD of chromosomal rearrangements. The majority of the probes used in this study were commercially available.
Discussion

All commercial or laboratory-prepared probes employed in this study were subjected to stringent individual and combinatorial quality control. The probes were tested on control lymphocytes to test their efficiency both as a combination and individually with respect to the presence and intensity of the signal. As part of the work-up, two hundred interphase nuclei were scored and some of the probes were subsequently tested on fibroblast cell lines and embryonic nuclei, to ensure optimisation of all protocols prior to diagnosis.

Testing the probes selected for nine PGD cases on two differently treated fibroblast triploid cell lines and comparing the hybridisation efficiencies, was an exercise aiming to delineate the effect that diverse fixation techniques have on hybridisation efficiency and subsequently on the accuracy of diagnosis (see section 3.3). The two different ways the fibroblast cell lines had been treated represented the two commonly used blastomere nuclei fixation techniques; methanol:acetic acid fixation and Tween fixation. The hypotonic solution treated culture presented with enlarged, swollen cells, bearing a resemblance to the appearance of blastomeres that had been fixed using the methanol:acetic acid technique as described by Tarkowski (1996) and modified for single blastomere use by Munne et al., (1998c). The PBS treated cell line revealed smaller, compact nuclei that preserved their three dimensional structure, resembling blastomeres that had been fixed using the Tween fixation technique. The results of this study showed that hybridisation efficiency was marginally but consistently higher in cell lines treated with PBS/air drying fixation (average hybridisation efficiency 85.6%), compared to the methanol:acetic acid fixed cell line (average hybridisation efficiency 81.8%). This supports the view that this alternative fixation technique resembling the Tween spreading method as described by Coonen et al., (1994a) is associated with more accurate FISH diagnosis. The signals of the probes tested on the PBS treated cell line appeared smaller but compact, brighter and distinct, ensuring a precise diagnosis. The signals on the hypotonic solution treated cell line appeared larger but diffuse, weaker than in regular sized nuclei, heterogeneous in consistency and in the case of sub-telomeric probes, split. In some instances split signals were observed, with more than a signal’s size distance between them, which according to Hopman’s criteria (Hopman et al., 1989) should be scored as two, rather than one signal. This last observation in some of the cases suggests avoiding the use of the methanol:acetic acid fixation technique for PGD for chromosome translocations employing the use of sub-telomeric probes.
A study performed by Velilla et al., (2002), compared three blastomere fixation techniques to determine the most efficient one to be used in the PGD setting for aneuploidy screening. Techniques evaluated were (1) the methanol:acetic acid method (Munne et al., 1998D), (2) the Tween 20 method (Coonen et al., 1994), and (3) a combination of the techniques 1 and 2 as described by Dozortsev and McGinnis, (2001). The comparison was done based on the number of cells lost after fixation, the average rate of informative cells, the rate of signal overlaps, and FISH errors. Technique 3, being essentially similar to method 2, employs methanol: acetic acid to (3:1) to remove excess cytoplasm, and it was shown to be more effective and presents with a lower misdiagnosis risk (Velilla et al., 2002). The study recognises the higher blastomere loss rate when using method 1 as well as its requirement for technical expertise giving a 3% chance of losing the cells even in the hands of an experienced practitioner (Velilla et al., 2002). With respect to method 2, the study reports cytoplasm interference with probe binding, leading to a possible increase in non-informative nuclei and more importantly misdiagnosis, even following modifications made by Xu et al., (1998) reducing the number of nuclei with cytoplasm to <5%. Reduced nuclear diameter has been linked to FISH errors (Munne et al., 1996). The study by Velilla and co-workers (2002) report that the larger nuclear diameter that the first fixation method provides, shows fewer overlapping signals as they lie on the same focal plane, in contrast to the small three dimensional nuclei obtained by the Tween 20 method, showing signals on different focal planes. The conclusions drawn by the study by Velilla and colleagues, disagree with the results of this study employing the differently treated fibroblast cell lines, which supports choice of the Tween 20 method over the methanol:acetic acid technique. It would be beneficial for a study to be conducted comparing the two techniques by results obtained from nuclei spread by two different practitioners, each being experienced in either fixation technique, rather than obtaining results from one person that has been introduced to both the techniques in a laboratory setting that specifically uses only the methanol:acetic acid method. This fact instantly raises questions regarding the quality of the results obtained from performing the hypotonic solution, Tween 20 technique, and consequently the validity of the study carried out by Velilla et al., (2002).

The study performed as part of this thesis does not suggest that the Tween 20 technique is better than the methanol:acetic acid one for spreading blastomeres. In
fact both methods have advantages and disadvantages. In the case of screening for more than three chromosomes, where more signals are present, and particularly in the case of using aneuploidy screening probe cocktails involving five probes, where DAPI staining is unfeasible, the preferable fixation method might be the methanol:acetic acid one. This way the larger nucleus is easily located and the signals are all in the same focus plane facilitating the diagnosis and avoiding misdiagnosis. However, aneuploidy screening of six chromosomes could be successfully performed on nuclei spread using the Tween method, opting for two sequential rounds and restricting to screening for three chromosomes in each round, and therefore minimising the chances of overlapping signals and misdiagnosis (Joy Delhanty, personal communication). In the case of applying probe combinations devised for PGD for chromosome rearrangements, the Tween 20 method is the one used by most groups worldwide (Conn et al., 1998; Van Asche et al., 1999; Coonen et al., 2000; Iwarsson et al., 2000; Scriven et al., 2001) as it ensures good quality of nuclei and clear compact, distinct, bright signals as suggested by the study performed on the two differently treated triploid fibroblast cell lines.

4.1.2 Chromosomal Analysis of Spare IVF Preimplantation Embryos Using FISH.

The poor fecundity characterising humans as a species is explained by the estimation that 70%-80% of all conceptions fail to develop to term and are lost before the pregnancy is clinically recognised (Edmonds et al., 1982; Ellish et al., 1996). This pregnancy wastage observed in humans is mainly due to chromosomal abnormalities, as over half of all spontaneous abortions are associated with abnormal karyotypes (Hassold, 1986; Eiben et al., 1990). The advent of IVF enabled the testing of human preimplantation embryos. Early studies using conventional cytogenetic techniques showed abnormalities ranging from 11-40%, commonly involving mosaicism with normal and aneuploid or polyploid cell lines (Angell et al., 1986; Plachot et al., 1987; Papadopoulos et al., 1989; Zenzes and Casper, 1992).

The data from FISH analysis of cleavage embryos came from work to develop PGD protocols for detecting chromosomal imbalance associated with parental chromosomal rearrangements. In this study, sixty-four surplus preimplantation embryos donated from nineteen patients were analysed using FISH and twelve probe
combinations devised for prospective PGD cases were applied as part of the work-up to assess hybridisation efficiencies. The chromosomes examined included 1, 2, 4, 5, 7, 8, 9, 11, 12, 13, 14, 16, 17, 19, and 22. Thirty-five normally developing embryos donated from fifteen couples (mean maternal age 30.8 years) were scored on day-three post insemination as grade II, or III. The embryos were categorised according to their chromosomal complement into: uniformly normal, uniformly abnormal, diploid mosaic (carrying a group of non-diploid cells), aneuploid mosaic, and chaotic. Categorisation of embryos was done according to (Delhanty et al., 1997), (see section 2.1.2.5). In total 77% of these embryos were uniformly normal for the chromosomes examined and 23% were chromosomally abnormal. This is in contrast with the results obtained from FISH analysis employing the same chromosome combinations on twenty-nine abnormally developing embryos donated by the same group of patients. This group showed 76% of embryos classified as chromosomally abnormal and 24% as uniformly normal for the chromosomes examined. Interestingly, the percentages are almost reversed between the normally and abnormally developing embryos and that is of great importance as both the normally and abnormally developing embryos derived from the same patient group and the same selection of chromosomes was examined. Overall in this series of embryos, excluding chaotic types, and using probes for three chromosomes, approximately 26.5% showed low level mosaicism, which is very close to the proportion found in previous studies (Munne et al., 1994a; 1994b; 1995a; Harper et al., 1995a; Delhanty et al., 1997). In agreement with the original karyotyping data, although a negligible fraction of these surplus embryos were uniformly abnormal (3%), the majority of abnormalities involved mosaicism with a minor aneuploid, polyploid or haploid cell line on a normal diploid background (Delhanty et al., 1993; Munne et al., 1993; 1995; Harper et al., 1994a; 1995a). What is most striking in the results is that predictably, abnormally developing embryos as a group, including those resulting from abnormal fertilisation, showing retarded cleavage or complete developmental arrest, were found to show much higher levels of chromosome abnormalities, in the order of 76%. These were more frequently embryos classified as aneuploid mosaic (34.5%), and chaotics (27.5%) commonly involving a chromosomally abnormal background. The relationship between chromosomal status and developmental potential has been studied extensively and results suggest that chromosomal abnormalities are greatly associated with a poor developmental potential translated as slow and arrested division (Munne et al., 1995a; Marquez et
This suggestion is verified by this study showing considerably higher percentages of chromosomal abnormalities seen in the group of abnormally developed embryos in comparison to the group of normally developing embryos.

Of the nineteen patients included in the study, patients a, c, d, e, f, i, and j donated embryos that were found to be uniformly normal for the chromosomes detected. Patient h provided two embryos both found to be fully chaotic, supporting the suggestion that chaotic embryos appear to be a strongly patient-related phenomenon, with some patients experiencing high levels of this type of abnormality over repeated cycles (Delhanty et al., 1997c). No abnormalities were observed for chromosomes 1, 4, 7, 11, 14, 16, and 17. However, as the probe combinations involving these chromosomes [combination for case 19 (chromosomes 4 and 11), probe combination for case 13-C (chromosomes 16 and 17), and the probe combination for case 40-F (chromosome 7)], were tested on embryos deriving from one patient each, the fact that no abnormality for these chromosomes was noted could be a patient related phenomenon, as all embryos donated from patients, c, i and j, respectively for case 19, 13-C, and 40-F, were found to be uniformly normal. Abnormalities on different embryos however, were noted for chromosomes 2, 5, 8, 9, 12, 13, and 22. The frequent observation of trisomies and monosomies involved all chromosomes except chromosome 5. The recurrent monosomic cells present in all abnormal embryos (b1, g1, h1, h2, k3, and n2) except in embryos m2 and o3 suggest that in some cases chromosome loss may be at least as important a mechanism for this post-zygotic mosaicism as mitotic non-disjunction, that would be expected to generate equal numbers of monosomic and trisomic embryos. Non-disjunction is seen for chromosome 2 in the case of embryo k3. Monosomy is the most frequent abnormality encountered in this series of embryos, the incidence of hybridisation failure, or overlapping signals, or two signals close to each other that would lead to the same mis-classification of monosomy (Munne et al., 1996b), should be taken into consideration in this study.

Haploid, triploid and tetraploid cells were frequently observed in this study in diploid/mosaic and chaotic embryos. This is in line with other studies reporting ploidy mosaics (Harper et al., 1995a; Delhanty et al., 1997c; Clouston et al., 1997; Munne et al., 1997; Staessen et al., 1999) with tetraploid or haploid cells predominating. Embryo m2 (diploid/mosaic) and embryo h2 (chaotic) showed two tetraploid cells each. Mosaicism including a tetraploid cell line is a very common finding in both human...
Discussion

blastocysts (Benkhalifa et al., 1993; Clouston et al., 1997) and those of other animal species (Long and Williams, 1982; Murray et al., 1986) and may well play a role in normal early development particularly when associated with TE lineages (Angell et al., 1987). Tetraploid trophoderm cells may arise as a result of endoreduplication or endomitosis and possibly play a role in embryo implantation (Drury et al, 1998). Laverge et al (1997) while studying 97 human cleavage-stage embryos reported that possible mechanisms for polyploid mosaics could involve endoreduplication of mononucleated blastomeres, or formation of a mitotic spindle during division of a binucleate cell which would subsequently form two mononucleate daughter cells with polyploid nuclei. The fusion of nuclei in binucleate blastomeres or less frequently blastomere fusion may also lead to polyploidy (Balakier et al, 2000). Mosaic tetraploidy has also been linked to poor quality arrested embryos (Wells and Delhanty, 2000). Two haploid blastomeres were part of chaotic embryo h1, while embryo b1 presented haploidy for all seven blastomeres. Haploid nuclei are a less common mosaicism finding as the presence of a haploid cell in a mosaic embryo is not clearly explained. However the underlying mechanism may be associated with binucleate cell production with a meiotic type of segregation (Delhanty et al, 1997b) or with incorporation of a polar body into the embryo (Staessen et al, 1999). Haploid/diploid mosaics can be found from pronuclear zygotes and are believed to arise due to the activation of the oocyte (Staessen and Van Steirteghem, 1997). Three triploid cells were observed in diploid/mosaic embryo o3, and two blastomeres were classified as triploid in chaotic embryo h2. The origin of diploid/triploid mosaicism is not clear. Incorporation of another gamete or its genome into one of the daughter cells derived after the first mitotic division or later, could offer logical explanation. The extra gamete might be a polar body (Muller et al, 1993). Moreover, fusion of a diploid zygotic nucleus with an extra sperm nucleus or the extrusion and degeneration of a haploid nucleus could theoretically produce a diploid cell line in a triploid embryo (Kuo et al., 1998). Haploid and triploid cells have been characterised as less viable and less actively dividing than tetraploid cells (Ruangvutilert et al, 2000a), with the exception of some triploid cells persisting until later in development as cases of diploid/triploid mosaicism (Edwards et al, 1994).

