Intracellular Trafficking, and Function of Receptor Tyrosine Kinases in Mammary Gland Development

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To my parents
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Abstract

Receptor tyrosine kinases (RTKs) have been implicated in the normal development and tumourigenesis of the mammary gland. This thesis investigates the roles of two RTKs, the fibroblast growth factor receptor 2IIIb (FgfR-2IIIb) and the Met receptor, in the development of the mammary gland. In addition, the signals that control the trafficking of the ErbB-2 RTK within polarised epithelial cells were examined.

Different experimental approaches were utilised to specifically abrogate receptor tyrosine kinase signalling within the mammary gland. Mice containing a genetic alteration of FgfR-2IIIb were used to investigate the physiological role of the receptor in the development of the embryonic and postnatal mammary glands. FgfR-2IIIb signalling was found to be crucial for the maintenance of embryonic mammary placodes. However, FgfR-2IIIb dependent and independent pathways were found to be necessary for the control of mammary placode induction. No role was identified for the receptor in postnatal mammary development. The function of the Met receptor in mammary gland development was also addressed by generating transgenic mice expressing a dominant negative form of the receptor in the mammary epithelium. These mice displayed no obvious abnormalities in mammogenesis.

ErbB-2 plays key roles in normal mammary gland development and tumourigenesis. In the mammary epithelium, ErbB-2 is localised to the basolateral membrane domain where it is able to receive signals from the underlying stroma. The mechanisms regulating this asymmetric distribution were investigated using an in vitro model of epithelial cell polarity. A 10 amino acid signal located within the intracellular juxtamembrane region of ErbB-2 was identified, and shown to be necessary and sufficient for its basolateral delivery. Furthermore, a second targeting signal in ErbB-2, previously demonstrated to control ErbB-2 basolateral delivery, was compared to the juxtamembrane signal. It was demonstrated that this second signal contained a cryptic basolateral targeting motif, but did not control the basolateral targeting of ErbB-2.
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>mA</td>
<td>Milli Amperes</td>
</tr>
<tr>
<td>AP</td>
<td>Adaptor protein complex</td>
</tr>
<tr>
<td>Ap</td>
<td>Apical</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BL</td>
<td>Basolateral</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CIP</td>
<td>Calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>kD</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>DABCO</td>
<td>1,4-diazobicyclo-[2,2,2,]-ocracne</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E4</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Fgf(R)</td>
<td>Fibroblast growth factor (receptor)</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FRS</td>
<td>FGF receptor substrate</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulphate proteoglycan</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>k</td>
<td>Kilo</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>m</td>
<td>Metre</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumour virus</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-morpholino]propane-sulphonic acid</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>Neo</td>
<td>Neomycin</td>
</tr>
<tr>
<td>NRG</td>
<td>Neuregulin</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS-tween</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95, DLG-1, ZO-1</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene tetraphthalate</td>
</tr>
<tr>
<td>Pfu</td>
<td><em>Pyrococcus furiosis</em></td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PLC-γ</td>
<td>Phospholipase C-γ</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethyl-sulphonylfluorid</td>
</tr>
<tr>
<td>PP</td>
<td>Postpartum</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PRL(R)</td>
<td>Prolactin (receptor)</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone related peptide</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RNasin</td>
<td>Ribonuclease inhibitor</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology domain 2</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>TBS-tween</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end bud</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethyl rhodamine isocyanate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>WAP</td>
<td>Whey acidic protein</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-Bromo-4-chloro-3-indolyl β-D-galactoside</td>
</tr>
<tr>
<td>ZO</td>
<td>Zona occludens</td>
</tr>
</tbody>
</table>
Chapter 1 | Introduction

1.1 Mammary Gland Development

The mammary gland is a unique organ that defines the Class Mammalia. The sole physiological function of the gland is to synthesise and deliver nutrient rich milk to newborn offspring. Since its complete anatomical description in the middle of the nineteenth century, the mammary gland has attracted attention from a broad range of scientific disciplines. During the past 100 years in particular, there has been an explosion of effort to characterise the anatomical and morphological design of the gland, as well as the regulation of its development. Endocrinologists, physiologists and developmental biologists have made key contributions over this period, and coupled now with new techniques in molecular biology, the complex and intricate web of signalling pathways that regulate the development of the mammary gland are beginning to be uncovered. Notably, the development of ‘transgenic’ and ‘knock-out’ mouse technologies has been instrumental in this advance.

The introduction of the mammary gland is a relatively recent event in evolution, probably occurring some 200 million years ago. As a consequence its development shares fundamental processes with a variety of other organs and is therefore shaped predominantly by genetic pathways that appeared earlier in evolution. However, the mammary gland does have several unique characteristics. Firstly, the majority of development takes place in postnatal life. Secondly, development occurs in phases that are linked to the reproductive cycle, and finally, both pituitary and ovarian hormones exert profound effects on the development of the organ by inducing and interacting with local signalling molecules.

In addition to understanding mammary gland development per se, studies have utilised the mammary gland to understand the general principles of organogenesis and tumourigenesis. The existence of powerful experimental technologies, such as transplantation and organ culture, and the fact that development occurs predominantly in the adult, make the mammary gland ideally suited for this (reviewed in Daniel and Smith, 1999; Medina, 1996). The field has also been strongly driven by the issue of breast cancer. During its development the mammary gland displays many properties associated with breast cancer, and numerous factors essential for normal development are also implicated in the disease (see Wiseman and Werb, 2002). Understanding how these factors
contribute to mammary gland development therefore leads to a better comprehension of breast cancer. This knowledge is already being translated into novel therapies, and will undoubtedly continue to do so, leading to lower incidences of, and higher success rates in the treatment of breast cancer.

For reasons outlined above, the majority of our knowledge stems from research on the murine mammary gland, the development of which is here considered in more detail.

The mammary gland consists of two primary cellular compartments, the epithelium (or parenchyma) and the surrounding mesenchyme (or stroma), which are derived embryologically from the ectoderm and mesoderm respectively. Development of the mammary gland can therefore be thought of as a remodelling of these two cellular compartments, and can be divided into two defined stages – embryonic and postnatal. Starting with the embryonic mammary gland, the next two sections of this chapter examine the morphogenesis of the mammary gland and the mechanisms that regulate its development throughout these two very disparate stages.

1.1.1 Embryonic Mammary Gland Development

1.1.1a General overview

*Mammary gland induction – embryonic day 10 - 12*

The first morphological signs of mammary gland development appears around embryonic day 10.5 (E10.5), with the formation of a ‘mammary line’ or ‘milk line’. The milk line is characterised by a localised thickening of the ectoderm ventrally along each flank of the embryo, connecting the fore- and hindlimbs. During mammary gland induction in the rabbit, the milk line is clearly visible as a raised crest above the epidermis (Propper, 1978), however in the mouse, there is much controversy as to whether a true milk line is formed. Whereas scanning electron microscopy of mouse embryos failed to identify a raised milk line (Mailleux et al., 2002), the structure was clearly visible in histological sections as a group of cells slightly elevated above the surrounding epidermis (Sakakura, 1987). This inconsistency could be explained by the relatively rapid development of the mouse embryo resulting in an extremely transient milk line.

By embryonic day 11 - 11.5 the first primordia of the glands are histologically visible as slight localised epithelial swellings, located at five distinct places in close proximity to the presumptive milk line. The precise location of the mammary
placodes is consistent between embryos - three thoracic and two inguinal - suggesting their induction is under tight genetic control (see Figure 1.1A). During the following day (E11.5 - E12.5), the placodes transform into lens-shaped buds of epithelial cells surrounded by a halo of layered, densely packed mesenchymal cells (Figure 1.1) and are outwardly detectable by scanning electron microscopy (Mailleux et al., 2002).

The formation of mammary placodes were originally thought to involve a localised increase in epithelial proliferation. However, Balinsky in the 1950s demonstrated that cells in the presumptive milk line of the mouse had a lower mitotic index compared to cells in the adjacent epidermis (Balinsky, 1950). This suggested the mammary placodes were generated by a displacement of cells, and not by a localised increase in proliferation of the epithelium. This idea was further substantiated by the studies of Propper in 1978, who described the presence of 'wandering' cells along the milk line in rabbits, based on morphological criteria (Propper, 1978); and more recently by analysing gene expression of mammary epithelial markers (Mailleux et al., 2002).

During the early stages of mammary gland development (E10 - E12), there appears to be no morphological distinction between the mesenchyme underlying the mammary epithelium and that underlying the surrounding epidermis. Nevertheless it is likely that a specialised mammary mesenchyme exists as mesenchyme surrounding the epithelial placode/bud is able to induce bud formation when cultured with non-mammary epidermis (reviewed in Sakakura, 1987). In the reverse experiment, prospective mammary epithelium was not able to induce bud formation when associated in culture with non-mammary mesenchyme. This strongly suggests mesenchymal cells induce mammary placode formation.

Sexual dimorphism and the 'resting phase' - embryonic day 12-16
A temporary decrease in DNA synthesis and absence of progressive differentiation, termed the 'resting phase', is observed from E12 - E16 (reviewed in Robinson et al., 1999; Sakakura, 1987). This term is slightly ambiguous as, despite the lack of proliferation and differentiation, it is a phase that is marked by profound morphological change, and incorporates the first signs of sexual dimorphism in the mammary gland.

At around E13 the gonads undergo sex-specific differentiation, leading to the determination of the sexual phenotype of the mammary gland at around E14 - 15. In male embryos, the mammary epithelium decreases in volume, transforming into
an irregular spindle shape (Figure 1.1B, and Sakakura, 1987). A day later, the gland rudiment detaches from the epithelial stalk that connects it to the epidermis, and then undergoes poor growth, or even degeneration (depending on the strain of mouse). In a series of elegant studies by Kratochwil and co-workers, the destruction of male mammary buds was attributed to the action of androgens produced in the testes (Durnberger and Kratochwil, 1980; Heuberger et al., 1982; Kratochwil, 1971; reviewed in Sakakura, 1987). The mammary mesenchyme was shown to express the androgen receptor and be a target tissue for testicular androgens. Upon androgen stimulation the mammary mesenchyme condenses around the epithelial bud, giving rise to the phenotype described above. The following descriptions are specific to female mammary gland development.

By E14.5, the mammary epithelium has invaded into the mesenchymal layers and transformed into a characteristic light-bulb shaped mass of cells connected to the surface of the embryo by a stalk-like epithelial structure (see Figure 1.1B).

A second type of mesenchymal tissue also appears during this phase. The fatty stroma or fat pad precursor tissue, is first detectable on the 14th day of gestation as a rather condensed tissue below the mammary mesenchyme that surrounds the epithelial compartment.