High levels of chromosomal abnormalities seen in this and other studies correlate with the relatively low success rate of IVF and human reproduction in general with only about one in four of conceptions resulting in implantation (Wilcox
et al., 1988). Whilst embryos showing highly abnormal chromosome constitutions are unlikely to develop further, it is unclear how the low level mosaicism observed here would affect further development as selective mechanisms to rescue less abnormal embryos may operate. Several scenarios have been suggested to play a role in embryo rescue as outlined in section 4.5.

All three chaotic embryos (n2, h1, h2) of the normally developing embryos group showed chromosomal complements implying the possibility of chromosome breakage. These observations were possible as the probe combinations applied to these three embryos involved two probes on chromosomes 5 and 22 respectively. Two cells in embryo n2 showed two copies for the control probe on chromosome 5 binding at 5q31, but only one copy for the locus specific probe binding on the short arm of chromosome 5 (5p15.2), indicating partial monosomy for this chromosome. This occurrence could be explained on the basis of chromosome breakage. However, no cells presented with three copies for the probe on 5p15.2, indicating that partial loss of the chromosome took place. Similarly, two cells in embryo h1 showed 2 copies for the probe binding at 22q11.2 but one and zero copies respectively for the probe on 22q13. Finally, embryo h2 showed two blastomeres carrying two copies for 22q11.2 but both were nullisomic for 22q13. These results equally imply chromosome breakage of 22 and subsequent loss of the resulting chromosomal fragments. As the partial losses noted were not complemented by reciprocal gains of the resulting fragments in other blastomeres of the same embryos, it was deduced that chromosome breakage was followed by loss of the acentric fragment in each case. Chromosome breakage has been recorded in karyotyping studies (Papadopoulos et al., 1989; Zenzes and Casper, 1992; Clouston et al., 1997), as well as in recent CGH studies (Wells and Delhanty, 2000; Voullaire et al., 2000). Partial chromosome loss and gain following chromosome breakage was observed in both studies by Wells and Delhanty, (2000) and Voullaire and co-workers (2000), as different cells contained the reciprocal products of the breakages, confirming that the losses were not experimental artefacts.

Whether the abnormalities observed in IVF generated embryos can truly reflect that which occurs following natural conceptions can only be debated. No increase in the rate of congenital abnormalities in live births resulting from IVF over those from natural conception has been observed (SART, 1992; FIVNAT, 1995). Evidence in favour comes from similar observations, dating back to the classical work of Hertig, of high levels of cleavage-stage arrest and nuclear abnormalities in in vivo
Discussion

It may be that certain ovarian stimulation or embryo culture protocols used for IVF exacerbate problems that exist in vivo (Wells and Delhanty, 2000). Various cellular stresses may be caused by inappropriate culture media and can result in chromosome damage (Martin et al., 1990). The impact, which sub-optimal in vitro factors have on post-zygotic chromosomal mosaicism, is unknown, though fluctuations in temperature and oxygen tensions have been shown to effect oocyte spindle formation and chromosome segregation (Almeida and Bolton, 1995; Van Blerkom, 1998). The use of different culture protocols has shown different cleavage rates (Scriven et al., 2000), while different drug and culture conditions have been suggested as affecting levels of mosaicism (Munne et al., 1997). Finally, it has been documented that some patients do inexplicably produce higher levels of aneuploid (Munné et al., 1996b), polyploid (Pergamont et al., 2000) or chaotic embryos (Delhanty et al., 1997c) than others even when subjected to identical IVF regimes and this patient-specific factor is difficult to allow for when comparing abnormality rates between centres.

4.2 The Clinical Application of PGD

4.2.1 PGD of Chromosome Abnormalities.

Over the years, reassuring data collected on the obstetric and neonatal outcome of PGD supports the safety of this procedure, showing no increase in major congenital malformations over standard IVF, ICSI or spontaneous pregnancies and births (Soussis et al., 1996a, b; ESHRE PGD Consortium, 1999; 2000; Strom et al., 2000a, b). With public awareness of PGD growing, patient demand has exponentially increased. One of the most common requests has come from couples at high risk of chromosomally abnormal pregnancy due to a parental chromosomal translocation. Chromosomal rearrangement carriers are considered to be the most motivated group for PGD as they experience infertility, a high incidence of miscarriage often recurrent, as well as recurrent TOPs as a result of a series of abnormal conceptions. This is illustrated by the reproductive histories of the forty-four couples referred to the UCL Centre over this study period, in which for a total of 93 previous conceptuses, only 7% resulted in a normal live birth. The vast majority of pregnancies in this patient group did not continue to term, presumably due to chromosome imbalance, 70% resulting in spontaneous abortion and 14% in TOP (see Fig.2.1, section 2.1.2.2.2). As
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a consequence, one of the main reasons for requesting PGD in this particular group was stated as recurrent miscarriage, with some individuals having experienced six consecutive pregnancies, which had all ended in spontaneous abortion in the first trimester. Although the primary role of PGD in these cases is obviously diagnostic to selectively transfer embryos with normal or balanced chromosome complements, the subsequent availability of unfertilised oocytes and surplus human embryos provides a unique and exclusive opportunity to study meiotic segregation patterns.

In response to the mounting demand for PGD for this patient group, a variety of approaches have been developed from a number of centres with the aim of helping such couples. Translocation carriers, the largest referral group, represent a challenge for PGD. As breakpoints can occur theoretically at any point on any chromosome, with the exception of the common t(11;22) translocation, practically all cases represent a unique event, which requires the development of a specific probe set for PGD. Two different approaches have been applied for the analysis of blastomeres, where probe combinations have been employed that either flank or span the breakpoints of a translocation (Conn et al., 1999, Munné et al., 1998c).

Prior to the commencement of this study, the task of obtaining suitable probes was time-consuming and laborious. However, with the growing availability and subsequent wide application of commercial probes, coinciding chronologically with initiation of this thesis in 1998, difficulties in developing PGD protocols have been eased considerably. Table 4.1 summarises the outcome of the referrals during the period from 1995-1998 (prior to initiation of this project and extensive availability of commercial probes) and compares it to the patient’s status during the period from 1998-2002 (following commencement of this study and extensive use of commercially available probes. The percentage of the total PGD cases completed or in progress has increased dramatically from 15% to 36.5%. This increase reflects the positive effect the evolution of technology had on the number of the cases that can be treated and the time taken to conclude the work-up for each case, allowing a greater number of patients to be offered treatment. As outlined in Table 3.19 there are several reasons why patients may decide against PGD, or why treatment could be discontinued. However, as data suggests after comparing the total discontinuance of the PGD work-up between the chronologically divided groups of the patients referred during 1995-1998 and 1998-2002, the percentage has dropped from 85% to 63.5% indicating a major improvement. The time taken to prepare cases undoubtedly played
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a large role in the loss of some couples from the referral list that opted for prenatal diagnosis or lost contact with the centre.

Table 4.1. Outcome comparison for patients referred before (1995-1998) and following commencement (1998-2002) of this study.

<table>
<thead>
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<tr>
<td>Reached Embryo Biopsy Stage</td>
<td>6 (15%)</td>
<td>9 (20.5%)</td>
</tr>
<tr>
<td>Awaiting PGD (Diagnosis Ready)</td>
<td>0 (0%)</td>
<td>7 (16%)</td>
</tr>
<tr>
<td>Total PGD completed or ready</td>
<td>15%</td>
<td>36.5%</td>
</tr>
<tr>
<td>Total PGD discontinued/cancelled</td>
<td>85%</td>
<td>63.5%</td>
</tr>
</tbody>
</table>

PGD will never be used as comprehensively as invasive prenatal diagnosis as there are several limitations involved. The couples have to go through IVF, the procedure is very expensive, and the success rate is perceived as low (Harper and Bui, 2002). One of the main prerequisites for PGD is a good cohort of embryos. A sufficient number and suitable quality of the embryos is essential for them to be subjected to biopsy. A low number of embryos translates into fewer embryos from which to select for transfer, while inadequate quality could mean a potential cancellation of biopsy. This was demonstrated in cases K and J where the unsuitable embryo quality reflected in the slow development of the embryos leading to cancellation of biopsy and subsequent transfer of embryos based solely on morphological criteria, without achieving a pregnancy.

For the series of PGD cycles reported here the, the clinical outcome was good; despite the poor reproductive histories in almost all cases three of the nine couples achieved their aim of a normal live birth, two after a single treatment cycle. This illustrates the value of PGD in enabling the selection of embryos with the best chance of implantation.

The expectancy of alternate, balanced, segregation patterns in gametes produced by reciprocal translocation carriers has been reported to be approximately 50% (Estop et al., 1995), and studies performed by Iwarsson et al., (2000) and Scriven et al., (2000) refer to the alternate segregation pattern as the most common

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mode of segregation. The results obtained from the analysis of 102 embryos in our group of translocation carriers, revealed that for those where meiotic origin could be determined, just over 50% (36/65) showed signal patterns consistent with alternate segregation. This compares with alternate segregation frequencies of 54% - 89% in the sperm of the three male carriers (cases: D, E, H). Signal patterns in 21/65 embryos (approximately a third) indicated that they were derived from gametes with an adjacent-1 segregation mode; this is the unbalanced type most likely to be compatible with postnatal survival of an abnormal fetus. Adjacent-2 segregation, which is considered to be a rather uncommon segregation pattern, (Estop et al., 1995; Gardner and Sutherland, 1996), gave rise to five of the 65 embryos. Of these, three were from couple G, a “whole-arm” translocation where the breakpoints were situated so close to the centromere that the translocated and the centric fragments included almost one chromosome arm each. Two previously karyotyped POCs from this couple had also been the product of Adjacent-2 segregation, indicating a predisposition towards this type of pattern in the maternal meioses. The rare 3:1 interchange and 3:1 tertiary segregation mode was detected in three of the 65 embryos. In the remaining 27 embryos the segregation pattern could not be determined due to the chaotic nature of their chromosomes. The breakpoint’s position has been shown to have a considerable effect on the segregation pattern involved (Escudero et al., 2000b). Two translocation cases have been reported with the same chromosomes but different breakpoints. Although very similar translocation breakpoints were involved the proportion of unbalanced types differed. In one case, 46,XX,t(2;14)(q23;q24), the predominant segregation mode was 3:1, whilst for the other 46,XX,t(2;14)(q31;q24), adjacent 1 segregation was more common (Escudero et al., 2000b). The results from the analysis of the embryos of the reciprocal translocation patients that underwent PGD are in line with the expected frequencies reported in other studies (Munne et al., 1998c, Iwarsson et al., 2000). Theoretically, with an alternate segregation frequency of at least 50% the chance of a normal pregnancy should be 1:1. As this is not consistent with the previous pregnancy rate of the examined patient group, other factors involved, even in natural cycles, should be considered as having a detrimental effect on the pregnancy rates of the particular patient group. Errors during post-zygotic events involving cell division and DNA replication are clearly evident in embryos derived from the PGD cycles performed for these couples.
A common outcome reported in most of these PGD cases is a high level of chromosomally abnormal embryos resulting in very few embryos for transfer. Using the diagnostic probe sets, results on the chromosomal constitution of 75 untransferred embryos were obtained of which 55, (73%) were mosaic. A high number of mosaic embryos, particularly chaotics, have been widely reported by most groups treating patients with a poor reproductive history who are carriers of chromosomal rearrangements (Conn et al., 1998, Munne et al., 1998c, Van Assche et al., 1999, Iwarsson et al., 2000). More recently, high frequencies of chaotic embryos have also been described in couples with repetitive implantation failure (Bielanska et al., 2002; Voulaire et al., 2002) providing evidence that it may be a factor in some of these cases. The data accumulating from molecular cytogenetic investigation of embryos derived from both patient groups adds weight to the earlier suggestion that carriers of chromosomal rearrangements who are unable to achieve a normal pregnancy outcome are the victims of two pathologies, namely the increased risk of an unfavourable meiotic outcome together with a predisposition to the production of embryos with the most extreme form of mosaicism (Conn et al., 1998). A high percentage of chromosomal abnormalities is common at the cleavage stage even in normally developing embryos from fertile patients (Harper et al., 1995a; Munne et al., 1995a). However, the chromosome abnormality rate appears to be so high in this particular group of chromosomal translocation PGD patients, that there are generally very few embryos to choose from for transfer, while in some cases it is possible for all embryos analysed to be chromosomally abnormal (Munne et al., 1998; 2000, Escudero et al., 2000b). A strong correlation between the proportion of chromosomally abnormal embryos and pregnancy rate has been suggested by early karyotyping studies (Zenzes et al., 1992), and supported by more recent FISH studies, suggesting that PGD patients with more than 50% chromosomally abnormal embryos achieved significantly fewer pregnancies following embryo transfer (Munne et al., 2000).