**Proliferative phase - embryonic day 16 - birth**

At E16 the buds undergo a transition into a third stage of embryonic development, characterised by the formation of a rudimentary ductal tree. Ductal sprouting involves, first the further elongation and invasion of the mammary bud into the underlying mesenchyme. This is followed by a limited amount of epithelial branching, commencing from E17 and ramifying out of the surrounding mesenchyme, toward the prospective mammary fat pad. Around this time the nipple forms by epidermal invagination at the surface of the skin. Continued proliferation towards the end of gestation leads to the formation of a small ductal tree consisting typically of 10-15 branches at birth, arising from a single duct that emanates from the nipple (Sakakura, 1987). The distal tips of these ductal branches are bulbous in structure and probably represent a rudimentary terminal end bud (TEB - the site of cell division in the mature mammary duct, discussed in detail in section 1.1.2).

Development of the fat pad precursor tissue is somewhat less dramatic. Between E15 and E17 it forms a looser tissue and consists mainly of preadipocytes. By birth,
A) E12.5 day mouse embryo showing the location of mammary placodes (wholemount in situ hybridisation using a Lef-1 probe). 10 placodes are generated (5 on each flank of the embryo); 3 in a thoracic location, P1 (behind forelimb), P2 and P3; and 2 inguinal placodes, P4 and P5 (behind hindlimb).

B) Schematic depiction of early mammary gland development: at E11 an epithelial placode (P) forms by epidermal thickening. Placodes form into an epithelial mammary bud (MB). At around E15 male mammary buds degenerate. By E18 a rudimentary ductal tree has formed in the female; concurrently, epidermal differentiation forms a nipple (N) connecting the surface of the embryo with the branched gland.
the majority of the fat pad precursor tissue is converted into white fat tissue, and forms a supportive substrate for epithelial growth.

1.1.1b Regulation of development

Although development of the mammary gland during embryogenesis is well characterised on a structural level, the molecular mechanisms responsible for the changes in morphology are poorly understood. Through the type of classic tissue recombination experiments described above, it is now widely accepted that the initial stages of mammary development are independent of systemic cues, and instead rely on instructive reciprocal interactions between the epithelium and mesenchyme. The formation of many organs and tissues, such as the lung, rely on such interactions where one cell lineage will signal to the other, setting up a chain of sequential instructive events (see Hogan, 1999). Interestingly, other ectodermal rudiments such as tooth buds, and hair and whisker follicles, also rely on epithelial-mesenchymal interactions for their induction and development, and show some parallels with the developing mammary gland.

A growing number of identified gene/gene families are expressed in the embryonic mammary epithelium or mesenchyme (summarised in Figure 1.2). These include transcription factors (e.g. Lef-1, Msx-1, Msx-2) and growth regulators (e.g. Fgf-7, Bmp-4) – a more comprehensive list is presented in Table 1.1. Surprisingly, gene deletion experiments have only implicated a few of these genes to be involved in mammary gland development.

Requirement for Lef-1 activity

Lef-1 (lymphoid enhancer-binding factor-1) is a member of the high mobility group (HMG) protein family of transcription factors, and is a key regulator of the β-catenin / Wnt signalling axis (see van de Wetering et al., 2002 for review). In the adult, Lef-1 is expressed in pre-B and T lymphocytes. In contrast, analysis of embryonic Lef-1 expression by in situ hybridisation revealed it to be expressed from the middle stages of embryogenesis (E10 – E11), in a wide variety of tissues – including the neural crest, mesencephalon, tooth germs, whisker follicles, pituitary gland and kidney. Importantly, Lef-1 is also expressed in the developing mammary gland (Kratochwil et al., 1996; Oosterwegel et al., 1993; van Genderen et al., 1994). Expression begins at around E11 in the mammary epithelium and persists for 3-4 days; by E14 – E15, two changes occur. Firstly, Lef-1 expression shifts from the epithelium to the mesenchyme (Foley et al., 2001; Satokata et al., 2000). An epithelial – mesenchymal shift in Lef-1 expression is significant during the
development of other skin appendages, for example, complete morphogenesis of
developing whisker follicles requires both epithelial and mesenchymal Lef-1
activity (Kratochwil et al., 1996). Secondly, in the epidermis destined to
differentiate into nipple sheath/skin, Lef-1 transcripts become undetectable by in situ
hybridisation (Foley et al., 2001).

In mice containing a germ-line deletion of Lef-1, the development of several organs
requiring inductive epithelial – mesenchymal interactions is impaired, suggesting
an essential role for Lef-1 in organ formation (van Genderen et al., 1994).
Specifically, these mutant mice lack teeth, whiskers, hair and have defects of the
trigeminal nerve; interestingly, there are no obvious defects in lymphoid cell
populations (the site of adult Lef-1 expression) at birth. Development of the
mammary gland is also impaired in these mice. Examination of newborn Lef-1
deficient females by histological staining showed an absence of mammary glands,
and a wholemount analysis of ventral skin from E13.5 day embryos revealed an
impairment in mammary bud formation compared to sibling wild type embryos.
Thus, mammary bud formation in the Lef-1−/− embryo is induced, but then arrests at
an early stage in organogenesis. It would be of interest to determine whether
mammary growth arrest coincides with the described shift in Lef-1 expression from
the epithelium to the mesenchyme.

This phenotype strongly implicates Lef-1 as a general regulator of epithelial-
mesenchymal interactions. The precise mechanisms by which this control is
effected remain unknown, but our understanding would undoubtedly be helped by
the identification of the downstream transcriptional targets of Lef-1. As mentioned
previously, Lef-1 is a mediator of Wnt signalling, and has been shown to be
downstream of Wnt activation (reviewed in van de Wetering et al., 2002).
Consequently, it has been proposed that Wnt signalling may contribute to
embryonic mammary development, although this has yet to be established.
Interestingly, of the 15 Wnt factors identified in the vertebrate genome, only
Wnt-10b expression has been confirmed in the embryonic mammary gland
(Christiansen et al., 1995).

Parathyroid hormone related peptide (PTHrP) signalling

PTHrP is a growth factor related to the parathyroid hormone (PTH). In contrast to
the systemically secreted classic peptide hormone PTH, PTHrP is expressed in a
number of foetal and adult tissues, and acts locally as a paracrine signalling
molecule (Wysolmerski and Stewart, 1998). Both molecules share a common family
of G-protein-coupled receptors, of which the prototype, PTH/PTHrP receptor
(PPR1), appears to mediate the majority of PTHrP functions. A variety of biological processes are regulated by, or at least involve PTHrP signalling events, and mice lacking PTHrP show severe skeletal dysplasia (Karaplis et al., 1994).

A role for PTHrP/PPR1 signalling in embryonic mammary development has recently been discovered. Both of these genes are expressed in the developing mammary gland (from E11 – E18), PTHrP in the epithelial bud and its receptor in the underlying mammary mesenchymal tissue (Wysolmerski et al., 1998). Their complementary expression patterns strongly infer signalling interactions between the two tissue types, and this was confirmed by Wysolmerski and colleagues using a homologous recombination approach (Wysolmerski et al., 1998). Targeted disruption of either PTHrP or PPR1 in the mouse leads to indistinguishable phenotypes in the mammary gland. Mammary bud formation proceeds normally until E15, at this point development arrests. Hence, mice null for the ligand or receptor fail to undergo the initial phases of branching morphogenesis, resulting in an epithelial bud that remains in the upper portions of the dermis. By reintroducing PTHrP as a transgene under the regulatory control of the keratin-14 promoter into the mammary epithelium of PTHrP null mice, mammary development could be rescued (Wysolmerski et al., 1998). This phenotype is consistent between males and females, thus PTHrP/PPR1 signalling is also required for androgen mediated sexual dimorphism (Dunbar et al., 1999). Indeed, PTHrP/PPR1 signalling induces androgen receptor expression in the male mammary mesenchyme, and transgenic expression of PTHrP in a PTHrP null background can rescue sexual dimorphic development (Dunbar et al., 1999). Additional roles for PTHrP/PPR1 signalling have been defined for nipple skin differentiation, and mesenchymal induction of Lef-1 (Foley et al., 2001).

In summary, PTHrP provides an instructive signal from the epithelium to the mesenchyme via PPR1 that determines mesenchymal cell fate. In turn, feedback from the mammary mesenchyme maintains the fate of the overlying mammary epithelium and triggers ductal morphogenesis, and nipple differentiation. The mammary phenotype of the PTHrP or PPR1 knock-out mice closely resembles that of mice containing a germline deletion of Lef-1. The question therefore arises whether PTHrP/PPR1 signalling and Lef-1 are part of the same genetic pathway in the developing mammary gland. The fact that Lef-1 appears earlier in the mammary gland than PTHrP suggests that Lef-1 would have to be upstream of PTHrP, however the studies of Wysolmerski and colleagues found that PTHrP signalling through PPR1 modulates mesenchymal, but not epithelial Lef-1 expression (Foley et al., 2001). A possible explanation for this apparent incongruity
Figure 1.2 - Gene expression during embryonic mammary gland development

Gene expression in the different mammary gland compartments during embryonic development. See Figure 1.1 for labels. Only confirmed gene expression data by in situ hybridisation is shown, therefore expression of certain genes may persist for longer, or be expressed earlier than is shown.
### Table 1.1 – Genes expressed during embryonic mammary development

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Mammary Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em><strong>Transcription factors</strong></em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hox-b9</em></td>
<td>Mesenchyme</td>
<td>Unknown</td>
<td>Chen and Capecchi, 1999</td>
</tr>
<tr>
<td><em>Hox-d9</em></td>
<td>Mesenchyme</td>
<td>Unknown</td>
<td>Chen and Capecchi, 1999</td>
</tr>
<tr>
<td><em>Irx-2</em></td>
<td>Epithelium</td>
<td>Unknown</td>
<td>Daniel and Smith, 1999</td>
</tr>
<tr>
<td><em>Lef-1</em></td>
<td>Epithelium &gt; Mesenchyme</td>
<td>K/O: Growth arrest at bud stage, absence of postnatal glands</td>
<td>van Genderen et al., 1994</td>
</tr>
<tr>
<td><em>Lmx-1b</em></td>
<td>Epithelium</td>
<td>Unknown</td>
<td>Robinson et al., 1999</td>
</tr>
<tr>
<td><em>Msx-1</em></td>
<td>Epithelium</td>
<td>K/O: No mammary phenotype</td>
<td>Phippard et al., 1996</td>
</tr>
<tr>
<td><em>Tbx-2</em></td>
<td>Mesenchyme</td>
<td>Unknown</td>
<td>Chapman et al., 1996</td>
</tr>
<tr>
<td><em>Tbx-3</em></td>
<td>Epithelium</td>
<td>Unknown</td>
<td>Chapman et al., 1996</td>
</tr>
<tr>
<td><em><strong>Growth factors/regulators</strong></em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bmp-2</em></td>
<td>Epithelium</td>
<td>Unknown</td>
<td>Phippard et al., 1996</td>
</tr>
<tr>
<td><em>Bmp-4</em></td>
<td>Mesenchyme</td>
<td>Unknown</td>
<td>Phippard et al., 1996</td>
</tr>
<tr>
<td><em>PPR1</em></td>
<td>Mesenchyme</td>
<td>K/O: Bud arrest, no sexual dimorphism</td>
<td>Dunbar et al., 1999; Wysolmerski et al., 1998</td>
</tr>
<tr>
<td><em>PTHrP</em></td>
<td>Epithelium</td>
<td>K/O: Bud arrest, no sexual dimorphism</td>
<td>Dunbar et al., 1999; Wysolmerski et al., 1998</td>
</tr>
<tr>
<td><em>Wnt-10b</em></td>
<td>Epithelium</td>
<td>Unknown</td>
<td>Christiansen et al., 1995</td>
</tr>
<tr>
<td><em>Ptc-1</em></td>
<td>Epithelium</td>
<td>Unknown</td>
<td>Lewis et al., 1999</td>
</tr>
</tbody>
</table>

Listed are the mammary compartments in which the genes are expressed, and an indication of their function as judged by the mammary phenotype in germline deletion experiments (K/O = knock-out).