It is notable that couple F who achieved a healthy live birth, were normally fertile and had only a single mosaic (chaotic) embryo. At present reports of PGD cases involving other structural chromosomal abnormalities are limited. Two groups reported on normal live births for PGD for pericentric inversions (Iwarsson et al., 1998; Escudero et al., 2001). In both cases almost 50% of the embryos analysed were found to be normal, acting as a good prognosis factor for the PGD outcome. Similarly, for case F 70% of the embryos were classified as normal providing a
positive prediction of the PGD result. Couple A, achieved a normal live birth despite the high percentage of chromosome abnormalities seen in the majority of the embryos. With the vast majority of the embryos generated by these couples found to be chromosomally abnormal ensuring a pregnancy can be difficult. A strong suggestion for those couples would be to perform several PGD cycles as required in order to increase their chances of implantation of a chromosomally balanced embryo, as seen for case A who following 3 PGD cycles accomplished a healthy live-birth. For this reason, counselling of prospective couples needs to emphasize that multiple IVF cycles are likely to be required in order to achieve the desired aim. With 50% of the embryos analysed found normal/balanced, couple C achieved a biochemical pregnancy. For couple E, a Robertsonian translocation carrier case, the problems were almost entirely post-zygotic; 89% of sperm and seven out of eight embryos were shown to be the product of alternate segregation. Significantly, no pregnancy was achieved for cases D, E, and I where the male carriers of the translocations presented with severe oligospermia (case D) and oligoasthenoteratozoospermia (case E, and I) respectively. Couples D and E produced a high number of chaotic embryos. This is consistent with the known effects of chromosome rearrangements on male fertility, (Faraut et al., 2000, and references quoted therein).

The abnormalities encountered in cleavage stage embryos have been reported to depend on various factors. Studies have shown that the risk of producing chromosomally unbalanced embryos depends upon the chromosomes involved in the translocation, the position of the breakpoints, and the sex of the translocation carrier (Faraut et al., 2000; Goldman and Hulten, 1992, 1993). In this series of patients, the couples achieving pregnancies involved a female rather than a male carrier. Moreover, with the exception of cases G and D, all remaining reciprocal translocations involve one or both chromosomes with a distal breakpoint. Although it is far from clear why specific translocations are prone to segregate in an unbalanced way during the first meiotic division, a frequently occurring factor is that the breakpoint in one of the chromosomes taking part in a reciprocal exchange is very close to the end of the chromosome. This may reduce the likelihood of crossing-over in the terminal region, which will have a secondary effect of reducing the chances of a balanced segregation, increasing the risk of unbalanced forms. Munné et al. (2000) have also considered this possibility, and have noted a significantly lower pregnancy
rate associated with a higher proportion of abnormal embryos from their cases involving at least one terminal breakpoint.

All the clinical pregnancies reported in this group of patients were achieved following changes to the biopsy protocol. The changes involved abandoning pre-incubation of the embryos in Ca⁺² Mg⁺² free medium for ten minutes in favour of biopsying the embryos immediately upon transfer to this medium and an increase in the size of the biopsy droplet. The reduction of the exposure time may help the reconstruction of the gap cell junctions involved in embryo compaction. The success of these latest cycles presumably reflects patient specific factors but may in part be attributable to the current high overall pregnancy rate seen in the routine IVF patient group at the unit (40% per ET). In case G, only one embryo out of a cohort of eleven (including chaotics) was diagnosed normal and transferred, and a pregnancy was achieved.

PGD centres have become reliant on commercially available subtelomeric probes in the treatment of couples being carriers of chromosomal rearrangements. However, it should be noted that these probes have in many cases considerably lower hybridisation efficiency than repetitive probes when used in interphase nuclei. Failure of hybridisation could lead to false conclusions indicating a signal pattern that corresponds to an unexplained segregation mode, or chromosome breakage. For this reason repetitive probes were employed where possible and the aim remained to obtain results from two cells per embryo. The possibility that FISH due to occasional experimental errors could artificially increase the perceived level of mosaicism should be further investigated (Wells and Levy, 2003).

Mosaicism has important implications for PGD based on the analysis of a single biopsied cell, posing a significant risk of misdiagnosis from inadvertent sampling of a minor cell line. The high percentage of mosaicism seen in this study has reinforced the aim to only recommend embryos for transfer on the basis of two biopsied cells, both diagnosed as unaffected. The debate over whether taking two cells rather than one is detrimental to embryo development is ongoing (De Vos and Van Steirteghem, 2001; Van Velde et al., 2000; Pickering et al., 2003). In a very recent study, Bielanska and co-workers, employed FISH to evaluate the potential of single blastomeres isolated from cleavage stage embryos and subsequently cultured and proliferated to provide more cells for analysis for PGD for chromosomal abnormalities. Blastomere culture would increase the number of cells obtainable for
chromosomal analysis, overcoming diagnostic problems arising due to unavailability of multiple cells. This promising method would also circumvent the issues raised regarding the detrimental effect on the biopsied embryo following removal of 1-2 blastomeres. Unfortunately, results indicate that blastomere culture leads to an increased rate of nuclear abnormalities and to difficulties in interpretation of polyploidy and mosaicism yielded by the cultured cells. This occurrence of polyploidy and mosaicism among the cultured cells, has discouraged the use of blastomere isolation and proliferation strategy for use in PGD (Bielanska et al., 2003).

It has been reported that, up to a quarter of a human embryo can be removed without impairment of its further in vitro development (Hardy et al., 1990). On the other hand, biopsying two instead of one blastomere could reduce the embryos capacity to implant, as disruption of the intracellular junctions could have a detrimental effect in the eight-cell embryo affecting its polarisation (Antczak and van Blerkom, 1999). Moreover, although it is believed that on day 3 the embryo is still totipotent, as the embryonic genomic has been activated the blastomeres may have already started to differentiate (Mottla et al., 1995; Braude et al., 1988). Van Velde and colleagues (2000) showed that taking two cells from six-cell stage embryos does not seem to affect their development as they cleave further during the day of the biopsy. Moreover, comparing embryos from which 1 or 2 cells have been taken suggested that the implantation and pregnancy rates are similar (Van Velde et al., 2000). By contrast, in a very recent study, Pickering and co-workers (2003) at the Centre for PGD, Guy's and St Thomas Hospital, suggest that as the potential of misdiagnosis cannot be eliminated totally, biopsying a single cell can result in a successful PGD program with encouraging pregnancy rates. In a letter commenting on laboratory diagnosis and the uses of error, by C. M. Ogilvie (2003), part of the same group, recognising the technically demanding and error prone nature of PGD, suggest that PGD patients, having undergone treatment where one cell has been biopsied, should further rely on prenatal diagnosis. In reply to this, JDA Delhanty (2003), as scientific director of the University College London Centre for PGD, suggests that the way forward is to increase accuracy of the tests, rather than to rely on a greater uptake of prenatal diagnosis. The easiest way to increase accuracy is to analyse two independent cells. Data presented by our group (University College London Centre for PGD), as well as the Centre for Medical Genetics, Brussels Free University, has shown that an acceptable pregnancy rate can still be achieved despite a policy of taking two cells

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from embryos consisting of at least six cells. Suggesting that patients should undergo prenatal diagnosis following PGD, would partly defy the point of PGD at the first place. It is important to remember that most couples opt for PGD to avoid prenatal diagnosis and the trauma of a termination of pregnancy, while others do not wish to jeopardise a much wanted pregnancy by having an invasive procedure (Delhanty, 2003).

Table 3.37 shows a summary of embryos biopsied in this series, and the results obtained. The results show that biopsying two cells did not lead to obtaining results for either of them for 30% of embryos biopsied. Interestingly, biopsying two cells and obtaining results from both of them, strengthening the validity of the diagnosis for the respective embryos, applied to another 30% of the biopsied embryos i.e. to the remaining half of the embryos from which two cells were biopsied. Biopsying a single cell and obtaining results, involved 28.3% of the embryos biopsied, while failing to get a result from the single biopsied cell was true for 11.5% of the embryos. Results from Table 3.37 fail to show conclusively whether biopsying one or two cells is preferable in terms of successfully obtaining the information required from the biopsied cell. In fact, percentages are similar (30%, 28.3%). Examining the aspect of complete failure to obtain results, for 8.8% of the embryos biopsied no result was obtained from either of the two blastomeres biopsied and similarly for 11.5% of the embryos no result was obtained from the single biopsied cell. As percentages are pretty similar for a conclusion to be drawn based on them, the focus on deciding between biopsying one or two cells was placed on the chromosomal constitution revealed by FISH analysis on the spare embryos. Detailed follow up results from the group of patients in this study re-affirms the view that it is advisable to obtain results from more than a single cell. In this series, definite evidence for the co-existence of chromosomally balanced and abnormal cells has been found in eight embryos, based upon signal interpretation (Table 3.39). In some cases, (embryos a, b and j) the embryos were prepared on day 5, which allowed a larger number of cells to be analysed confirming the nature of the mosaicism. Four of the eight embryos appeared to have been the product of an adjacent 1 segregation (embryos a, b, d, and i) and four appear to have been initially balanced (embryos c, e, g, and j). For embryos b, d and i where three cells in the untransferred embryo (including the biopsied cell in embryos b, a and i) appear to be balanced due to lack of a subtelomeric signal, hybridisation failure cannot be ruled out. However, this is unlikely to be the cause in embryo a.
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since 7 of 23 cells showed a balanced signal pattern. In this case, chromosome breakage and loss of the segment is indicated since loss of the whole chromosome 11 would involve loss of the centromeric probe as well, which was not observed. Increasing amounts of data from CGH analysis of individual blastomeres shows that chromosome breakage is not a rare event in human preimplantation embryos, occurring in as many as 10% of embryos (Voullaire et al., 2000, Wells and Delhanty, 2000, Voullaire et al., 2002) (see section 4.5). Embryos from translocation patients may be at greater risk than average in this respect. Evidence for chromosome breakage and reciprocal loss and gain of the fragment was obtained from analysis of 44 nuclei from the previously frozen ‘Aneuploid mosaic’ (Table 3.38) embryo of couple H, as described in the Results section. Embryos c, e, g and j that started as balanced, became aneuploid mosaics due to chromosome loss in most cases, with one example of chromosome gain (biopsied cell of embryo g). It is important to note that for four of these embryos (a, b, d and i), if a single cell had been taken for diagnosis this cell could have been balanced (or apparently so), leading to the transfer of an embryo that is in fact the product of unbalanced, Adjacent-1, segregation, likely to survive with an abnormal outcome, as has been reported previously (Munne, 2002). In the other four cases, where the embryos were initially balanced, the presence of 30-50% aneuploid cells would have reduced the chances of implantation and survival; biopsy and analysis of two cells reduced the risk of this happening. The three live births achieved for the nine couples treated in this series, despite the poor history in most cases, is further proof that biopsy of two cells per embryo is compatible with a positive outcome.

4.2.2 Ethical Concerns and Current Status

The ability to select an embryo following genetic testing sometimes raises questions of choosing a child to order, as a commodity that has been designed simply to meet the needs and desires of the parents. PGD has been applied to selecting a child to provide a cure for an already existing affected child (Verlinsky et al., 2002). Ethical concerns are however, that it is not acceptable to select a child solely for the purpose of treating a child who is already alive (Harper and Bui, 2002). The added problem in this HLA typing case, is that if treatment using haemopoietic cell stem cells obtained from the cord blood is unsuccessful, the second child will be required to donate bone marrow, a painful procedure with no direct benefit to the donor.
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(Harper and Bui, 2002), leading to issues of consent and protection of the child's autonomy. Although the merits of saving a sibling can be rationalized and commended this process has been met with great controversy, bearing in mind that there is only 25% chance of an embryo to be a suitable match, with an expectation that 75% of the created embryos might be unsuitable and possible discarded (Braude et al., 2002). PGD is already being used for sexing (gender determination for social purposes) (ESHRE PGD Consortium, Data collection III, 2001; Malpani et al., 2002) fuelling substantial debate and controversy (Savulescu and Dahl, 2000), and many consider that this is not a legitimate use of PGD (Robertson, 2002). We live in an age where there is decreasing tolerance of disability and abnormality, as modern medicine is promising “perfect babies” people are concerned about eugenics (King, 1999), or offering PGD for characteristics such as intelligence, height etc (Berkowitz, 1999). However, the genetics of most of these characteristics are multifactorial and the diagnosis would be very complicated, especially from a single cell. In general most centres agree, that PGD should not be conducted for non-medical purposes (Nagy et al., 1998). Of course parents want healthy and happy children. But there is a limit to what medicine should do to achieve this.