It could be that *Lef-1* expression is induced by two independent pathways in the mammary epithelium and mesenchyme. It would be interesting to compare Lef-1 activity in these two cell lineages, to determine whether transcriptional activation of downstream Lef-1 targets is different.

**Transcription factors**

A variety of transcription factors have been implicated in embryonic mammary gland development. Foremost in this list are members of the homeobox gene family
of transcription factors (reviewed in Lewis, 2000). The homeobox genes share a relatively conserved 61 amino acid homeodomain that functions as a helix-turn-helix DNA-binding domain, and have key regulatory roles in organogenesis. Notably, the homeobox genes Msx-1 and Msx-2 are required for mammary gland development (reviewed in Bendall and Abate-Shen, 2000).

At E13.5, Msx-1 and Msx-2 are expressed in the mammary epithelium as determined by in situ hybridisation (Phippard et al., 1996). The expression pattern of Msx-2, but not Msx-1, shows a great deal of similarity to Lef-1, exhibiting a shift in gene expression from the epithelial to mesenchymal mammary compartments at E14.5 (Satokata et al., 2000). Expression of Msx-1 persists until E16.5, but surprisingly is not required for early mammary development as mice containing a homozygous deletion of Msx-1 have normal mammary glands at birth (Phippard et al., 1996; Satokata et al., 2000). By contrast, a germline deletion of Msx-2 leads to an arrest in epithelial sprouting at E16.5 (Satokata et al., 2000). Growth arrest at this stage coincides with the downregulation of Msx-1 transcripts, suggesting that Msx-1 and Msx-2 may be functionally redundant during the early stages of mammary organogenesis. Indeed, this would explain the lack of a mammary phenotype in mice deficient for Msx-1. Further to this hypothesis, a double knock-out mouse lacking both Msx-1 and Msx-2 fails to form mammary buds at E12.5 (Satokata et al., 2000). Msx-2 may also be important for the differentiation of fat pad mesenchyme as it is also expressed in this tissue at E13.5 (Phippard et al., 1996).

The downstream targets of Msx-1 and Msx-2 remain unknown in the mammary gland. Analysis of Msx gene function in tooth development has shown that ectopic bone morphogenic protein 4 (Bmp-4) can partially compensate for Msx-1 deficiency. This suggests that Bmp-4 is a downstream component of the same genetic pathway as Msx-1. It is tempting therefore to speculate this is also true for the mammary gland for two reasons, i) the tooth and mammary gland undergo a similar process of epithelial budding during their early development, and ii) Bmp-4 is expressed in the developing mammary gland (Phippard et al., 1996). However, the type of rescue experiments described above for the tooth bud have yet to be performed in the mammary gland, and also there appears to be subtle differences in Msx function between these two tissue types. For instance, whereas Msx-1 is expressed in the epithelial compartment in the mammary gland during bud formation, in the structurally analogous developmental stage in the tooth, Msx-1 is found in the mesenchyme (Chen et al., 1996; Phippard et al., 1996). Also, Msx-1 is dispensable during embryonic mammary gland formation, but its deficiency results in bud arrest during tooth development. Thus, despite both ectodermal rudiments sharing
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a common set of regulatory genes essential for their development, there are significant tissue-specific differences in their function.

A number of other transcription factors have expression patterns suggestive of prominent roles in early mammary development (listed in Table 1.1). However, apart from limited data on their embryonic expression, little is known about their function.

**Growth factors/ regulators**

Despite the elegant studies by Wysolmerski and co-workers delineating the effects of growth signals through PTHrP/PPR1, our knowledge of growth factor signalling in the embryonic mammary gland remains sparse. Few growth factors, or their cognate receptors, have been implicated in early mammary development. Fibroblast growth factor 7 (Fgf-7, also known as keratinocyte growth factor (Kgf), see section 1.2.2) and its receptor Fgf Receptor 2IIib (FgfR-2IIib, or Kgf receptor) are expressed in the E14 mammary mesenchyme and epithelium respectively (Cunha and Hom, 1996). Although Fgf-7 can stimulate proliferation of rodent mammary epithelial cells *in vitro* (Imagawa et al., 1994) and *in vivo* (Ulich et al., 1994), mice lacking Fgf-7 surprisingly do not have a known mammary phenotype (Guo et al., 1996).

Expression of the *Irx-2* transcription factor in the mammary epidermis and sprout is an interesting observation (Daniel and Smith, 1999). In *Drosophila*, the homologue of this family of transcription factors acts downstream of the Hedgehog signalling pathway, and participates in wing vein formation and neural patterning (Gomez-Skarmeta et al., 1996). Although the three members of the mammalian hedgehog family (sonic, desert, and indian) could not be detected by *in situ* hybridisation (Veltmaat and Spencer-Dene, personal communication), the hedgehog receptor *Patched-1* (*Ptc-1*) is expressed in the mammary epithelium (Lewis et al., 1999). Members of the Gli family of transcription factors, which also act downstream of hedgehog signalling can also be detected in mammary gland at E11.5 – E12.5. Furthermore, the naturally occurring ‘extra-toes’ mutant mouse, which contains a functional deletion of *Gli-3* has placode induction defects (Dr. J. Veltmaat, personal communication). Intriguingly, the majority of genes listed in Table 1.1 have been shown to be downstream of Hedgehog signalling in other mammalian systems. Hence, if Hedgehog signalling through Patched-1 is essential for mammary development, then it could provide a unifying scaffold, co-ordinating many of the pathways described within this section.
1.1.2 Postnatal Mammary Gland Development

The development of the postnatal mammary gland is just as complex as its embryonic counterpart. Starting with an overview of postnatal mammary development, this section will discuss some of the known genes controlling mammary development, giving particular emphasis to the different types of signalling pathways and interactions that occur. As the male mammary gland degenerates during its embryonic development, the following account represents development of the female gland only.

1.1.2a General overview

Pre- and postpubertal development

The postnatal mammary gland undergoes very little development until the onset of puberty. Following birth, the bulbous end buds present at the end of each rudimentary duct disappear concurrent with the loss of circulating maternal hormones. During the first 3 weeks of life, ducts slowly elongate and invade into the mammary fat pad, however, growth increases only isometrically in association with overall body growth. Surprisingly, growth at this stage is dependent on ovarian hormones as removal of the ovaries arrests ductal penetration of the fat pad (reviewed in Daniel and Silberstein, 1987).

Between 3 - 4 weeks of age, the onset of puberty is marked by the production of ovarian and pituitary hormones. The rate of ductal growth and epithelial branching increases accordingly, and by 5 weeks of age the ducts have extended approximately two-thirds into the fat pad. The fat pad acts both as a supporting substrate for growth, and as a restrictive pocket for ductal elongation. By 10 weeks of age the ducts have reached the limit of the fat pad and ductal elongation is inhibited (Figure 1.3).

Unlike the development of other glandular tissue, such as the pituitary or prostate gland, ductal morphogenesis requires a coordinated site of elongation, where the addition of new cells coincides with forward progressive growth and canalisation - the process of lumen formation. In the mammary gland, the terminal end buds (TEBs) perform this function (reviewed in Daniel and Silberstein, 1987; Silberstein, 2001a). The TEBs form in response to ovarian hormones just prior to puberty, and are present during ductal elongation, disappearing again as ductal growth is inhibited at the periphery of the fat pad. That the TEBs are the site of mammary epithelial proliferation became apparent when mammary glands pulsed with $^{14}$C-thymidine were exposed to autoradiography (Daniel and Silberstein, 1987).
these experiments, DNA synthesis was shown to be greatest in the end buds, whilst the subtending ducts were relatively quiescent. Additionally, the TEBs act as control points for bifurcation (end bud branching) and ductal pattern formation.

Architecturally, the TEBs range in size from 0.1 to 0.5mm in diameter and consist of 4-6 layers of cuboidal epithelia. Luminal epithelial cells from the subtending duct are continuous and are derived from the luminal cells of the TEB (see Figure 1.3C). These cells possess microvilli on the luminal surface and exhibit an apico-basolateral polarity (see section 1.3). Immediately beneath the basal lamina, at the leading end of the TEB, are a group of specialised cells, the cap cells, that are morphologically distinct from other mammary cells. Cap cells exhibit a lack of differentiated structures, do not show cytoplasmic polarity and do not form junctional complexes with neighbouring cells (Daniel and Silberstein, 1987). As the cap cells are followed laterally around from the tip of the TEB towards the neck, they exhibit structures characteristic of another mammary cell type, the myoepithelium. Myoepithelial cells form a contractile sheath around the ductal epithelium that is responsive to oxytocin, and hence are responsible for the movement of milk out of the ductal network. Additionally, they generate the basal lamina surrounding the mammary ducts. Cap cells produce the TEB basal lamina and are also observed migrating away from the TEB tip towards the luminal ductal epithelium where they are interspersed with another cell type of the TEB, the body cells. Thus, the cap cells may represent a stem cell population that ultimately gives rise to the entire epithelial population of the gland (see section below on 'Mammary stem cells', Daniel and Silberstein, 1987; Silberstein, 2001a; Williams and Daniel, 1983). Within the TEB, mitosis is seen in the cap and body cells.

In response to ovarian hormone secretion associated with the oestrus cycle, the ducts develop lateral and alveolar buds. These lateral buds are competent to form branches, and they develop structures at their growing tips resembling TEBs (Daniel and Silberstein, 1987; Richert et al., 2000). The differentiation of alveolar buds is less complete. As post-pubertal growth continues, a rudimentary alveolar structure consisting of a single layer of epithelia surrounding a hollow lumen develops. However, progression of rudimentary alveoli into fully functioning milk secreting units does not occur during post-pubertal development as pregnancy is an absolute requirement for complete alveolar differentiation (Robinson et al., 1995).