The most recent report by the European Society for Human Reproduction and Embryology (ESHRE) PGD Consortium reveals the current status in this field, with data on a total of 1561 referrals, 370 regular PGD cycles and 334 PGD-AS and for the first time reports on 78 cycles for social sexing from 24 centres and 215 pregnancies and 117 babies from 12 centres. The majority of the patients referred for social sexing were fertile and the pregnancy rate was 35% per egg collection, which is higher than any other PGD pregnancy rate. The consortium showed a growing number of PGD cases being performed, while the increase of pregnancies following FISH diagnosis is attributed to the large number of babies born after PGD-AS. Finally, for this data collection again a number of misdiagnoses identified both prenatally and post-natally were reported. The work of the ESHRE PGD consortium has proved to be of great importance to PGD for the follow-up of cases and pregnancies (ESHRE PGD consortium. Data collection II, 2000; ESHRE PGD consortium. Data collection III, 2002) and is the only reliable means of providing international data on PGD. The consortium was established to survey the availability of PGD for different conditions, to collect data on the accuracy, reliability and efficiency of PGD, to initiate follow-up studies, to promote best practise, and to produce guidelines and recommendations.
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(Geraedts et al., 2001). It was noted that PGD is more widely known in the reproductive medicine community than in the genetic circles, therefore a closer collaboration, co-operation and interdisciplinary communication between fertility units and genetic centres is highly justified and recommended (Geraedts et al., 2001).

4.3 The Wolf-Hirschhorn Syndrome (WHS) Project.

A patient was referred to the UCLH Cytogenetics Unit showing some features of WHS. However, WHS does not present with a standardised phenotype, as numerous characteristics are associated with it, and additional malformations are constantly added to its phenotypic description (Kohlschmidt et al., 2000). It would therefore be premature to assume its involvement based on phenotypic features. To add to the complexity, the very gene dense critical chromosomal region (4p16.3) of the syndrome is not yet defined with certainty, as new critical regions are being proposed (Zollino et al., 2003) and several genes are continually being reported as strong candidates in the causation of WHS (Stec et al., 1998; Wright et al., 1999; Endele et al., 1999). While progress was being made on the molecular refinement study undertaken, extensive cytogenetic analysis (performed by cytogeneticists at UCLH and in the Royal Free Haematology Department) revealed the complex karyotype of a rare type of de novo non-reciprocal chromosome rearrangement involving formation of isochromosome 20 by the whole 20p arm and translocation of the residual 20q arm to the short arm of chromosome 4 without any concomitant loss of material from 4p. The karyotype was interpreted as, XY,der(4)t(4;20)(p16.3;q11.1),i(20)(q11.1). However, as the size of the deletion and the mechanisms leading to WHS can vary considerably (Rauch et al., 2001) it is sensible that in conjunction with FISH and high resolution banding, molecular techniques should be employed to promote further delineation of this complex syndrome. The polymorphic markers used in this study (D4S127, D4S43, D4S169) examined in a more detailed molecular aspect the possibility of the WHS involvement in this case and the parental origin of the abnormality.

Numerous protocols were employed and various parameters were modified accordingly to ensure optimal conditions for the corresponding primers with analysis carried out using either the ABI PRISM™ or standard gel electrophoresis. Use of the extremely sensitive ABI PRISM™ system allowed the number of PCR cycles to be
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kept to a minimum. However, the number of cycles was increased when products were analysed by gel electrophoresis, as was the case at one point for polymorphic marker D4S43 seen in protocol 4 (see Section 3.9.3). Although less sensitive in detecting amplification (detected in form of bands on an agarose gel rather than peaks on the ABI PRISM™) electrophoresis was preferable in some cases as a simpler and less expensive detection system. However, when no bands were visible by gel electrophoresis as seen in protocols 4, 5, and 6 (see Section 3.9.3) for marker D4S43, a possible explanation was the lack of sensitivity of the system compared to the fluorescent detection one. The first PCR reaction for all primers was performed testing several annealing temperatures within the range suggested by published data, to select the most favourable to ensure best amplification. This ‘gradient’ of annealing temperatures was also employed at later stages in the experiments in cases where failure of amplification was thought to be a result of an unsuitable annealing temperature, as seen for the experiments carried out for marker D4S43, where a second gradient was performed in protocol 4. The annealing temperatures were increased to avoid non-specific amplification or complete failure of amplification as seen in protocol 6 carried out for marker D4S43 and protocol 3 carried out for marker D4S169. The volume of DNA in a PCR reaction was increased or decreased according to the original concentration of the sample. An increase in the concentration of DNA in the PCR reaction was used as a means to circumvent amplification failure, as seen in protocol 3 (see Section 3.9.3) for marker D4S127. The concentration of primers was increased to achieve extra specificity in amplification as seen in protocol 4 for marker D4S43. In this case it was thought that amplification of a non-variable region occurred, as six different DNAs (BW, JE, F, M, S, P) were all found to be heterozygous for the same alleles, which was rather improbable since D4S43 is a highly polymorphic marker. Prior to reaching the conclusion that non-specific amplification was taking place, contamination was considered to be a possible explanation for the heterozygocity for the same alleles found in six different DNAs. Changing all the reagents used and employing a “cold start” as seen in 3 for marker D4S43 excluded the possibility of contamination. The so called “hot start” was employed for most protocols meaning that the polymerase was added to the PCR reaction mix when the temperature of the latter had almost reached denaturation point so as to ensure the enzyme’s most advantageous action. However, this technique carried contamination risks in contrast to the so-called traditional “cold start” where
the enzyme was added to the PCR reaction mix before the samples were loaded on the PCR machine.

Analysis performed for polymorphic marker D4S127 (Taylor et al., 1992) was uncomplicated and straightforward, the optimal annealing temperature was identified without difficulty and the few obstacles encountered in the analysis involved enhancement of the DNA concentration in the original samples and in the PCR reaction. As the patient was found to be heterozygous for the same alleles as the sister and the father, no deletion for this marker was shown, minimising the possibility of the WHS involvement.

In the case of polymorphic marker D4S43 (Horn et al., 1991), the possibility of contamination as an explanation for classifying six individuals as heterozygotes for the same alleles was excluded, as new reagents and "cold start" were employed for protocol 3. As a result it is likely that the same alleles were amplified indicating that non-variable amplification was taking place. The concentration of primers in the reaction, as well as the number of cycles was greatly increased as seen in protocol 4, in order to achieve further specificity and the products were analysed on agarose gels. However, no bands were detected. As this detection system lacks the sensitivity of the fluorescent one, this fact did not imply failure of amplification. The products were consequently analysed on the ABI PRISM™, which confirmed the failure of satisfactory amplification. Further, investigation of the sequence to be amplified by this set of primers, revealed a very rich GC sequence. This information, suggested the problem to be possible failure of denaturation due to the nature and the strength of the bonds formed between guanosine and cytosine in this sequence. Substitution of 60% of the dNTPs mix with deaza-dGTPs ensured a less strong bond between G and C, allowing the successful denaturation and subsequent amplification of the sequence as seen in protocol 7. Results demonstrated no deletion for D4S43, as the patient and the sister were both heterozygous inheriting one maternal and one paternal allele.

The PCR product of the primers for polymorphic marker D4S169 (Pritchard et al., 1991) was very large (1.6Kb). However, it was only a fragment of this 1.6Kb sequence that contained the polymorphism and hence was of interest to the project. This fragment being as small as 214bp could arise following RsaI digestion. PCR was performed according to the published data suggestions, and both the digested and the undigested products were analysed by agarose gel electrophoresis as well as by the ABI PRISM™. However, no bands or peaks were obtained. The activity of RsaI was
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tested on other PCR products and the enzyme was proven to work satisfactorily. The fact that there were no bands when the products were run on agarose gel could be due to the lack of sensitivity of the detection technique with respect to the Rsal fragment's size, and the fact that the original PCR product was too big to be detected by standard electrophoresis. Similarly, the ABI PRISM™ could not detect a product of this size (1.6Kb) and it was speculated that the reason why the 214bp Rsal fragment was not detected by the ABI PRISM™ was the fact that the fluorescently tangled fragment of the 1.6Kb product was not the 214 bp fragment but the reciprocal Rsal fragment which would not be expected to be detected by fluorescent analysis due to its size. Therefore, no conclusion was drawn from this polymorphic marker with respect to the WHS involvement.

The results obtained by this molecular refinement study support the cytogenetic evidence according to which there is no WHS involvement in this case, since there is no deletion detected within the WHCR according to the polymorphic markers employed. However, there are numerous markers located within the WHCR and their deletion could hypothetically explain the feature of WHS in this case. The markers employed for this study were selected based on criteria that published data suggested. All were highly polymorphic and were the most common markers within the WHCR that have been involved in several cases of WHS reported in the literature.

Following exclusion of the WHS involvement based on this molecular refinement, this case was published as the first reported centromere telomere fusion resulting in pure trisomy 20p (Sidwell et al., 2000). FISH analysis showed both centromeric repeat sequence and interstitial telomeric sequence at the t(4;20) breakpoint junction. The CGH analysis (see fig 3.26) performed as the final part of the investigation of this patient confirmed the cytogenetic findings of the publication that the patient was indeed carrying pure trisomy of the short arm of chromosome 20. Importantly, in fact these CGH results were able to provide additional information by excluding any other chromosome involvement, ensuring that the clinical manifestations of this case were a consequence of this rare de novo non-reciprocal rearrangement, involving formation of an isochromosome. In addition, CGH confirmed the FISH study carried out by Sidwell and colleagues (2000) and did not detect any loss of material of 4p, as was originally suspected, adding to the complexity and rareness of this case. The results of this study support the interpretation of a break in the pericentric long arm of chromosome 20 close to the
end of the alphoid sequences, leading to formation of an isochromosome with fused centromeres and therefore a monocentric appearance. The absence of mosaicism involving normal cells or telocentric 20p or 20q, supports a single step aetiology.

4.4 Molecular Cytogenetic Analysis of Fetal DNA (ICH Project)

Touch preparations and DNA were provided from fetal tissues from induced abortions. Ninety-five per cent of these selective abortions were performed by surgical termination between the 9th – 12th week of pregnancy.

CGH was employed to check for deletions and/or duplications on all chromosomes, and FISH on the touch-preparations offered information on the respective ploidy status of the fetal samples. Forty-six DNA samples were provided and for eighteen of these results were obtained from CGH analysis. Eleven samples were female and seven were male, but all were disomic for all autosomes. No deletions or duplications, partial or whole were observed. These results were supported by the FISH results on the respective touch-preparations. For all eighteen samples classified as normal from CGH analysis, the FISH analysis on the corresponding touch-preparations indicated a normal copy number of the chromosomes examined. The sex classification according to the CGH data was also confirmed by FISH results, with the exception of two samples (498 and 525) for which the results of the sex classification were inconsistent. According to FISH, sample 498 was a male/female mosaic but CGH results supported a male fetus, while sample 525 was classified as male by FISH and female by CGH. These discrepancies could be explained by the possibility of maternal cell contamination or an initial twin pregnancy. Maternal contamination represents the main likely discrepancy between conventional cytogenetic analysis and CGH analysis (Lomax et al., 2000).

Out of fifty touch-preparations, eight failed to provide results following FISH application, as the DNA of the cells was degenerated and therefore not suitable for any analysis. From the remaining fifty samples twenty-six were classified as female disomic for chromosome 18, and twenty as male disomic for chromosome 18. Two samples, one of which was sample 525, were mosaic carrying both male and female cell lines both disomic for chromosome 18. Samples 542 and 546 were classified as complex aneuploid mosaics carrying three and four cell lines respectively. The
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majority of the cells analysed were male or female, disomic for chromosome 18, and only a small fraction of cells presented with monosomy 18 in both samples, and with trisomy X in sample 546.

Chromosome abnormality is a major cause of pregnancy loss, with more than half of the spontaneous abortions in the first trimester being karyotypically abnormal. However, that is not the case in studies performed on fetuses arising from non-medical reason terminations. A study examining the extent of chromosomal anomalies in 944 induced abortions revealed a chromosome abnormality rate of 3.2% with non-mosaic and mosaic trisomies and triploidies being the commonest anomalies found (Kajii et al., 1978), while in a more recent study Burgoyne et al., (1991) estimated 4.7% numerical chromosome anomalies for women with a clinical recognized pregnancy at the end of the 7th week after the last menstrual period.

As expected in this study, the vast majority of fetuses selectively aborted for non-medical reasons were found to be chromosomally normal. In the very few cases where an abnormality was detected, it was present only in a restricted selection of cells with the majority of the cells analysed showing a normal chromosomal complement. Monosomy of autosome 18, the main aneuploidy encountered in this study, has not yet been reported to be compatible with live birth. As the number of cells analysed and found to carry a single copy of chromosome 18 was very small (7, 3 in sample 546 and 4 in sample 542) and with a probe combination efficiency of 96%, it would be sensible to consider that the single signal for chromosome 18 was not reflecting the true chromosomal complement of those cells but could have arisen as a FISH artefact, possibly due to failure of hybridisation, or overlapping signals. In the case of sample 542, 6 cells out of 50 were found to be trisomic for chromosome X (Table 3.41). In some instances mosaicism is present in the fetus and may persist in postnatal life, or it could be confined to a specific organ or present in various percentages in different organs (Shashi et al., 1996), which could explain some cases. Studies also suggest that there is a selective advantage for the euploid cell line with time (Gravholt et al., 1991; Petit and Fryns, 1994), helping mosaic fetuses to survive to term. Unfortunately, for the cases in this study classified by FISH as complex aneuploid mosaics (542, 546) no DNA was available for further analysis employing CGH, suggesting limited availability of fetal tissue.

The majority of samples subjected to CGH did not provide analysable results. Considering the fact the CGH involves technical complexities that cause it to be a
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labour intensive and temperamental assay that requires expertise with several
cytogenetic and molecular genetic techniques, this was expected to a certain extent.
For the ten samples classified in category B (52, 59d, 60d, 69d, 489, 523, 535, 539,
540, 546) the CGH had worked efficiently for the reference DNA, implying that the
DNA for these samples was either degenerate or of very low molecular weight and
therefore not suitable for analysis. This was confirmed following assessment of the
size and quality of those DNA samples by gel electrophoresis. In the case of category
A, complete failure of CGH had occurred as a result of uneven hybridisation, weak
fluorescence, speckling signals produced and poor chromosome morphology. Further
investigation of these DNA samples employing gel electrophoresis identified some of
them to have deteriorated classifying them as unsuitable for any analysis (506, 516,
517, 518, 546). For the remaining samples (69, 495, 497, 499, 526, 529, 536, 537,
538, 550, 552, 553) good DNA profiles were obtained following gel electrophoresis
and therefore CGH was repeated for these samples. The application of CGH on these
samples was still unsuccessful without apparent reason. This was initially unexplained
and therefore attributed to the complexity of the technique. However, continuous
investigations, performed on all steps involved in the assay, and comparable studies,
including control DNAs tested together on the same slides indicated that
contamination of the test DNAs should be considered, possibly occurring during the
DNA extraction. This was suggested, as it was the combination of the test DNA with
the reference DNA that caused the latter to fail to hybridise, while the reference DNA
hybridised successfully against itself pointing to contamination as the possible cause
of the failure of the experiment. Although the DNA-based approach avoids the
problems associated with tissue culturing, the integrity of the analysis depends on
extracting DNA efficiently, from an appropriate tissue (Lomax et al., 2000). The
nature and source of contamination remain unknown.

Conventional cytogenetic analysis of fetal material depends on tissue culturing
and karyotyping. This technique has some problems, including external
contamination, culture failure and selective growth of maternal cells (Eiben et al.,
1990). CGH has been employed in numerous studies to determine the true extent of
chromosomal anomalies in spontaneous abortions. Fritz et al., (2001) showed that the
incidence of aneuploidy is still clearly underestimated since potential abnormalities in
culture failures have not yet been evaluated. Their study re-evaluated culture failures
in early spontaneous abortions and estimated the contribution of chromosome
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anomalies to the first trimester being nearly 70%. When employed for the analysis of different chromosome imbalances, CGH provided additional information to complement conventional cytogenetic investigations and refined the description of the aberrant chromosome segments allowing further characterisation of the underlying mechanisms involved, (Tonnies et al., 2001). Teamed with conventional cytogenetic techniques such as FISH (Tonnies et al., 2001) or flow cytometry (Lomax et al., 2000), CGH exhibits a lower failure rate and higher accuracy in determining chromosomal anomalies, than conventional cytogenetic analysis alone (Lomax et al., 2000). In fact, the advantages of CGH analysis of abortions are broader than diagnostic accuracy. Technically, CGH analysis allows collected tissues to be stored in the freezer and batching of DNA extraction, labelling, and hybridisation, thereby increasing technical efficiency. However, the presence of high levels (60%-70%) of maternal cells in a tissue sample may render CGH analysis ineffective in the determination of fetal aneuploidy or accurate gender determination of a male fetus. Proper identification and separation of fetal/placental tissues is essential if this approach is to be effective (Daniely et al., 1998).

This project in collaboration with the ICH provided valuable experience concerning technical problems related to CGH. Problems encountered in this study include; granular hybridisation signals, insufficient quantity of test DNA used for the CGH experiment, and poor suppression of the interspersed repetitive sequences present in both the test and reference DNAs. The accuracy of the hybridisation signals should be uniform, smooth and intense. Indeed, any decrease in hybridisation efficiency has a dramatic effect on the ratio profile (du Manoir et al., 1995). Careful adjustment of several parameters such as the quality of metaphase spread preparations, extracted genomic DNA concentrations, fragment lengths of each labelled genomic probe, suppression of interspersed repetitive DNA sequences and image analysis are all extremely important for the outcome of CGH experiments (Kallionemi et al., 1994; du Manoir et al., 1995). Chromosome morphology before and after denaturation, and the quality of hybridisation should be assessed (Karhu et al., 1997), which can be time-consuming. However, even when all these parameters have been assessed and all fulfil the requirements for a successful application of CGH, analysis is known to be sub optimal for several chromosomal regions. These include regions of the genome with high concentrations of repeat sequences such as heterochromatic regions 1qh, 9qh, and Yqh, and the centromeric regions of acrocentric
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chromosomes 13, 14, 15, 21, and 22 (Moore et al., 1997). Telomeres of most chromosomes have also been reported to be unreliably evaluated by CGH (du Manoir et al., 1995). Variability of the ratio profiles near the telomeres has been attributed to the difficulty of locating precisely the chromosome ends during the segmentation process because the fluorescent intensities gradually decrease at these regions (Kallionemi et al., 1994). Telomeric regions should therefore be interpreted with caution.

During this part of the work for this thesis all technical aspects of CGH were investigated. As various protocols were optimised throughout this study to achieve good results, the technique was finally perfected for use at the single cell level, as a research tool for preimplantation genetic analysis.

4.5 Assessing CGH as a diagnostic tool on untransferred embryos.

CGH performed on donated embryos from a routine IVF patient classified two of them (W1, W3) as uniformly normal male embryos, while both blastomeres from W2 were shown to be monosomic for autosome 16 and the sex chromosome X (Turner's syndrome). Turner's syndrome has an incidence of 1 in 5,000-10,000 female births. This represents only a fraction of conceptions with this condition, as 98%-99% are spontaneously aborted and only 0.3% are born alive (Hassold et al., 1996). Monosomy 16, also found in embryo W2, has been previously identified in preimplantation embryos using FISH (Munne et al., 1998), but is not seen in miscarriages indicating that this embryo would not have survived. These results demonstrate that the protocol developed will detect monosomies of whole chromosomes in single blastomeres. CGH was then applied to 50 blastomeres from 30 day-four untransferred embryos donated by five couples undergoing PGD for chromosomal rearrangements (described in section 3.4, 3.5, and 3.6). These embryos had been diagnosed as unsuitable for transfer after diagnosis.

In previous single cell CGH analyses of spare IVF embryos, non-mosaic aneuploidies, chromosome breakage, and a high degree of mosaicism were detected. Seventy five per cent of the IVF embryos were mosaic or chaotic (Vouillaire et al., 2000; Wells and Delhanty, 2000). These studies were performed on spare embryos of good morphology without any predisposition to chromosomal imbalance. Following
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commencement of this project, Malmgren et al., (2002) published results of CGH analysis of single blastomeres from non-transferred embryos generated by couples undergoing PGD for reciprocal or Robertsonian translocations. Application of CGH on all the blastomeres made available from each embryo was carried out to verify the PGD results and detect the overall genetic balance in each cell. Similarly the blastomeres examined in this project belonged to untransferred embryos generated by PGD patients. However, in this study the embryos were re-biopsied on day-four and 1-2 blastomeres were used for CGH analysis while the remainder of the embryo was spread and subsequently subjected to FISH analysis to confirm the diagnosis.

Information was collected evaluating CGH as a diagnostic tool, being the main aim of this project, following direct comparison of CGH to FISH, which is the current diagnostic technique employed for PGD for chromosomal rearrangements.

Limitations of CGH and Inconsistent CGH and FISH results

CGH is a technique with several difficulties due to the complexities of its nature. The internal control and reliability criterion for each CGH experiment was the consistency of the determination of the sex chromosomes. The euchromatic region of the Y chromosome is at the limit of size resolution for which aneuploidy can be detected by this procedure (Voullaire et al., 1999). All cells from each embryo were consistent for the determination of the sex chromosomes and all were in agreement with the respective FISH results. However, it should be noted that a possible case of mosaicism for a sex chromosome abnormality would negate this suggestion of an ‘internal control’. In addition, the chromosomes present in a normal diploid copy number can be used as an internal negative control for assessment of efficiency of hybridisation (Malmgren et al., 2002). It has been reported that small deletions or amplifications of the telomeric regions are difficult to interpret by CGH analysis and might be missed (Malmgren et al., 2002). Therefore, it was expected that imbalances involving a translocation with a very distal breakpoint could be missed as the resolution of CGH is limited and unreliable ratio rate changes may appear at the telomeric regions (Kallionemi et al., 1994).

The efficiency of single blastomere CGH for this study was 82% comparing well with the CGH efficiencies reported in the studies carried out by Wells and Delhanty, (2000) where the efficiency reported was 88%, and Voullaire et al., (2000) where the respective efficiency was 89%. However, those studies employed IVF
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embryos of good quality while the spare embryos included in this study had already been classified as chromosomally unbalanced with some of them being arrested and therefore expected to give a lower efficiency. The efficiency for the study performed by Malmgren et al., (2002) also using spare embryos from PGD cases was 70%, considerably lower than the one reported in this project. The fact that Malmgren et al., (2000) used a different type of WGA than the one used by Voullaire et al., (2000), and Wells and Delhanty (2000), could account for the lower efficiency seen in their study, as the protocol used, based on linker-adapter mediated PCR, might be less reliable or amplify DNA less efficiently leading to their poorer results (D. Wells, personal communication). Another explanation for the failure of CGH is the presence of large anuclear fragments, a relatively common finding in human embryos. It has been shown that 5% of good quality embryos and 12% of poor quality embryos contain anucleated blastomeres (Hardy et al., 1993). The embryos analysed in this study were genetically imbalanced and highly mosaic and therefore the degree of anucleation might be high, as also suggested by Malmgren et al., (2002).

Evaluating results obtained for the same embryos from both FISH and CGH application requires thorough consideration of all the limitations of each of the techniques. For example, in the case of embryo E9, all three blastomeres subjected to CGH were found to be normal, contradicting the only FISH result supporting nullisomy 13. The FISH result was re-evaluated as it was based only on the biopsied cell, and the possibility of failure of hybridisation for the probe for chromosome 13 was considered. The accuracy per probe per cell has been determined to be 93% for euploid samples. Therefore, in this case it is much more likely that embryo E9 was mosaic as this was frequent in embryos from this couple.

Another explanation for the inconsistency between FISH and CGH results is nuclear fragmentation, otherwise known as micronuclei, often seen in blastomeres from preimplantation embryos (Munne et al., 1998d; Wilton et al., 2002). These fragments can be lost when the cell is spread on a microscope slide for FISH resulting in loss of chromosomes. For CGH the intact blastomere is lysed in the PCR tube and any nuclear fragments are included in the analysis. Therefore FISH may overestimate the frequency of monosomy and nullisomy in single blastomeres (Wilton et al., 2003a). One of the major weaknesses of CGH is its inability to detect abnormalities of ploidy, for example haploidy or polyploidy, however, it has been shown that these
abnormalities are relatively uncommon in normally fertilized human embryos (Delhanty et al., 1997c; Iwarsson et al., 1999).

FISH would not detect many aneuploidies detected by CGH, as the number of probes is limited (i.e. too few spectrally distinct colours), and FISH is subject to other limitations (e.g. overlapping signals, split signals, etc). In fact, in the extensive aneuploidies detected by CGH in blastomeres A1.1, F6.1, and H11.1 (Tables 3.46, 3.48 and 3.50) several chromosomes are involved along with the ones involved in the chromosomal rearrangements for which PGD was performed. Autosomal monosomies such as monosomy 18 identified in A1.1 as well as monosomies 10, 13, 16, and 20 as seen in H11.2 are associated with early fetal lethality. Sandalinas and co-workers, cultured embryos that had been found to be aneuploid by PGD, until the blastocyst stage, thus revealing which ones arrested during preimplantation development. Almost all the embryos carrying complete monosomies were arrested prior to blastocyst stage (Sandalinas et al., 2001). However, these cells (A1.1 and H11.2) exhibited a wide array of chromosome abnormalities, suggesting that chromosomes had been segregated in a random, chaotic manner (Wells and Delhanty, 2000). Extensive partial loss and gain of test DNA as seen in these three instances can be extremely difficult to interpret and the precise nature of the aneuploidy cannot always be defined (Voullaire et al., 2000).