**Developmental changes during pregnancy**

Gestation in the mouse usually lasts around 19 days. During this time the mammary gland undergoes its most rapid phase of proliferation and
Figure 1.3 - Postnatal mammary gland development

A) Diagram showing development of the adult mammary gland (adapted from Hennighausen, 2001). The fat pad is represented by the oval shape. Black circles represent TEBs, greens circles represent alveoli, and red circles represent lobulo-alveoli. Changes in ductal morphology shown as wholemount preparations of the mouse mammary gland are detailed in (B). C) A simplified terminal end bud (TEB). Direction of growth is shown by the bold arrow. Multi-potent cap cells are present at the tip of the TEB and can give rise to myoepithelial cells through a series of differentiative steps along the lateral edge of the TEB. Cap cells can also migrate in towards the lumen and produce body cells and luminal epithelia.
differentiation, leading to an 8 - 12 fold increase in the epithelial content of the gland (Medina, 1996). Growth during pregnancy is hormonally induced and initially is characterised by an enhancement of ductal branching, and the formation of alveolar units as seen in the post-pubertal gland. The second half of pregnancy sees further ductal branching and the cleavage, and differentiation, of alveolar buds to form numerous individual alveoli. Myoepithelial cells also differentiate and form a loose basket-shape of cells around the alveoli that assists lactation (Daniel and Silberstein, 1987; Richert et al., 2000). Towards the end of pregnancy, alveoli are grouped into functional lobules (lobulo-alveoli) and these structures fill the majority of the fat pad by the 18th day of gestation (Figure 1.3).

These events are accompanied by further cellular differentiation leading to the development of secretory epithelial cells lining the alveoli and ducts that are capable of producing and secreting milk proteins and lipids. Structural analysis of the ductal cells one day prior to parturition shows an accumulation of rough endoplasmic reticulum and secretory vesicles consistent with this function (Mather and Keenan, 1998). In preparation for birth and subsequent suckling by the young, milk is actively secreted into the lumen of the lobulo-alveolar units and this process is aided by a decrease in tight junction permeability which prevents secreted milk leaking through into the extracellular milieu (Nguyen et al., 2001).

A number of different milk proteins and lipids are produced by the secretory epithelial cells. Their production follows a characteristic time course. For instance, \textit{WDNM1} expression commences early during pregnancy (day 9) and its transcription increases dramatically towards the 14\textsuperscript{th} day, then remains high throughout the rest of pregnancy and lactation (Morrison and Leder, 1994). \textit{\(\beta\)-casein} RNA is expressed earlier (day 11) than whey acidic protein (\textit{WAP}) (day 14), and \(\alpha\)-lactalbumin is only transcribed late in pregnancy (day 16) (Robinson et al., 1995).

Although the differentiated secretory epithelial cells express high levels of milk proteins during pregnancy, maximal expression only occurs throughout lactation. Interestingly, low levels of milk protein expression can be detected in the cycling mammary gland in response to hormones released during the oestrus cycle. Hence, the mammary epithelia can undergo a transient differentiation stage during the oestrus cycle, but requires pregnancy to undergo terminal differentiation (Robinson et al., 1995). Transient differentiation of luminal mammary epithelia is probably a very early feature of the mammary gland, as newborn mice are able to secrete a product known as ‘witch’s milk’ in response to circulating maternal hormones.
Lactation and involution

Lactation involves the continuous synthesis and delivery of milk and lasts for approximately three weeks after parturition. A process called involution, involving apoptosis of the lobulo-alveoli and gland remodelling then arises and is triggered by milk stasis once the pups have weaned (Quarrie et al., 1996). The majority of our knowledge on involution is based on experimentally controlled, forced weaning studies where the pups are removed from the mother after a brief period of lactation. However, natural weaning leads to a similar physiological change in the mammary gland, albeit with a slower time-course (Quarrie et al., 1996). Initially, involution involves limited alveolar cell death and is mediated by local intra-gland cell death signals. This stage of involution is reversible as lactation can be reinitiated upon suckling, due to the suppression of death signals by circulating lactogenic hormones (Li et al., 1997).

If suckling is not reinitiated after 48 hours then a second, non-reversible, stage of involution ensues; systemic hormone levels drop dramatically and the gland is restructured (Li et al., 1997; Lund et al., 1996). Apoptosis starts soon after weaning and peaks at day 4 of involution, by which time the alveoli have collapsed into epithelial cell masses that are eventually cleared away by invading macrophages or viable neighbouring epithelial cells (Fadok, 1999). As the epithelial content of the gland decreases, cell proliferation increases in the adipocyte and stromal compartments of the gland (Richert et al., 2000). By the 8th day of involution the majority of cell death has occurred and a remodelling of the ducts and surrounding stroma is apparent. Involution is complete after approximately three weeks after weaning. Although it is often described in the literature as a return of the mammary gland to its pre-pregnant morphology, the remodelled gland appears somewhat more differentiated than the virgin gland. Instead, it resembles a gland at the early stages of pregnancy when alveolar differentiation has just commenced. The complete developmental cycle of the mammary gland is presented diagrammatically in Figure 1.3.

Mammary stem cells

The dynamic growth, differentiation and recycling of the mammary epithelial compartment requires the existence of multi-potential stem cells within the mammary gland. As mentioned briefly above, the tip of the TEB contains undifferentiated pluripotent cap cells that have the potential to adopt alternate cell fates — if cap cells enter the TEB body they become luminal epithelia, whereas lateral migration results in a myoepithelial cell fate (Williams and Daniel, 1983).
However, both cap cells and TEBs disappear once the fat pad has been completely filled by the ductal tree (see Figure 1.3), and therefore cannot act as a stem cell population for the changes induced during pregnancy and glandular remodelling. Moreover, all portions of the ductal epithelium are capable of reconstituting the entire epithelial component of the mammary gland when transplanted into a cleared mammary fat pad (a mammary fat pad in which the epithelial cell population has been removed); amazingly an entire functional mammary gland may comprise of the progeny from a single cell (Kordon and Smith, 1998). This indicates other cells with pluripotent properties must exist within the mammary gland.

Despite extensive research in this area, molecular markers for mammary stem cells have not been identified. Gilbert Smith and colleagues have recently used light and electron microscopy in an attempt to identify the different epithelial cell types in the mammary gland, based on characteristics such as size, nuclear morphology and nuclear and cyto-matrix density (reviewed in Chepko and Smith, 1999; Smith and Chepko, 2001). Their work suggests the existence of five epithelial cell types within the mammary duct. Of these, only two could enter into mitosis, the other three contained no mitotic chromosomes and were postulated to be terminally differentiated. The two candidate stem cell types, the small light cell (SLC) and the undifferentiated large light cell (ULLC), can both undergo asymmetric division, but have different ductal locations. The SLCs have a basal location and never touch the lumen, conversely the ULLCs are found mainly, but not exclusively, adjacent to the lumen. As previous *in vitro* studies have demonstrated the mammary luminal cell population contains the multi-potent epithelium, it is thought the ULLCs represent the active functional mammary stem cell and the SLCs represent a quiescent form (Smith and Chepko, 2001).

1.1.2b Regulation of development

A large volume of literature has emerged over recent years implicating the involvement of a disparate list of genes in the control of postnatal mammary gland development. Despite this, it is apparent we are a long way away from integrating these different genes into functional signalling pathways that are active during the different stages of mammary development. In this review I will focus primarily on the more recent findings that have utilised transgenic and knock-out technologies to build upon knowledge gained from traditional hormonal ablation and tissue recombination studies. Special emphasis is given to cases where functional signalling pathways have at least been partially elucidated.
Introduction

Oestrogen signalling

It has been established for a long while that oestrogen influences mammary ductal growth, since ovariectomy in pre-pubescent mice inhibits ductal invasion into the fat pad (Daniel and Silberstein, 1987). Moreover, oestrogen pellets implanted into the fat pad of ovariectomised mice promote TEB formation and DNA synthesis (Daniel et al., 1987). Accordingly, mice deficient for the alpha form of the oestrogen receptor (αERKO' mice) are infertile and suffer from a severe ductal elongation defect, possessing only a primitive ductal tree at the nipple that is devoid of TEBs (Bocchinfuso and Korach, 1997; Korach et al., 1996). The beta form of the oestrogen receptor is dispensable for mammary gland development (Shyamala et al., 2002).

Immunohistochemical analysis of oestrogen receptor α distribution in the mammary gland revealed a stromal and luminal epithelial location (Shyamala et al., 2002). Thus, oestrogen could act on the receptors in the epithelial, stromal or both compartments to regulate mammary ductal growth. To establish which receptors were key for ductal growth, Cunha et al. performed tissue recombination experiments with αERKO and wild type mice (Cunha et al., 1997; reviewed in Bocchinfuso and Korach, 1997; Imagawa et al., 2002). Wild type or αERKO mammary epithelium was transplanted under the renal capsule of female nude mice with wild type or αERKO mammary stroma, and allowed to grow for four weeks. During this time the αERKO mammary epithelium underwent ductal morphogenesis when associated with wild type stroma. In the reverse experiment, αERKO stroma could not support the ductal outgrowth of wild type epithelium. These results were surprising as the distribution of oestrogen receptors strongly implicates a direct mode of oestrogen action on the mammary epithelium. Instead, a critical role for stromal oestrogen receptors was identified, suggesting an indirect, paracrine action for stimulating epithelial growth. In agreement with this finding, cap cells within the TEB are oestrogen receptor negative, whereas the surrounding stromal tissue is positive for the receptor (Daniel et al., 1987). The function of epithelial oestrogen receptors has yet to be fully established, although one report suggests they may be required for ductal development in the adult mouse (Mueller et al., 2002).

Analysis of the αERKO mouse has also demonstrated that oestrogen influences mammary development by an indirect mechanism. Oestrogen can enhance progesterone levels and induce progesterone receptor expression in the mammary gland (reviewed in Imagawa et al., 2002). In addition, by binding to responsive elements in its promoter, activated oestrogen receptors enhance prolactin synthesis.
and secretion from the pituitary gland. The importance of this is demonstrated by grafting heterozygous αERKO pituitary glands onto homozygous αERKO mice (Bocchinfuso et al., 2000). Grafted αERKO mice had increased circulating prolactin and progesterone levels, and this was permissive for mammary ductal development, indicating that local direct effects of oestrogen on the mammary gland are not as crucial for ductal growth as originally conceived.

A potential paracrine mediator of epithelial proliferation in response to stromal oestrogen receptor activation is the epidermal growth factor (EGF). Slow release implants of EGF in the mammary glands of ovariectomised mice can stimulate TEB formation, demonstrating that a growth factor can substitute for oestrogen (Coleman et al., 1988; see also Silberstein, 2001a). Oestrogen has also been shown to activate EGF expression (Kenney and Dickson, 1996). However, tissue recombination experiments demonstrated that EGF signals through stromal EGF receptors (EGFRs) and not through epithelial EGFRs, indicating that other mammotrophic factors downstream of EGFR activation exert the defined oestrogen-mediated effect on epithelial cells (Wiesen et al., 1999).