Some regions of the karyotype including the telomeric, centromeric, and heterochromatic regions show variation in the profile and these regions should be excluded for CGH analysis (Kallionemi et al., 1994). Moreover, certain chromosomes (1p, 17, 19, 22) were prone to show frequent enhancement of the test signal, and these regions have also been suggested to be excluded from the analysis (Malmgren et al., 2002). Based on the results of this study it was concluded that PGD results for case H [46,XY,t(1;18)(p32;q23)] could not be entirely confirmed by CGH due to the distal breakpoints on both chromosomes involved in the translocation, as well as the involvement of the short arm of chromosome 1 reported in literature as presenting artefacts. The frequent enhancement of the test signal for the short arm of chromosome 1 was present in seven (H2.1, H2.2, H3.1, H3.2, H7.1, H7.2, H11.1) out of the eleven blastomeres examined by CGH. This partial trisomy as detected by CGH was partially confirmed for six of the blastomeres (H2.1, H2.2, H3.1, H3.2, H7.1, H7.2), by FISH analysis on the respective embryos. For all these embryos FISH also detected partial monosomy of the translocated segment of chromosome 18. However,
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this was not picked up by CGH presumably due to its size. As the short arm of chromosome 1 is prone to artefacts it is very difficult to determine with certainty whether this partial trisomy is an artefact or indeed reflects the chromosomal constitution of the blastomeres. However, as it is also confirmed by FISH results, the argument that the partial trisomy of the short arm of chromosome 1 detected by CGH is indeed authentic is strengthened. In the case of embryo H11 the CGH and FISH results contradict each other as FISH detects monosomy rather than trisomy of the region (1p31→pter). In this case either the enhancement of the test signal is an artefact as reported in literature, or it is genuine and justified based on the extensive chaotic chromosome segregation observed in this embryo. Malmgren et al., (2002) also showed the presence of different distributions of chromosomes involved in the translocation within an embryo as a result of mitotic malsegregation. Similarly for embryos 8 and 10 from patient A [46,XX,t(5;11)(q34;q25)], resulting from an adjacent-1 segregation pattern according to FISH analysis, the finding of normal cells following CGH suggests that imbalances of distal segments of 5q and 1q are not detected.

The minimum size of an imbalance that can be detectable by CGH is likely to be different for gains or losses (Malmgren et al., 2002), as deletions are harder to identify than duplications of the same size. In fact the only time when CGH detected deletions in this series was in the three blastomeres (A1, F6 and H11.1) that presented with extensive aneuploidy involving a variety of chromosomes. The fact that CGH detects deletions with greater difficulty than duplications was widely seen in case G [46,XX,t(8;12)(q11.2;q12)]. Although this was a so called: “whole arm translocation” meaning that the size of the imbalances should be well within the limits of CGH resolution, however abnormalities detected in blastomeres biopsied from these embryos were limited to partial duplications involving the short arms of the chromosomes involved in the translocation. No partial deletion was suggested by CGH results. The inability of CGH to identify deletions was clearly demonstrated in embryo G6 where FISH detected only deletions while both cells subjected to CGH were classified as normal in an otherwise aneuploid/mosaic embryo (according to FISH). Embryos G2, G4, G7 and G10 presented with a selection of cells as balanced and classification of the respective blastomeres biopsied by CGH as normal was reasonable (Table 3.49). The single cell biopsied from embryo G3 and both blastomeres from embryo G15 that were described as normal based on CGH, while
being classified by FISH as fully chaotic could be explained on the basis of selecting the best cells morphologically for CGH analysis, and therefore it is not unexpected for these cells to be chromosomally normal. However, another explanation that was considered was the inability of CGH to detect possible monosomies in these cells. In the case of cells G3.2, G5.1, G9.1, and G13.1, the partial trisomy detected by CGH is confirmed by FISH results (Table 3.49). However, in all these cases some of the cells analysed by FISH exhibiting the respective partial trisomy on either of the chromosomes also show partial monosomy of the other chromosome. The fact that CGH detects partial trisomies of either of the chromosomes involved in the translocation failing to show partial monosomy of the other chromosome (as seen in FISH analysis in some of the cells) leads to the conclusion that either CGH fails to detect partial monosomies or presents evidence of chromosome breakage. Chromosome breakage evidence, has also been presented in case G from FISH results seen in embryos G3, G5, G9, and G15 (see section 4.4). Chromosome breakage has been reported in karyotyping studies (Clouston et al., 1997), while Wells and Delhanty, (2000) and Voullaire and co-workers (2000) presented data supporting chromosome breakage in their CGH study. It is proposed that chromosomal fragile sites exist and are prone to breakage as reported in de-novo chromosome rearrangements (Warburton, 1991). Martin et al, (1990), suggested fragile sites to be induced by depletion of certain nutrients from the culture medium. However, in case G [46,XX,t(8;12)(q11.2;q12)], FISH results on embryo G13 suggest that it is failure of CGH to detect partial monosomies rather than chromosome breakage that results in CGH analysis on blastomeres failing to detect partial monosomies. This was implied as embryo G13 was classified as uniformly aneuploid by FISH, with all 8 cells analysed presenting with partial trisomy of the short arm of chromosome 12 (as confirmed by CGH) and partial monosomy of the translocated segment of chromosome 8. Considering the size of the translocated and centric fragments of this translocation it is surprising that CGH failed to detect any deletions in this whole arm translocation, adding to the procedure’s inaccuracy.

In summary, this study shows that CGH carried out on single blastomeres detected trisomies of chromosome regions; 1p31→pter, 3, 5p21→q35, 6p12→p25, 6q24→q26, 7p11.2→p21, 7p21→q11.1, 7q31→q34, 8p21→q11.2, 12p11.1→p13, 12p12→q11, 12q12→q24.3, 16p13.2→p13.3, 16q23→q24, 17p11.2→p13, 18p11.1→p11.3, 18q12→q21 and trisomies of whole chromosomes 5, 12, 18, and 22.
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CGH also detected monosomies of chromosome regions; 1p31.2→qter, 17p12→p13, 17p11.2→q11.2 and of monosomies of whole chromosomes 10,13,16,18, and 20. However, in case A [46,XXt(5;11)(q34;q25)] due to the distal nature of the breakpoints in both chromosomes, CGH failed to detect monosomies or trisomies involving the either of the translocated segments 5q34→qter and 11q25→qter. In case F [46,XX,ins(7)(p22q31.1q32)] , CGH failed to detect monosomy of the inserted region 7q31.1→q32. In case G [46,XX,t(8;12)(q11.2;q12)] CGH failed to detect monosomy of the translocated segment 12q12→qter and monosomy of the centric segment 8pter→q11, 8q11.1→qter. Finally in case H [46,XY,t(1;18)(p32;q23)] CGH failed to detect monosomy of the translocated segment 1p32→pter as well as monosomy and trisomy of the translocated segment 18q23→qter.

Diagnostic efficiency of CGH

This study intended to assess the diagnostic efficiency of CGH. At this point it is important to separate the potential use of CGH in PGD for chromosomal rearrangements and its clinical application for PGD-AS. CGH of blastomeres has been applied clinically for the determination of aneuploidy and resulted in the birth of a healthy baby (Wilton et al., 2001). Wilton et al., (2003) demonstrated that CGH can be used to obtain a complete karyotypic diagnosis of embryos from patients with recurrent implantation failure, improving management and treatment of many IVF patients by increasing the chances of transferring viable embryos more likely to result in a successful pregnancy. The same group argued that a higher implantation rate could be achieved when employing CGH instead of FISH, as the FISH diagnosis was shown to miss many chromosome abnormalities that would be lethal at early stages of development and could explain recurrent implantation failure in these patients. CGH has not been yet used for diagnostic purposes for detecting chromosomal rearrangement in a PGD setting. Malmgren and colleagues (2002) employed CGH on human embryos from patients with balanced structural chromosomal aberrations and showed that CGH verified the PGD results in most cases (except from those involving distal breakpoints) and showed a high degree of numerical aberrations, including monosomies, trisomies and duplications or deletions of parts of chromosomes that would not have been detected by FISH analysis.

The present study shows that CGH failed to verify the PGD results in many cases, which is attributed to the weaknesses of the technique and the subsequent
inaccuracy of the procedure. The data from this study supports that CGH is not an alternative to FISH, at least for PGD of many chromosomal rearrangements. One reason for this is the sensitivity of CGH, which has been estimated at around 3 Mb. (Protocols that can detect aneuploidy not exceeding 3 Mb in a single cell embryo biopsy have been described.) Considering that the reciprocal form of translocations are the most common structural abnormality in chromosomes seen in live births, accurate determination of this anomaly represents a significant issue (Hill, 2003). CGH presents with numerous disadvantages that would dictate its selective application on the diagnosis of chromosomal rearrangements excluding those involving distal breakpoints, or any of the chromosomes reported in literature as presenting artefacts. Application of CGH for the diagnosis of such cases could potentially lead to misdiagnosis due to the limited resolution of CGH in regions of the karyotype that are subjected to CGH artefacts leading to unreliable ratio changes. The fact that the minimum size of an imbalance that can be detectable is likely to be different in gains and losses, with deletions being more difficult to detect, as well as the ability of different chromosomal regions to hybridise better than others, highlight the inappropriate nature of CGH for diagnostic purposes.

Apart from the inability of CGH to provide any information regarding the ploidy status of an embryo, the major disadvantage of the technique holding it back from extensive clinical application is the fact that the time required for its performance is impossible to fit into the PGD situation with day-four transfer. An approach to fit the time frame required for CGH in a clinical setting has been attempted by Wilton and colleagues (2001), with embryos being cryopreserved until results are available. However, even with a high survival rate it has been estimated that cryopreservation causes a 30% loss of embryo viability (Edgar et al., 2000). This requirement for cryopreservation can be viewed as a limitation in the use of CGH for clinical PGD, as any improvement in implantation rates gained after embryo biopsy and CGH screening maybe counterbalanced to some extent by a reduction in the number of viable embryos following cryopreservation. A modified cryopreservation protocol designed to optimise protection of cell membranes during freezing and thawing demonstrated an improved embryo survival, similar to that for intact embryos using routine techniques (Jericho et al., 2003). Munné and Wells argue that for the patients who only have a handful of embryos to begin with, losing 46% or more to cryopreservation is a major concern, and it is likely to significantly offset any
improvement in pregnancy rates achieved by screening a larger number of chromosomes (Munné and Wells, 2003). In a letter by Wilton, as a reply to these concerns, it is stated that the negative impact of cryopreservation is overstated in the concerns raised by Hill and Munné and Wells, as the survival rate of cryopreserved embryos has been shown to be 75% thus identical to that of intact embryos (Jericho, et al., 2003). In addition, the majority of embryos in the study conducted by Wilton and co-workers would have been cryopreserved whether or not CGH was performed. Any detrimental effect of cryopreservation would only apply to the one or two embryos per patient that would have been transferred fresh if there had been no CGH analysis (Wilton, 2003b).

Performing a blastocyst transfer might be an option addressing the problem arising by the need for cryopreservation. Shortening of the hybridisation time or the use of DNA microarrays instead of metaphase chromosomes as the template may enable full karyotypic analysis of a single cell in 2-3 days which could be compatible with extended in vitro culture and transfer of embryos on day-five or six (Wilton et al., 2003). However, a day-five or day-six transfer would mean that several embryos would not be available for transfer, as only a small percentage of them would reach the blastocyst stage. In addition, as mosaic and chaotic embryos have been reported to reach the blastocyst stage (Ruangvutilert et al., 2000b) the results obtained from the biopsied blastomeres from day-three may not be representative of the embryo on day-five of development, especially as mitotic segregation may be disturbed by the environmental conditions of further culture of the embryos (Munné et al., 1997; Malmgren et al., 2002) affecting mosaicism levels.

Another strategy to address the problem of the time required for CGH results to be obtained in the clinical setting of PGD-AS, is applying an accelerated CGH protocol to polar bodies that are available for biopsy on the day of fertilisation (Wells et al., 2002). The polar body biopsy approach fits well time-wise in a clinical setting, without necessitating cryopreservation of the embryos and therefore is advantageous with respect to avoiding invasive procedures on the embryo. However, there are limitations to the technique, the main one being that any imbalances arising in male meiosis cannot be detected, however, it should be noted that these are infrequent compared to those of maternal origin (Wells and Levy, 2003). Another disadvantage of the polar body biopsy technique is that any mitotic errors, or effects of environmental factors on the embryo’s chromosomal constitution will not be detected
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(Wilton, 2003). Additionally, in a review by Verlinsky and Kuliev (2003) it is stated that limiting the CGH analysis to the first polar body may have lead to a misdiagnosis due to undetected meiosis II errors, which could have been identified only through sequential first and second polar body analyses. Both methodologies of cleavage stage biopsy and subsequent cryopreservation, as well as polar body biopsy have been clinically applied (Wilton et al., 2001; Wells et al., 2002), the most efficacious method still remains to be determined (Wells and Levy, 2003).

Verlinsky and Kuliev in a recent review (2003) suggest that the reported CGH data are not sufficient to reach a conclusion on the aneuploidy detection rate. Despite the expected higher detection rates by CGH, it was in the same range of aneuploidy rates, detected earlier in thousands of oocytes and preimplantation embryos tested by FISH in the practise of PGD (Munné et al., 2002, Kuliev et al., 2002, Gianaroli et al., 1999). However, there was a difference in the type of aneuploidy, mainly attributable to a high frequency of complex errors (involving the errors of different chromosomes) in oocytes and mosaicism in preimplantation embryos. Therefore, it cannot be excluded that a complete karyotype by CGH or other techniques may lead to an increased detection rate of embryos with complex errors, rather than significantly increasing the overall proportion of embryos with aneuploidies (Verlinsky and Kuliev, 2003). At this time FISH provides the same level of diagnostic accuracy in delineating embryos deemed safe for transfer (Hill, 2003). The method of FISH is rapid and less labour intensive than CGH. Second, chaotic mosaics, which account for approximately 30% of preimplantation embryos, display aneuploidy affecting multiple chromosomes per cell, in this case assessing a handful of chromosomes by FISH should be enough to classify them as abnormal (Munne and Wells, 2003). However, this is contradicted by the data presented by Wilton, demonstrating that at least 25% of embryos that were found to be abnormal by CGH would have been misdiagnosed as normal by the most extensive FISH screen currently available (Wilton, 2003b).

The only conclusion that can be derived from this extremely limited data may be that the standard CGH procedure seems impractical for clinical application, requiring further improvement of the technique to overcome its limitations (Verlinsky and Kuliev, 2003). The fact that only a small sample of embryos have been subjected to CGH studies, adds extra value to the current project as more information is provided aiding to classify the importance and potential of CGH as a diagnostic tool.
**Extent and significance of mosaicism**

One of the aims of this overall project was to investigate the extent of mosaicism in untransferred embryos from couples undergoing PGD for a chromosomal rearrangement. Fifty percent of the embryos analysed in this series were classified by CGH and FISH analysis as chaotic (14 embryos) and 14% were categorised as mosaics (4 embryos). The overall combined frequency of mosaicism and chaotic mosaicism in this study was estimated to be 64% (18 out of the 28 embryos for which results were obtained), and surprisingly half of these mosaic/chaotic embryos presented with a selection of cells as balanced. The remaining 46% of the embryos were either uniformly normal or balanced, or uniformly aneuploid. The incidence of normal blastomeres analysed by CGH is unpredictably high.

The finding of uniformly normal embryos (F3 and G10) in this study is of high significance as it is likely that they have a superior potential for post-implantation development. This is substantially proven as in fact both patients F and G achieved pregnancies that resulted in two healthy deliveries in the first cycle of treatment, following PGD. More than one third of the embryos analysed in this project (32%) presented with a selection of cells as balanced based on the FISH results. Sixty-six percent of these were classified as balanced/chaotic (A2, G2, G4, G7, H8 and H10) and 34% were classified as balanced/aneuploid (A8, A11 and H7). With the exception of blastomeres H7.1 and H7.2, all the other blastomeres biopsied from the embryos carrying a balanced cell line were classified as normal according to the CGH analysis. Therefore, the cells biopsied from the vast majority of balanced/mosaic or balanced/chaotic embryos were of normal chromosomal complement.

These observations lead to the suggestion that possibly in some cases, as the cells biopsied for CGH were selected on morphological criteria, their good morphology reflected their normal chromosomal constitution. Most studies support that as a whole normal embryos could not be distinguished from the aneuploid, mosaic, or chaotic ones based on morphological criteria (Wells and Delhanty, 2000; Voullaire *et al.*, 2000) and morphology does not necessarily dictate a balanced chromosomal complement (Malmgren *et al.*, 2002). However, complete chromosome analysis using CGH has recently demonstrated that twice as many grade 1 embryos...
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were chromosomally normal having a lower frequency of chromosome abnormality compared to grade 3 (Wilton et al., 2003a), while poor quality, arrested or slow embryos have a high frequency of aneuploidy (Munné et al., 1994b; Jericho et al., 2003). Based on that data, it could be extrapolated that similarly to whole embryo morphology, morphologically superior cells could have a greater chance of having a chromosomally normal complement when compared to blastomeres of inferior morphology, which could partly explain the high number of chromosomally normal blastomeres analysed by CGH.

Contamination with normal DNA could also explain the high incidence of blastomeres found to be normal by CGH analysis. Although, the frequency of amplification in the negative controls, tested in the preliminary study for this project (see section 3.11), was found to be only >5% accounting for contamination with normal DNA, it should still be considered as an unlikely but nonetheless potential explanation.

The proportion of embryos that are aneuploid declines throughout pregnancy, presumably due to strong selection against those with unbalanced chromosome constitutions. At three weeks 9% of embryos are aneuploid, this falls to 5% at 10 weeks and to only 0.5% at full term (Lubs and Ruddle, 1970; Boue et al., 1985). During the first few days though following conception it is clear that much higher rates of abnormality are tolerated and there is evidence that selection against abnormal cells only begins at the morula-blastocyst transition (Evsikov and Verlinsky, 1998, Sandalinas et al., 2001). The high incidence of mosaic and chaotic embryos in this series raises the question of whether these embryos carrying a selection of balanced cells can continue to divide and differentiate in order to give rise to a vital embryo. Many different scenarios have been speculated with respect to the population of chromosomally normal cells and their role in the embryo’s survival and viability. Possibly, the abnormal cells could be directed towards the trophectoderm lineage (James and West, 1994) where they will contribute to the extra-embryonic membranes where chromosome imbalance can be tolerated somewhat better (Wolstenholme, 1996). However, the existing data from human embryos suggests that there is no difference in the frequencies of euploid and aneuploid cells between the inner cell mass and trophectoderm (Evsikov and Verlinsky 1998). Another theory, is programmed cell death (apoptosis) of abnormal blastomeres and selective survival (growth advantage) of normal cells leading to a core of normal cells and subsequent
"embryo rescue". Even embryos with non-mosaic aneuploidy due to a meiotic error can potentially give rise to a normal fetus (e.g. loss of one copy of a trisomic chromosome or doubling of a monosomic chromosome). The euploid cells could then outgrow the original aneuploid cells (Wells and Delhanty, 2000). Finally a third scenario is that the aneuploid/mosaic embryo fails to develop beyond a point and is eliminated prior to blastocyst formation or at a later developmental stage (Malmgren et al., 2002), however, natural selection to the blastocyst stage is not effective for all chromosomal abnormalities as some still persist and extensive mosaicism has been detected in blastocysts (Sandalinas et al., 2001; Ruangvutilert et al., 2002b).

Understanding the importance of the contribution of normal cells in the fate of a mosaic/chaotic embryo, and accepting the speculation that blastomeres of good morphology that are biopsied are more likely to have a normal chromosomal complement, the question of the subsequent fate of the embryo is raised; "How could any of the above mentioned scenarios regarding embryo rescue function, if the normal/balanced cell(s) that could allow the embryo to develop further is removed? Following removal of 1-2 normal blastomeres for the purpose of diagnosis or research, the effect on a mosaic embryo is likely to be detrimental, as only abnormal cells may remain thus compromising viability and survival. Therefore, as biopsy could be most damaging to the embryo it should be avoided where possible. Although this is not feasible for PGD for chromosome abnormalities or single gene defects where biopsy is performed as means to diagnosing a healthy embryo, however, it should be reconsidered for the purpose of selecting the best embryo for transfer to improve implantation rates. In contrast, existing data suggests that embryo biopsy and chromosome screening provide a statistically significant increase in implantation, decrease in spontaneous abortion and decrease in aneuploid pregnancy (Gianaroli et al., 1999; Munne and Wells 2002, Wilton et al., 2002). Multiple studies by these groups support that implantation rates are almost doubled for women over 37, and their data suggests that whatever the detrimental effects of embryo biopsy, it is more than compensated for by the benefits of identifying the most viable embryos. The debate regarding the benefits of biopsy for the purpose of improving implantation rates is ongoing. A recent study, suggests that non-invasive amino acid profiling has the potential to select developmentally competent single embryos for transfer, thereby increasing the success rates and eliminating multiple births in IVF (see section 1.1.4.4) (Houghton et al., 2002). This technique employs liquid chromatography to
measure the turnover (depletion/appearance) of a physiological mixture of 18 amino acids. Further non-invasive techniques to predict developmental capacity should be explored.

To overcome the resolution limitation of CGH, an innovative strategy, called matrix or microarray-CGH (M-CGH), has been devised. In M-CGH, chromosomal targets are being replaced by arrays consisting of well-defined genomic clones (Typically BCA, PAC, or YAC clones), which are spotted onto a glass microscope slide using automated robotic devices. The array may consist of thousands of clones, each corresponding to a small region of the genome. This allows for genomic imbalances to be elucidated with much higher resolution (100-200kb) (Pinkel *et al*., 1998; Bruder *et al*., 2001; Wessendorf *et al*., 2002). With the exception of hybridisation of differentially labelled specimen (test) and reference DNA onto slides with DNA arrays instead of metaphase spreads, the major steps in CGH are also featured in M-CGH (Wells and Levy, 2003). Laser scanning replaces fluorescent microscopy and results are summed up as a separate logarithmic signal ratio values for each of the tested clones on a given array. M-CGH has recently been proven successful for the detection of whole chromosome aneuploidy and microdeletions (Pinkel *et al*., 1998; Bruder *et al*., 2001; Albertson *et al*., 2000; Cai *et al*., 2002). As a more automated technique, if M-CGH can be successfully adapted to the analysis of single cells it will offer the possibility of wide application for identification of the most viable embryos (Wells and Levy, 2003).

### 4.6 Conclusions

One of the main objectives of this study was to develop reliable FISH-based protocols to be applied to biopsied blastomeres from day-three cleavage embryos for the purpose of diagnosing chromosomally normal embryos generated by patients carrying a chromosomal rearrangement. DNA probes and strategies were developed and clinically applied in PGD treatment for nine couples. The poor reproductive history of the majority of these patients underlined the fact that PGD was the only option available to help them in their quest for a healthy family. This study showed the feasibility of the clinical application of PGD for chromosome abnormalities, successfully resulting in three normal live births (and one biochemical pregnancy, from eight embryo transfers), a pregnancy rate of 37.5% per ET. Being, the first live births to couples carrying a chromosomal rearrangement reported in our group, this...
fruitful outcome reflects the impact of the evolution of technology on the practicability of PGD. The availability of commercial probes replacing the laborious and time-consuming laboratory-prepared probes had a constructive effect on our PGD programme. A greater number of couples were offered treatment and fewer cases were discontinued or lost contact with the centre. The success of the PGD programme is a collective result of the efficient work performed by the Human Genetics Group, as well as the Assisted Conception Unit. As this is a team-effort it is important to recognise the important input of the IVF unit. Changes in protocols and culture media implemented during this study have contributed significantly in this gratifying outcome. It seems hopeful that with the combination of less-labour intensive commercial FISH probes and a good overall IVF pregnancy rate, this success will continue. One of the conclusions of this study is that multiple PGD cycles can also contribute to an improved PGD pregnancy rate. Multiple attempts can improve the chances of a healthy embryo implanting and lead to a clinical pregnancy (as seen for patient A). For this reason it is imperative that all prospective PGD patients for chromosomal abnormalities undergo counselling, emphasizing that several IVF cycles could be required to achieve the desired aim, especially in view of the fact that the vast majority of embryos may be chromosomally abnormal as a result of both abnormal meiotic segregation and post-zygotic mosaicism (Iwarsson et al., 1998a,b; 2000; Munné et al., 1998b; 2000; Pierce et al., 1998; Van Assche et al., 1999; Coonen et al., 2000; Escudero et al., 2000a; 2001; Evsikov et al., 2000; Scriven et al., 2000; Vandervorst et al., 2000; Fridstrom et al., 2001). This high percentage of chromosomal abnormality encountered in embryos generated by PGD patients is reflected in the slightly poorer pregnancy rate for this particular patient group than for other genetic disorders (ESHRE PGD Consortium, 2000).

The significance of this research project is not measured only by its clinical success. Even in cases where no suitable embryos for transfer are available, the information on meiotic segregation obtained by the analysis can help the patients make an informed decision on their options concerning future assisted reproduction. Information on meiotic outcomes and segregation patterns is of great consequence for PGD patients. Sperm studies on male chromosomal rearrangement carriers are possible outside a clinical setting for the purpose of obtaining formation on meiotic behaviour. However, PGD is the only approach for investigating meiotic segregation
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patterns of female translocation carriers (Conn et al., 1995; 1998; 1999; Cozzi et al., 1999), adding extra value to the contribution of this project to embryo research.