**Progesterone signalling**

The steroid hormone progesterone has profound effects on mammary gland development (reviewed in Silberstein, 2001a). The physiological effects of progesterone are mediated, at the cellular level, by the progesterone receptor (PR), which interacts with regulatory sequences of target genes upon its activation. As with oestrogen, functional knock-out models of the hormone receptor (in this case the progesterone receptor knock-out ‘PRKO’ mouse) have been especially informative in defining a role for progesterone in the mammary gland. PRKO mice are sterile and show severe developmental defects in a variety of organs associated with the female reproductive system, including an inability to exhibit sexual behaviour (the lordosis response) (Lydon et al., 1995). Importantly, progesterone has a proliferative function in the mammary gland. Consequently, PRKO mice show less extensive ductal development, as well as a complete absence of lobulo-alveolar structures when mice were administered with pregnancy levels of oestrogen and progesterone (Humphreys et al., 1997b; Lydon et al., 1995).

An analysis of PR localisation by immunohistochemistry found it present in approximately 55% of adult female mammary luminal epithelial cells, but not in the mammary stroma (Shyamala et al., 2002). Previous reports however, described the stromal presence of PR (Haslam and Shyamala, 1981), and further to this have shown a requirement for stromal PR in mammary development (Humphreys et al.,
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1997a; Humphreys et al., 1997b), although these studies did not identify stromal PRs directly in mammary tissue. Additional studies have since determined that PR in the mammary stroma is not essential for full mammary development (Brisken et al., 1998).

The requirement of mammary epithelial PRs has been well characterised (Brisken et al., 1998). As progesterone receptor synthesis is influenced by increased oestrogen levels during pregnancy, Brisken et al. performed tissue recombination experiments using PRKO mammary epithelium in wild type cleared fat pads to address the function of epithelial PRs during pregnancy (as PRKO mice are infertile). These experiments demonstrated that PRs were required in the mammary epithelium to induce side branching and lobulo-alveolar development during pregnancy. Another interesting observation was that PR-negative epithelial cells could contribute to lobulo-alveoli if they were in close vicinity to PR positive cells (Brisken et al., 1998). This strongly suggested that a paracrine signalling factor, later identified as Wnt-4, acts downstream of progesterone receptor activation in the mammary epithelium to induce side branching and alveolar development (Brisken et al., 2000; Brisken et al., 1998).

The progesterone receptor exists as two isoforms, PR-A and PR-B, that are initiated from alternative start codons of the same gene. Both isoforms are expressed in the mammary epithelium and are virtually identical except for the presence of an additional 165 amino acids in the amino terminus of the B isoform (reviewed in Hennighausen and Robinson, 2001). As the PRKO mouse contains a functional deletion of both isoforms of the receptor, a PR-A isoform knock-out mouse ('PRAKO' mouse) was generated by homologous recombination (Mulac-Jericevic et al., 2000). Analysis of mammary glands from PRAKO mice revealed that the B isoform was sufficient for normal ductal growth and alveolar budding. However, overexpression of the A isoform causes excessive lateral branching in the mammary gland and so may also influence mammary development (Shyamala et al., 1998).

Although Wnt-4 has been identified as a paracrine, or to be more specific an epithelial juxtacrine, mediator of progesterone's proliferative effects (Brisken et al., 2000), the genes acting upstream and downstream of the progesterone receptor are poorly defined. One potential candidate, cyclin D1, is involved in the control of cell cycle progression from G1 phase, and its involvement in progesterone dependent cell division has been postulated (Humphreys et al., 1997b). Evidence for this however is sparse, but mice containing a functional deletion of cyclin D1 show defects in mammary gland development that overlap with the PRKO mouse,
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namely a delay in lobulo-alveolar development during pregnancy (Fantl et al., 1999; Fantl et al., 1995; Sicinski et al., 1995). Interestingly, when the levels of progesterone receptor were assessed in these mice, an elevation of the B isoform (PR-B) during pregnancy was apparent (Fantl et al., 1999). This is particularly striking as PR-B is sufficient for ductal growth and alveolar development (Mulac-Jericevic et al., 2000). If cyclin D1 does contribute to the proliferative effects of progesterone, then it would need to be determined whether cyclin D1 is downstream of the receptor (probably PR-B), or is activated indirectly, perhaps through the paracrine signalling factor Wnt-4.

Mice containing an inactivating mutation of the transcription factor C/EBPβ also have a mammary phenotype that resembles the PRKO mouse, with a reduction observed in ductal branching and an impairment of pregnancy induced development (Robinson et al., 1998; Seagroves et al., 2000). Of particular interest in the context of progesterone signalling, C/EBPβ null mammary glands have a much higher number, and altered distribution of progesterone receptor positive epithelial cells (Seagroves et al., 2000). These data suggest that C/EBPβ may regulate cell fate decisions to determine whether an epithelial cell will become PR positive or negative. Hence, C/EBPβ may act upstream of the progesterone receptor and cyclin D1 may be a downstream mediator of progesterone action. Interestingly, the mammary defects observed in PR, cyclin D1 and C/EBPβ null mice are all epithelial cell autonomous (Brisken et al., 1998; Fantl et al., 1999; Robinson et al., 1998)

Prolactin signalling

Prolactin (PRL) is a pituitary hormone that signals through the prolactin receptor (PRLR) and has important functions in the mammary gland (Daniel and Silberstein, 1987). Analysis of PRL and PRLR deficient mice show a similar mammary phenotype and identify two principal roles for prolactin signalling (Horseman et al., 1997; Ormandy et al., 1997a; Ormandy et al., 1997b). Firstly, PRLR null mice have reduced ductal side branching after puberty with a persistence of TEBs that is not evident in wild type controls. Secondly, during pregnancy the mammary gland lobulo-alveoli units fail to differentiate. Furthermore, mice that are heterozygous for PRLR fail to lactate their young after the first litter (Ormandy et al., 1997a). As mice homozygous for the PRLR null mutation are infertile, the significance of prolactin signalling in mammary development during pregnancy was demonstrated in studies using PRLR negative epithelium transplanted into wild type fat pads (Brisken et al., 1999).
Of special relevance in these transplantation studies was the demonstration that the ductal side branching defect was due to the indirect actions of prolactin on the mammary gland, as transplants grew normally in nulliparous wild type mice (Brisken et al., 1999). Thus, PRLRs expressed outside the mammary epithelium (but not in the mammary stroma) are essential for side branching. This could be explained by prolactin increasing the levels of systemic progesterone, providing a possible link between the prolactin and progesterone dependent pathways in the mammary gland. Although there is no direct evidence for this link, the levels of progesterone in PRLR null mice were significantly lower than in wild type controls (Brisken et al., 1999). An epithelial cell autonomous signal involving the direct action of PRL on the mammary epithelia was shown to be responsible for lobulo-alveolar development (Brisken et al., 1999).

Prolactin binding to its receptor leads to receptor dimerisation and activation of janus kinase 2 (JAK2). This in turn phosphorylates the signal transducer and activator of transcription 5 (STAT5) (reviewed in Hennighausen et al., 1997; Shillingford and Hennighausen, 2001). Once phosphorylated, STAT5 forms homo- or heterodimers (STAT5 exists as two isoforms - a and b) and is translocated to the nucleus where it activates genetic programs of cell proliferation and differentiation (see Hennighausen and Robinson, 2001). STAT5a is the more important protein for mammary development (Liu et al., 1997), although STAT5b can also partially compensate for STAT5a loss (Liu et al., 1998). Analysis of mammary glands from STAT5a deficient mice show defective mammary development that resemble the PRLR null phenotype (Liu et al., 1997; Ormandy et al., 1997a). Moreover, transplantation of STAT5 null mammary epithelia (STAT5 null mice are infertile) into wild type mammary fat pads fail to form functional alveoli during pregnancy. Taken together, these data demonstrate the prolactin – prolactin receptor – JAK2 - STAT5 pathway is critical for the functional differentiation of mammary alveolar cells (Miyoshi et al., 2001). It should be noted however, that STAT5 can be activated by other signalling pathways in the mammary gland (See Shillingford and Hennighausen, 2001).

**Growth hormone signalling**

Growth hormone (GH) is another secreted pituitary hormone that is absolutely required for mammary development. It’s receptor (GHR) is localised within the epithelial and stromal compartments of the mammary gland and GHR activation is necessary for TEB formation (Reviewed in Kleinberg, 1997). GH action in the mammary gland is probably mediated by insulin-like growth factor 1 (IGF-1), as...
IGF-1 transcripts are immediately upregulated upon GHR activation (Kleinberg et al., 1990). Also, Ruan and colleagues demonstrated, firstly, that addition of IGF-1 could mimic the actions of GH and induce TEB formation in the mammary gland (Ruan et al., 1992), and secondly, that mammary development in mice containing a targeted deletion of IGF-1 could be rescued by exogenous IGF-1 but not GH (Ruan and Kleinberg, 1999). Finally, TEB formation induced by the GH – GHR – IGF-1 signalling axis can be enhanced by a synergistic mechanism with oestrogen which, as described earlier in the chapter, also contributes to TEB formation (reviewed in Kleinberg, 1997).

Osteoprotegerin-ligand and RANK signalling

One of the more interesting and unexpected signalling pathways controlling mammopoiesis involves the osteoclast differentiation factor osteoprotegerin-ligand (OPGL). This protein is involved in stimulating the activation of osteoclasts, and hence is essential for bone remodelling and calcium mobilisation. OPGL is a member of the tumour necrosis family (TNF) of molecules and exerts its effects on osteoclasts by binding to the TNF receptor family member receptor activator of NFκB (RANK) (Kong et al., 1999). OPGL activity can be reduced by a soluble competitor protein, osteoprotegerin (OPG), that competes with RANK for OPGL binding. Intriguingly, OPGL and OPG expression is under the control of the female sex hormones oestrogen, progesterone and prolactin, and is also regulated by PTHrP activity (see Fata et al., 2000). During female menopause, the level of systemic hormones in the body decreases dramatically, and this shifts the balance between OPGL and OPG, resulting in activation of RANK and osteoclast differentiation, that can lead to osteoporosis. It is of interest that osteoporosis in animal models can be reversed by treatment with OPG (Simonet et al., 1997). Generation of OPGL and RANK germline deletions in mice has provided an elegant, and unpredicted, evolutionary explanation for why, i) OPGL and OPG expression are under hormonal control, and ii) there is a gender bias for osteoporosis. The explanation involves the ‘molecular hijacking’ of the evolutionary conserved OPGL/RANK signalling pathway by the recently evolved, and hormonally controlled, mammary gland to ensure its full development during pregnancy.

The mammary defect in OPGL or RANK mice phenocopy each other (Fata et al., 2000). Although the mice develop normal glands during puberty, indicating that the signalling pathway is not required for ductal elongation, during pregnancy they fail to undergo lobulo-alveolar development and cannot suckle their young. As with many of the pathways described so far, this defect is epithelial cell
autonomous, and can be rescued in OPGL null mice by local administration of recombinant OPGL (Fata et al., 2000). *In situ* hybridisation of mammary gland sections using anti-sense probes to OPGL or RANK showed an exclusive epithelial expression for both RNA species, which was restricted to the alveolar cells in the case of OPGL, suggesting an autocrine/juxtacrine mode of action. Further experiments demonstrated that OPGL is required for the proliferation and survival of mammary epithelial cells, and more specifically that OPGL is a critical regulator of alveolar cell proliferation (Fata et al., 2000). So by sequestering OPGL/RANK signalling, the mammary gland seems to have found a way to ensure that lactation-competent mammary epithelia develop concurrently with skeletal calcium release. This control guarantees the successful transmission of high levels of essential maternal calcium to neonates through the process of lactation.

More recently, Cao and colleagues have shown that the action of IKKα, an intracellular kinase, degrades IkB leading to the activation of NFκB. NFκB in turn was shown to induce levels of cyclin D1 leading to increased proliferation. More interestingly, IKKα is a critical mediator in the mammary gland that controls epithelial cell proliferation in response to RANK signalling via cyclin D1 induction (Cao et al., 2001). This is further substantiated by the similarity in mammary gland development in mice containing homozygous germline deletions of *RANK, OPGL, IKKa* and *cyclin D1* (Cao et al., 2001; Fantl et al., 1995; Fata et al., 2000; Sicinski et al., 1995). Importantly, these findings also imply that cyclin D1 can be activated by a number of pathways in the mammary gland (see section entitled 'Progesterone signalling'). Finally, as progesterone induces synthesis of OPGL during pregnancy, and as mice null for *PR, OPGL* and *RANK* also show similar mammary phenotypes, it would be of interest to determine if OPGL/RANK signalling controls the effects of progesterone during late pregnancy, just as Wnt-4 mediates progesterone induced ductal side branching in early pregnancy.

*Other factors controlling mammary development*

A number of other genes have important functions during mammary development. For example, members of the MMP/TIMP family of proteins are involved in extracellular matrix remodelling, essential for ductal growth and gland remodelling (Simian et al., 2001; Reviewed in Wiseman and Werb, 2002). Colony stimulating factor induction of macrophages, together with eosinophil recruitment to the TEBs have an essential and complementary function in branching morphogenesis (Gouon-Evans et al., 2000). Important roles for the transforming growth factor β (TGFβ) family have been reported for the inhibition of lateral branching and TEB
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growth (reviewed in Pollard, 2001; Silberstein, 2001a); although other family
members are necessary for ductal elongation and epithelial differentiation
(Robinson and Hennighausen, 1997). Finally, cell adhesion is an important
mediator of cell signalling and controls mammary cell survival. Hence, cell
adhesion may be a particularly crucial factor during involution (Streuli and
Gilmore, 1999).

Summary

Mammary gland development is controlled by a dynamic and diverse array of
factors. In general, mammopoiesis is under the inductive control of systemic
hormones, that initiate the distinct phases of development described in section
1.1.2a. The resultant dramatic remodelling of the gland seen at these phases is
mostly due to locally-induced factors downstream of the hormone receptor that
initiate juxtacrine or paracrine signalling events in the epithelium and mesenchyme.
Finally, it is becoming more apparent that many of the signalling pathways
converge to use a similar set of regulatory genes, cyclin D1 is a particularly good
example of this.

1.2 Receptor Tyrosine Kinases (RTKs)

Originally identified as the transforming gene products of some oncogenic viruses,
the RTKs now form a large group of membrane spanning cell surface receptor
enzymes. These receptors are endowed with intrinsic tyrosine kinase activity, and
participate in the control of fundamental cellular processes, including migration,
proliferation, differentiation, quiescence and survival or death. Around 17 RTK
families have been identified and share a similar domain structure: a ligand binding
extracellular domain, a single transmembrane-spanning domain, and an
intracellular domain encompassing the tyrosine kinase. Ligand binding to the
extracellular domain of the RTK by secreted or membrane-bound peptides leads to
a number of physiological responses that can be generalised as follows
(summarised in Schlessinger, 2000). Firstly, the receptor becomes activated in
response to the ligand by undergoing homo- or hetero-oligomerisation (usually
dimerisation). This results in the activation of the kinase and autophosphorylation
of conserved tyrosine residues in the cytoplasmic domain. Secondly, some of the
tyrosine residues, usually situated outside the catalytic core of the enzyme, become
primed as protein docking sites as a result of phosphorylation. These
phosphorylated residues function as binding sites for the Src homology 2 (SH2) or
phosphotyrosine binding (PTB) domains of a variety of signalling and adaptor proteins, and thus provide a scaffold for the assembly of signalling complexes that is dependent on receptor activation (see Yaffe, 2002). Thirdly, intracellular signalling pathways are activated from the receptor complex, leading to specific cellular responses, such as transcriptional activation of target genes. Finally, as the signals originating from the activated RTK can be very potent, the cell needs to tightly regulate its activity by attenuating the signal. This usually occurs by receptor endocytosis and degradation (Di Fiore and De Camilli, 2001). This last point is particularly important, as dysfunction (especially constitutive activation) of RTKs is responsible for a plethora of diseases and developmental disorders (reviewed in Robertson et al., 2000).

Three RTKs, all from distinct families, have been studied in this thesis: the hepatocyte growth factor receptor (also known as the Met receptor – a Type VI RTK); ErbB-2 (a Type I RTK); and finally the fibroblast growth factor receptor 2 (FgfR-2 – a Type IV RTK). After a brief description of the structure and signalling activity of each RTK family, the role of each receptor in development (with particular emphasis on mammary development) will be discussed.

1.2.1 The Met Receptor

Met is the prototype receptor of the Type VI family of RTKs, which to date contains just two members – Met and Ron (also known as the macrophage stimulating protein receptor). These receptors show a high degree of homology (63% at the amino acid level), and also elicit similar cellular responses upon activation (Medico et al., 1996). Met was identified in 1984 as the product of an oncogene in a chemically transformed human cell line (Cooper et al., 1984). This oncogene, Tpr-Met, resulted from a chromosomal translocation that fused the intracellular domain of Met to that of Tpr, a protein of unknown function, leading to a constitutively active homodimeric chimeric receptor with strong transforming ability (Park et al., 1986). Subsequent molecular cloning identified Met as a putative cell surface tyrosine kinase receptor for growth factors (Dean et al., 1985; Park et al., 1987).

The activating ligand for the Met receptor, hepatocyte growth factor/scatter factor (HGF/SF), was also discovered in 1984 by two independent groups, firstly as a strong mitogenic serum factor for hepatocytes (Nakamura et al., 1984), and secondly as a fibroblast secreted factor causing dissociation/scattering of epithelial cell colonies (Stoker and Perryman, 1985). However, it was not until 1991 that HGF
and SF were definitively shown to be identical proteins that activate the Met proto-
oncogene (Bottaro et al., 1991; Naldini et al., 1991). Structurally, HGF/SF shows a
great deal of homology to the plasminogen family of proteins and is composed of
an α- and β-chain linked by a single di-sulphide bond. Encoded by a single gene on
chromosome 7, HGF is synthesised initially as an inactive 728 amino acid single
chain inactive form that undergoes proteolytic cleavage to form the active α-β
heterodimeric molecule. The most interesting feature of HGF is the presence of 4
kringle domains in the α-chain that are involved in protein:protein interactions.
Alternative splicing of HGF transcripts can lead to the generation of peptides
containing varying numbers of kringle domains, that induce agonistic or
antagonistic responses upon Met receptor binding (Montesano et al., 1998, reviewed
in van der Voort et al., 2000). Hence, the kringle domains of HGF may provide a
degree of signalling specificity that determines the response of the activated Met
receptor.

In addition to binding its high affinity receptor (Met), HGF can also bind with low
affinity to cell surface-associated heparan sulphate proteoglycans (HSPGs) (Lyon et
al., 1994). Extensive site-directed mutagenesis has been used to elucidate both the
physiological roles of the HGF-heparan interaction, and also to map the heparan
binding sites on HGF. These studies have shown that the second kringle domain on
the α-chain of HGF is essential for heparan binding, and in the absence of this
interaction the potency of HGF is decreased significantly (Zioncheck et al., 1995;
reviewed in Trusolino et al., 1998; van der Voort et al., 2000). Further, unlike the
interaction between Fgfs and HSPGs that is required for Fgf receptor activation
(discussed in section 1.2.2b), HGF does not require a HSPG to activate Met. So in
the absence of a direct requirement for HSPGs in Met activation, a number of
different roles for HSPGs in Met signalling can be postulated that are not mutually
exclusive: i) HSPGs may promote dimerisation of HGF and thereby promote
receptor interactions, ii) HSPGs may present HGF to Met in the correct
configuration to allow receptor activation, iii) HGF molecules may be locally
concentrated by HSPGs in a way that could potentiate Met signalling, or
conversely, iv) HSPGs could act to sequester ligand away from the Met receptor
and so may also play a regulatory role in receptor activation. Recent evidence
suggests direct interactions between the Met receptor and HSPGs may also facilitate
signalling (Rubin et al., 2001).
1.2.1a Met receptor structure

The structure of the Met receptor is presented diagrammatically in Figure 1.4. It consists of a membrane spanning β-chain connected by a di-sulphide bridge to an extracellular α-chain that is heavily glycosylated during its processing. The protein is synthesised as a single chain inactive precursor that undergoes proteolytic cleavage in a post-Golgi compartment to form the active heterodimeric configuration that is detected at the cell surface (Comoglio and Boccaccio, 2001).

The extracellular moiety of the mature receptor constitutes the ligand binding region and contains two domains of unknown function, a cysteine-rich motif (MRS – Met related sequence) embedded within a ‘Sema’ domain, that is also present in the signalling molecules semaphorins and in their receptor plexins (see Trusolino and Comoglio, 2002). Analysis of the intracellular region of the β-chain has uncovered a number of interesting regulatory domains. Within the juxtamembrane domain a serine residue (Ser775) has an inhibitory effect on receptor activity following phosphorylation by protein kinase C or calcium-calmodulin-dependent kinase (Gandino et al., 1994). The catalytic kinase core of the receptor comprises two critical tyrosine residues (Tyr1224 and Tyr1235). Phosphorylation of both these residues is required for full receptor activation (Longati et al., 1994) and the resultant phosphorylation of the carboxy-terminal tyrosine residues (Tyr1349 and Tyr1356) is permissive for the binding of SH2/PTB domain-containing proteins (reviewed in Bardelli et al., 1994; Comoglio and Boccaccio, 2001). Unlike the docking sites identified in other RTKs that utilise multiple tyrosine residues to recruit specific signalling proteins, these two residues and the adjacent sequences (Y1349VHVNY1356VNV) are sufficient to mediate high-affinity interactions with multiple SH2/PTB domain-containing cytoplasmic effector molecules (Ponzetto et al., 1994).