The collective results of embryo analysis following PGD for these nine couples, showed that only 29% were normal for the chromosomes tested while 71% were abnormal with high levels of mosaicism or chaotic division on a background of aneuploidy. Our group has provided evidence that some couples are particularly prone to produce chaotic embryos (Delhanty et al., 1997). Several groups have since reported that this tendency is frequent in carriers of chromosomal rearrangements that have been referred for PGD because of poor reproductive histories (Conn et al., 1998; Munné et al., 2000; Iwarsson et al., 2000; Malmgren et al., 2002). Comparing the percentage of abnormal embryos (23%) seen in preliminary PGD study carried out on surplus normally developing embryos, to the percentage of chromosomally abnormal embryos (71%) encountered in this cohort of PGD patients, it is reasonable to propose that these couples referred for PGD demonstrate a combination of high frequency of both meiotic and post zygotic errors, which would explain their poor reproductive histories.

The detailed follow-up analysis performed on the embryos subjected to PGD for this study, revealed a cohort of embryos presenting with some balanced cells. This considerable proportion of aneuploid/balanced mosaic embryos could without doubt lead to a possible misdiagnosis in the case of a single cell biopsy. One of the most important conclusions from the clinical application of PGD in this study was the advisability of biopsying two cells instead of one cell where possible, reducing the risk of misdiagnosis in cases of aneuploid/balanced mosaics. The three live births achieved for the nine couples treated in this series, despite the poor history in almost all cases, is further proof that a policy of biopsying two cells from embryos consisting of 6 or more cells and a single cell from 4 or 5 cell embryos is compatible with a positive outcome.

The constant evolution of molecular, cytogenetic and combinatorial techniques aims to facilitate and enrich PGD. Novel techniques such as interphase conversion allowing the entire karyotype to be screened in one step have been developed in the recent years and have found clinical application. Their principle involves fusion of polar bodies or blastomeres with enucleated human or bovine oocytes to induce mitosis (Verlinsky and Evsikov, 1999; Willadsen et al., 1999). Interphase conversion methods for inducing metaphase in biopsied blastomeres have

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been successfully applied in a number of translocation cases to date with subsequent
analysis by standard chromosome painting (Verlinsky and Evsikov, 1999a; Evsikov et al., 2000) or SKY FISH (Willadsen et al., 1999). For both these methods however
there remains the difficulty inherent in working with single metaphase spreads of
limiting artefactual chromosome loss. Although such novel methods can engender
great enthusiasm, it is important for all factors to be assessed before clinical
application is attempted, especially in cases where ethical concerns are being raised.
Currently, neither of the techniques is being clinically applied. Their usefulness in
PGD has yet to be determined, as more data needs to be collected.

CGH now seems to be the method of choice for enumerating the whole
chromosome set in blastomeres (Voullaire et al., 2000; Wells et al., 2000). A very
important part of this thesis was dedicated to assessing the feasibility of CGH as a
diagnostic tool. Following numerous laborious modifications and time-consuming
attempts at several protocols, types of hybridisation, and labelling, CGH was
successfully applied to single blastomeres from day-four embryos generated by
couples undergoing PGD for chromosomal rearrangements. The contribution of this
study to research is very important, as only one group has applied CGH on
blastomeres from embryos from PGD translocation carriers. This study concluded that
CGH is a temperamental, complicated method with considerable limitations and
requiring expertise in various molecular and cytogenetic aspects. Diagnostic methods
should ideally be simple, robust and straightforward in their application and CGH
does not fall into this category. Theoretically it is feasible, but practically it is not,
especially in cases where specific rearrangements need to be diagnosed. In the case of
screening for aneuploidy, the setting is slightly different as the technique attempts to
screen for random chromosomal abnormalities in order to improve pregnancy rates.
However, that is quite different from PGD for a specific chromosomal abnormality
where the technique is employed to diagnose a chromosomally normal embryo,
avoiding an unbalanced pregnancy. This study confirmed that CGH should not be
employed to diagnose translocations involving distal breakpoints (Malmgren et al.,
2002), or small structural aberrations, or chromosomes reported in literature to present
artefacts (Kallionemi et al., 1994), due to its limited resolution.

Perhaps the most striking result of this study was the fact that the majority of
the blastomeres analysed by CGH were found to be normal. The lack of sensitivity of
the technique in detecting small aberrations could lead to a false classification of cells
as normal. However, as the finding of normal cells was extensive and present in all patients irrespectively of the rearrangement involved, it was mainly attributed to the fact that the cells biopsied were selected based on morphological criteria, and were therefore of a normal chromosomal constitution, but in most cases derived from a mosaic embryo. This finding is very important, especially as this is the only CGH study performed on biopsied cells rather than whole embryos. On the whole, the CGH study validated the FISH analysis, as in almost all embryos presenting with some apparently balanced cells (as seen by FISH results) this was confirmed by CGH, supporting the suggestion that the good quality of cells selected for CGH was reflected in their normal chromosomal constitution.

DNA microarray or microarray CGH (M-CGH) analysis is a rapid evolving method of molecular analysis that could find several potential uses in PGD (Maughan et al., 2001; Clarke et al., 2001). Following in the steps of CGH, microarrays could replace the metaphase spreads that are used now to assess chromosomal imbalance in CGH. It is likely that their versatility will make them an attractive option for PGD. However, at present, technical imitations such as paucity of material available for hybridisation, sensitivity and reliability of the data, and the cost of producing appropriate microarrays are likely to hinder their application in PGD for some time (Braude et al., 2002).

Rapid advances in molecular genetics will stimulate further the use of PGD. It is likely that a combination of approaches will enable the molecular examination of the entire chromosome complement, at the same time as testing for common genetic diseases, such as cystic fibrosis (Wells et al., 1999). The challenge will be to regulate the use of the constantly evolving PGD technology for medical purposes and to limit or prevent its use for eugenic selection.
CHAPTER 5

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Appendix

APPENDIX

1A Cell Culture Media.

All media were prepared with de-ionised double distilled water, immediately autoclaved at 15psi 121°C for 30 minutes and stored at room temperature (15-25°C) under sterile conditions.

IA.1 Iscove's/10%FCS/GPS/PHA - for peripheral lymphocyte culture.
Sterile Iscoves modified Dulbeccos medium; 10% v/v heat-inactivated fetal calf serum; 10μl/ml phytohaemagglutinin (PHA); 20μl/ml GPS. [GPS: 0.2M L-Glutamine, 300mg/ml Penicillin, 500mg/ml Streptomycin monosulphate; filter-sterilised and stored at -20°C.]

IA.2 2xTY medium - for bacterial culture.
16g/l Bacto tryptone; 10g/l Bacto yeast extract; 5g/l NaCl; 1g/l glucose.

IA.3 Glycerol stocks.
250μl of bacterial or yeast overnight culture; 650μl 100% sterile glycerol in 1ml cryo-tubes. Stored at -20°C overnight and -70°C thereafter.

IA.4 YPD.
10gr/l yeast extract, 20gr/l peptone. Autoclave for 30 minutes, when the temperature is around 50°C add 50ml/l glucose (40% sterilised by filtration)

IA.5 Medium for agar plates/stabs.
1% w/v Bacto-agar added to the culture medium (SD, LB or 2xTY) and autoclaved. For agar plates/stabs this was heated until liquification, cooled before addition of required antibiotic supplement and poured immediately into sterile petri dishes or bijou’s and left to dry under aseptic conditions.

IA.6 SD medium - for yeast culture.
7g/l Bacto yeast nitrogen base (without amino acids); 20g/l glucose; 55mg/l adenine and tyrosine. After autoclaving, 1% v/v filter-sterilised casamino acid solution added.

2A Solutions and Buffers.

All solutions and buffers were prepared with de-ionised distilled water, autoclaved at 15psi 121°C for 30 minutes and stored at room temperature unless otherwise stated.

2A.1 General solutions.

2A.1.1 PBS: 10mM phosphate buffer; 2.7mM KCl; 137mM NaCl; pH7.4.

2A.1.2 20xSSC: 0.15M NaCl; 15mM Sodium citrate; pH7.

2A.1.3 10xTBE: 90mM Tris-HCl pH8; 90mM Boric acid; 2mM EDTA.

2A.1.4 TE: 10mM Tris-HCl, pH8; 0.1mM EDTA.

2A.1.5 10xTN: 0.1M Tris; 1.5M NaCl; pH8.
Appendix

2A.1.6 Loading buffer for agarose gel electrophoresis: 40$\%$ (W/V) sucrose, 4mM bromophenol blue, 4mM xylene cyanol.

2A.2 Plasmid/Cosmid/Yeast DNA miniprep/maxiprep solutions. (Stored at 4°C).

2A.2.1 GTE: Glucose 50mM, Tris 25mM pH 8, EDTA 10mM.

2A.2.2 Denaturation solution: 0.2N NaOH, 1% SDS

2A.2.3 Resuspension solution: 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 μg/ml, RNase A 0.2M NaOH; 1% SDS.

2A.2.4 Neutralisation solution: 1.32 M potassium acetate pH 4.8

2A.2.5 Lyticase solution: 1 M NaH$_2$PO$_4$; 25U/μl desiccated lyticase.

2A.3 Alu PCR reagents. (Stored at -20°C).

2A.3.1 10x PCR buffer: (HT Biotechnology Ltd): 0.1M Tris-HCl, pH 9; 0.5M KCl; 15mM MgCl$_2$; 1% Triton X-100; 0.1% w/v gelatin.

2A.3.2 Alu oligonucleotide primers:

CL1: TCC CAA AGT GCT GGG ATT ACA
CL2: CTG CAC TCC AGC CTG GG (Oswell) (Lengauer et al., 1992).

2A.4 Nick translation reagents. (Stored at -20°C).

2A.4.1 dNTP mix: 0.2mM each dATP, dCTP, dGTP; 0.1mM dTTP; 0.1mM label-dUTP.

2A.4.2 10x nick translation buffer: 0.5M Tris-HCl, pH 7.5; 0.1M MgSO$_4$; 1M DTT.

2A.4.3 Nick translation enzyme: DNA polymerase I, DNase I in 50% glycerol, 50 mM Tris-HCl, pH 7.2, 10 mM MgSO$_4$, 0.1 DTT, 0.5 mg/ml nuclease free BSA.

2A.5 FISH solutions.

2A.5.1 Pepsin buffer: 0.01N HCl; 0.1mg/ml pepsin.

2A.5.2 Paraformaldehyde buffer: 1xPBS; 1% paraformaldehyde.

2A.5.3 Hybridisation buffer - Locus-specific probes: 50% deionised formamide; 10% w/v dextran sulphate; 2xSSC pH 8. Stored at -20°C.

2A.5.4 Hybridisation buffer - α-satellite probes: 60% deionised formamide; 10% w/v dextran sulphate; 2xSSC pH 8. Stored at -20°C.

2A.5.5 SSCT: 4xSSC; 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate).

2A.5.6 Antifade medium: 1.25μg/ml 4',6-diamidino-2-phenylindole (DAPI) in Vectorshield mounting medium (Vector). Stored at 4°C, protected from light.

2A.6 DNA extraction solutions.

All solutions were prepared with de-ionised double distilled water, immediately autoclaved at 15lbs psi 121°C for 30 minutes and stored at room temperature (15-25°C) under sterile conditions.
Appendix

2A.6.1 Lysis buffer I: In 100ml 1.21g Tris, 0.19g/100ml EDTA, 0.2g SDS, 1.17g NaCl, 10mg/ml of proteinase K added after autoclaving.

2A.6.2 TKM1 (low concentration salt buffer): 10mM Tris-HCl, pH 8.0, 10mM KCl, 10mM MgCl₂, 2mM EDTA.

2A.6.3 TKM2 (high concentration salt buffer): 10mM Tris-HCl, pH 8.0, 10mM KCl, 10mM MgCl₂, 2mM EDTA, 0.4M NaCl.

2A.6.4 Lysis buffer II: 2μl of 125μg/ml Proteinase K (PK), 1μl of 17mM sodium dodecyl sulphate (SDS).

2A.7 CGH solutions.

2A.7.1 Proteinase K buffer (10xPKB): 20mM calcium chloride, 200mM Tris-HCl. PH7.5.

2A.7.2 Magnesium chloride buffer: 1xPBS; 1% MgCl₂.

2A.7.3 Paraformaldehyde MgCl₂ buffer: 1xPBS; 1% paraformaldehyde; 1% MgCl₂.

2A.7.4 Denaturation solution: 70% deionised formamide/2XSSC.

2A.7.5 TNT: 1xTN; 0.05% Tween 20.

2A.7.6 10xreaction buffer mix: 500mM Tris-HCl-pH7.5-, 50mM MgCl₂, 100 μg/ml BSA, and β-mercaptoethanol.

2A.7.7 A4 Nucleotide mixture: 0.2mM dNTP, dATP, dCTP, dGTP, 0.03mM dTTP, 500mM Tris pH 7.5, 50mM MgCl₂, 100mM β-mercaptoethanol, 100 μg.ml BSA).