1.2.1b Met receptor signalling

Activation of the Met receptor by HGF, at least in epithelial cells, triggers a unique morphogenetic program leading to ‘invasive growth’ (reviewed in Bardelli et al., 1997; Trusolino and Comoglio, 2002). This phenotype results from the integration of separate HGF responses, among which are cell proliferation, motility, migration, cell:cell dissociation (scattering), protection from apoptosis and ECM degradation. Invasive growth is a fundamental cellular process witnessed in tissue remodelling, as seen during branching morphogenesis (discussed in section 1.2.1c) and wound healing, but is also an absolute requirement for the metastatic progression of
Domain structure of the Met receptor, FgfR-2IIIb, and ErbB-2. All three RTKs have a similar domain structure consisting of an extracellular domain (containing unique structural features as indicated) involved in ligand binding, a transmembrane domain, a tyrosine kinase domain and a regulatory domain involved in effector molecule recruitment. The regulatory domains of FgfR-2IIIb are present within, and either side of the tyrosine kinase domains.
tumours. Hence, the Met receptor provides an excellent model system to study the signalling pathways involved in tumour progression.

The complexity of HGF's pleiotropic effects is reflected in the scaffold of signalling molecules and associated pathways that are coupled with Met receptor activation. Ras activity plays a central role in the invasive growth program as evidenced by the use of dominant negative Ras mutants to abolish these responses (Hartmann et al., 1994). Activation of Ras controls both proliferative and motile responses to HGF through the well characterised Raf-MEK-MAPK pathway. Association of Ras with the activated Met receptor is mediated by the Grb-2-Sos complex that interacts directly with the Met receptor on Tyr\(^{1356}\) (Ponzetto et al., 1994). An alternative, indirect mechanism of Ras activation also occurs via the adaptor protein Shc that binds both the Met receptor and Grb-2-Sos directly. This indirect interaction amplifies the mitogenic and motogenic responses to HGF (Pelicci et al., 1995). A further Ras activation pathway that is Grb-2-independent also exists as a Met receptor mutant defective for Grb-2 binding can still activate Ras (Tulasne et al., 1999).

The recruitment of Grb-2-associated binding protein-1 (Gab-1) to Met (either indirectly via Grb-2, or directly to Met via Tyr\(^{1349}\)) provides another major branch of the Met signalling repertoire. Indeed, overexpression of Gab-1 alone is sufficient to induce some of the invasive growth phenotypes observed after HGF stimulation (reviewed in Furge et al., 2000; van der Voort et al., 2000). Amongst others, Gab-1 recruits phosphatidylinositol 3-kinase (PI3K) which has a prominent regulatory role in Met induced mitogenesis, motility and morphogenesis, and may be required for the scattering response. Interestingly, PI3K can also bind Met directly, and in addition interacts with Ras, which may be particularly essential for its activity as a Met mutant that cannot bind Gab-1/PI3K upon HGF stimulation can still cause cell scattering (Tulasne et al., 1999). Noteworthy from these studies is that simultaneous activation of the Ras and PI3K pathways is required and sufficient for the full activation of the invasive growth program elicited by the transforming chimeric receptor Tpr-Met (van der Voort et al., 2000). This however does not hold true for the wild type Met receptor as other signalling effectors are required. For instance, a sustained recruitment of phospholipase C-\(\gamma\) (PLC-\(\gamma\)) to Gab-1, and the recruitment and phosphorylation of STAT3 to the Gab-1/Met receptor is obligatory for HGF-induced branching tubulogenesis (Boccaccio et al., 1998; Gual et al., 2000). Also, Rac and Rho participate in the motogenic response to HGF, probably via modification of the cytoskeleton (Ridley et al., 1995; van der Voort et al., 2000).
Presented in Figure 1.5 is a simplified representation of the signalling pathways associated with Met receptor activation.

Intriguingly, many of the signalling pathways and adaptor proteins that are utilised by the Met receptor are shared by many other RTKs; for example the epidermal growth factor receptor recruits both Gab-1 and STAT3 and activates Ras. Yet surprisingly, only Met is competent to induce tubule formation and activate the invasive growth program. A major question for the future is to address how the same set of effector molecules differentially alter the morphogenetic specificity of signalling pathways emanating from different RTKs (see Pawson and Nash, 2000; Simon, 2000 for reviews).

Recently, a number of interesting observations have been made that clarify some of the more confusing aspects concerning Met signalling. The discovery that α6β4 integrin can act as an adaptor molecule for HGF-dependent invasive growth, at least in cancer cells, is a prime example of such work (Trusolino et al., 2001). This work described a direct association between Met and α6β4 integrin that is essential for the observed cellular responses to HGF stimulation. Interestingly, following Met activation, α6β4 integrin is phosphorylated resulting in the direct recruitment of Shc and PI3K, thus providing an alternative platform that potentiates HGF-triggered activation of the Ras- and PI3K-pathways. As well as defining a novel role for α6β4 integrin in the invasive growth process (in addition to its role in adhesion), this study may also explain the observation that Ras activation following Met receptor phosphorylation occurs in the absence of Grb-2-Sos binding to the active Met receptor (Trusolino et al., 2001; Tulasne et al., 1999).

Another novel interaction involving the Met receptor has given insights into the paradoxical observation that HGF can act as both a pro- and anti-apoptotic factor (at high and low HGF concentrations respectively). The expression of anti-apoptotic signals following Met activation were attributed to cell survival factors such as Bag1. However, Met also cooperatively and directly interacts with the death receptor Fas through its extracellular domain, an interaction probably involving the α-chain of Met. This interaction prevents the pro-death signal, Fas-ligand, from binding to and activating Fas, providing a novel mechanism for cell survival (Wang et al., 2002). Upon HGF activation, a conformational change in the Met receptor releases Fas sequestration, and provides a platform for Fas-ligand activation of apoptotic signalling pathways. The authors speculate that when HGF concentrations are low, sufficient Met:Fas complexes exist to prevent cells undergoing apoptosis, however, when HGF levels are high, cells become sensitised.
to Fas-ligand activity once a threshold of orphan Fas receptors is reached (Wang et al., 2002).

1.2.1c Roles in development

It is now well established that signalling through the HGF/Met axis is essential for mouse development. This is hardly surprising given the pleiotropic nature of the cellular responses to HGF and the multitude of signalling pathways that it activates. Perhaps what is surprising though is the multifarious requirement for HGF/Met signalling in the development of a variety of diverse tissues and organs.

Analysis of the expression patterns of HGF and Met during embryogenesis using in situ hybridisation and RNase protection assays revealed a unique and complimentary pattern of expression (Sonnenberg et al., 1993). First and most notably, the Met receptor is expressed in cells of epithelial origin in a variety of organs including the kidney, tooth bud, lung, pancreas, intestine, mammary gland and salivary gland (Sonnenberg et al., 1993; Tsarfaty et al., 1992). Expression of the ligand, HGF, is always found in cells of mesenchymal origin, and importantly in cells adjacent to the sites of Met expression. These data strongly suggest a paracrine mechanism of action, where HGF/Met interactions mediate a signal exchange between the mesenchyme and epithelium, with potentially important roles in development. Secondly, Met and HGF transcripts were detected in myogenic precursor cells and in motor neurons, implicating a role for Met receptor activation in muscle and neuronal development (Sonnenberg et al., 1993). Taken together with the identification of HGF as a fibroblast-derived morphogen that induces tubular morphogenesis of epithelial cells (Montesano et al., 1991a; Montesano et al., 1991b), these expression patterns principally implicate HGF and Met in a developmental program that regulates branching morphogenesis in a variety of epithelial organs. Further to this, Met receptor activation was hypothesised to act in the formation of lumen in mammary ducts (Tsarfaty et al., 1992).

That signalling through the HGF/Met axis is important for embryogenesis was demonstrated convincingly with the generation of mice containing targeted germline mutations of HGF or Met (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). Homozygous disruption of either HGF or Met in the mouse leads to a similar embryonic lethal phenotype between E12.5 and E15.5, owing to abnormal development of the placenta and liver characterised by a severe reduction in the number of trophoblasts and epithelial cells respectively. Similarly, expression of a dominant negative Met receptor in Xenopus laevis embryos resulted in hypoplasia of
Diagram depicting the signalling pathways associated with Met receptor activation. In the absence of HGF, Met is associated with α6β4 integrin and Fas, this interaction prevents Fas-ligand activating Fas. HGF presented by HSPGs causes phosphorylation of the tyrosine kinase, priming of the docking site, and the dissociation of Fas and Met, allowing Fas-ligand to bind to Fas. Multiple signalling molecules are recruited to the Met docking site and α6β4 integrin as indicated. Dotted arrows indicate indirect interactions.
liver, intestine and kidney (Aoki et al., 1997). Consistent with the epithelial scattering effect of HGF observed in vitro and the sites of HGF/Met expression described above, these mutant mice also have a complete absence of muscle groups of the limb, diaphragm and tongue that derive from migrating myogenic precursor cells (Bladt et al., 1995, reviewed in Birchmeier and Gherardi, 1998). The transcription factor Pax-3 was shown to modulate the expression of Met in these myogenic precursor cells, as mice deficient for Pax-3 show a similar migration defect during limb development and do not express the Met receptor (Epstein et al., 1996; Yang et al., 1996). Whether Pax-3 resides upstream of the Met receptor in other organs remains to be determined. By performing placental tetraploid rescue experiments that allow Met deficient embryos to proceed to term, it was demonstrated that HGF/Met signalling was not required for the development of the skeletal musculature (Dietrich et al., 1999). Further requirements for HGF/Met signalling in developing neurons (as a chemotactic factor for axonal outgrowth and a survival factor for motor neurons), angiogenesis and tissue repair have been described (Maina and Klein, 1999; van der Voort et al., 2000).

Although establishing a definitive role for HGF and Met in a vast array of developmental processes, the use of ligand/receptor null mouse mutants has not been informative in linking particular effector molecules and downstream pathways associated with Met to the defects described above (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). The generation of 'knock-in' mice models containing mutated versions of the Met receptor have begun to couple these defects with specific signalling pathways. Mice containing a knock-in Met receptor that is unable to bind Grb-2, due to a mutation in the docking site, proceed to term (Maina et al., 1996). However, despite normal placental and foetal liver development, these mice possess the migratory muscle defect observed in the full receptor knock-out mutant (Bladt et al., 1995; Maina et al., 1996). Thus, the requirement for HGF in the liver and placenta can be fulfilled by a lower threshold of Met-mediated Grb-2-Sos-Ras signalling. Conversely, myogenic precursor cells require a higher threshold of Grb-2 mediated signalling from the Met receptor, consistent with the requirement for Ras signalling for the motogenic responses to HGF (Hartmann et al., 1994; Maina et al., 1996). It would be interesting to determine whether ablation of indirect Ras activation via the α6β4 integrin/Met receptor interaction would uncover additional developmental roles for HGF/Met signalling. In another knock-in Met receptor model, containing an enhanced binding motif for PI3K in an otherwise inactive docking site, the axonal outgrowth defect was rescued. The control knock-in model containing a fully inactive docking site phenocopied the
Met receptor knock-out mouse (Maina et al., 2001). This study suggests that the PI3K arm of the Met receptor signalling complex is required for the chemotactic response to HGF in vivo.

Mammary development

Since the discovery in 1991 that HGF can induce branching morphogenesis (the generation of epithelial tubules) in kidney epithelial cells (Montesano et al., 1991a), a great deal has been learnt about this process. A number of other epithelial cell types, including mammary epithelia, have been shown to exhibit a similar branching phenotype in response to HGF when grown in collagen gels (Brinkmann et al., 1995; Matsumoto and Nakamura, 1996; Soriano et al., 1995). In the case of mammary epithelial cells, many of the branches in one study possessed structures resembling TEBs, indicative of a prominent role for HGF/Met in ductal branching and elongation (Brinkmann et al., 1995), however in an independent study using primary mammary epithelial organoids, TEB structures were not detected (Niranjan et al., 1995). In addition, branching morphogenesis has been shown to require activation of the invasive growth program that is unique to this family of RTKs. Despite this, scant information exists to implicate HGF/Met signalling in the process of branching morphogenesis in vivo. This is probably due in part to the majority of branching morphogenic programs being activated at the later stages of embryogenesis, subsequent to the observed embryonic lethality of HGF and Met null mice. The use of tetraploid rescue experiments using HGF or Met null mice, or the generation of conditional Met receptor knock-out mice may eventually define a general requirement for HGF/Met in this process. Until these experiments are achieved, the mammary gland provides an accessible model to study these physiological processes in vivo.

Analysis of gene expression in mammary glands by RNase protection assay (Pepper et al., 1995) and Northern blotting and RT-PCR (Niranjan et al., 1995) confirmed that Met was expressed in the mammary epithelium, and HGF was produced by mammary stromal fibroblasts. Both HGF and Met are co-ordinately expressed in the mammary gland throughout the developmental stages described in section 1.1.2a. Furthermore, the stages of maximal expression correlate with the stages of development where mammary branching is at its most intense. Transcripts are first detected at 6 weeks of age in the virgin mouse, and are increased further as the mammary epithelia fills the fat pad up to 12 weeks of age. During pregnancy, levels of expression remain high for the initial phases of increased branching and alveolar development, but decrease rapidly in the second half of pregnancy from
day 12.5, concurrent with the cessation of branching and the initiation of lobulo-alveolar development and milk protein synthesis (Niranjan et al., 1995; Pepper et al., 1995). After lactation expression of neither gene is detectable, but HGF and Met transcription is once again upregulated to pre-pregnancy levels towards the end of involution.

Further evidence for a role in mammary development came from transgenic studies. Overexpression of the ligand or Tpr-Met (Liang et al., 1996; Takayama et al., 1997) led to the development of mammary tumours in mice, however, these studies did not address the consequences of HGF/Met overexpression on normal mammary development. Using organ cultures of dissected mouse mammary glands, the Birchmeier laboratory in Berlin found that exogenous HGF application resulted in ductal branching (Yang et al., 1995). A follow-up study using cultured mammary epithelia established a requirement for Met activation of Gab-1 and PI3K in this process (Niemann et al., 1998). In yet a further study, primary mouse mammary epithelial cells were retrovirally infected to overexpress HGF and injected into cleared fat pads. The reconstituted glands showed hyperplastic branching and an increase in the size and number of TEBs (Yant et al., 1998). Although informative, these studies fail to address a number of issues: firstly, only reconstituted or organ cultured glands were examined, secondly, the effects of pregnancy on HGF/Met function were not examined (this is particularly important as expression of both HGF and Met appears to be under endocrine control, see Kamalati et al., 1999; Soriano et al., 1998). Finally, the effect of Met receptor abrogation was not investigated; this experiment is particularly important as it would allow an examination of mammary development in the absence of, instead of during the hyper-activation of, HGF/Met signalling.

1.2.2 The Fibroblast Growth Factor Receptor-2

FgfR-2 is the second member of a group of 5 structurally related fibroblast growth factor receptors, which together comprise the Type IV RTKs (Powers et al., 2000; Sleeman et al., 2001). These receptors, perhaps with the exception of FgfR-5 which does not possess a tyrosine kinase domain (Sleeman et al., 2001), transduce signals from a family of approximately 22 fibroblast growth factors (Fgfs) (see Ornitz and Itoh, 2001; Powers et al., 2000 for comprehensive reviews of the Fgf family). In Caenorhabditis elegans just two Fgfs have been identified (Egl-17 and Let-756), and one receptor (Egl-15), conversely, in Drosophila, Fgf signalling is mediated by one ligand (Branchless) and two receptors (Breathless and Heartless) (Borland et al., 2001). Thus a relatively simplistic invertebrate signalling system has diverged
through evolution to generate a highly complex signalling network consisting of at least 22 ligands and 5 receptors in mammals.

Most Fgfs are readily secreted from cells, either by means of a classic amino terminal signal peptide that is cleaved, or as in the case of Fgf-9, a non-cleaved signal peptide (Revest et al., 2000). Notable exceptions are: Fgf-1 and Fgf-2 which can be found on the cell surface, high molecular weight forms of Fgf-2 and Fgf-3 that reside in the nucleus, and Fgf-11 – 14 which retain an intracellular location (Ornitz and Itoh, 2001; Powers et al., 2000). Further divisions of the family can be made according to their evolutionary relatedness (Ornitz and Itoh, 2001), and each subfamily tends to share a similar spatial and temporal expression pattern (although each Fgf also has unique sites of expression). Some Fgfs are expressed exclusively during embryonic development (e.g. Fgf-3), whilst others are expressed in both embryonic and adult tissue (e.g. Fgf-7). Furthermore, the Fgfs have an overlapping pattern of receptor binding (i.e. most Fgfs will bind to all four receptors (FgfR-1 – 4) – FgfR-5 has yet to be characterised), which strongly implies a high degree of functional redundancy within the system. Nevertheless, targeted gene-inactivation of separate Fgfs or FgfR family members yields specific phenotype for each factor (see section 1.2.2c, and Ornitz and Itoh, 2001 for a summary of phenotypes).

Following the identification of the first FgfR (FgfR-1) in chicken by affinity chromatography using immobilised Fgf-2 (Lee et al., 1989), Dionne et al. cloned the human homologue of FgfR-1 and a novel receptor with a high affinity to FgfR-1, designated FgfR-2 (Dionne et al., 1990). Subsequently, FgfR-3 and 4 were identified, and a number of splice variants were shown to exist in the family creating numerous soluble or membrane-bound receptor isoforms, each potentially possessing a different biological activity (Johnson and Williams, 1993). Despite the existence of these isoforms, Fgf signalling is now thought to be principally mediated by just 7 FgfRs (excluding FgfR-5) – two receptor isoforms each for FgfR-1 – 3, and one isoform of FgfR-4. The significance of alternate splicing on FgfR-2 function is discussed below.

1.2.2a FgfR-2 structure

FgfR-2 shares a similar structural organisation to the other members of the family, and is depicted in Figure 1.4. The extracellular domain of the receptor consists of two or three immunoglobulin (Ig)-like domains, termed Ig-I, Ig-II, and Ig-III, that are generated by alternative splicing. The functional significance of this splicing
event, that results in the loss of Ig-I, is unknown. A row of 8 acidic amino acids, the 'acid box', is situated between Ig-I and Ig-II and appears to be important for receptor function. A typical single transmembrane domain connects the extracellular portion of the receptor to the intracellular juxtamembrane domain, which contains phosphorylatable tyrosine residues (these are absent in FgfR-3 and FgfR-4). The tyrosine kinase domain contains an insert of approximately 15 amino acids containing two phosphorylatable tyrosine residues that are important for mitogenic activity in FgfR-1 (Wang and Goldfarb, 1997). Further tyrosine residues are present in the carboxy-terminal tail (Klint and Claesson-Welsh, 1999). In contrast to the Met receptor, the distinction between those tyrosine residues integral to the kinase activity of the receptor and those involved in effector molecule recruitment is not clear.

As alluded to briefly above, a specific alternate splicing event leads to the generation of two functionally distinct FgfR-2 receptors (see Figure 1.6A). This mechanism, which is conserved between FgfR-1, -2, and -3, relies on differential exon usage for encoding the carboxy-terminal half of the third immunoglobulin-like domain (Ig-III). Ig-III is encoded by three exons, designated Illa, Illb, or Illc, that are situated contiguously in the coding region of FgfR-2 (Johnson et al., 1991). The amino terminal half of Ig-III is encoded by the invariant exon Illa, however, the second half can be encoded by either the Illb, or Illc exon. Importantly, Ig-III is involved in ligand binding. Thus, alternate splicing of Ig-III leads to the generation of two distinct receptors, termed FgfR-2Illb and FgfR-2Illc, that show different ligand-binding specificities (Miki et al., 1992; Ornitz and Itoh, 2001). In addition, each isoform of FgfR-2 is expressed in a different cell lineage: FgfR-2Illb is expressed in epithelial tissues, whilst the FgfR-2Illc tends to be expressed in mesenchymal cell lineages (Orr-Urtreger et al., 1993). Ligands specific for these receptor splice forms are expressed in adjacent tissues, resulting in a directional signalling event between the mesenchyme and epithelium or vice versa. For instance, cells expressing FgfR-2Illb can be activated by mesenchymal Fgf-3, -7 or -10, whereas these ligands cannot activate cells expressing FgfR-2Illc. Conversely, epitheliaIlb ally expressed Fgf-8 can activate mesenchymally expressed FgfR-2Illc, but not the epitheliaIlb ally expressed FgfR-2Illb receptor (Igarashi et al., 1998; Ornitz and Itoh, 2001; Ornitz et al., 1996). Finally, Fgf-7 appears to be a unique stromal mediator of FgfR signalling as, in contrast to other Fgfs which show a broad overlapping pattern of receptor binding, it can only activate the Illb isoform of FgfR-2 and not other receptors (Ornitz et al., 1996).
1.2.2b  FgfR-2 signalling

Most studies on FgfR-mediated signal transduction have been performed on the prototype family member FgfR-1, however, due to the high degree of similarity between receptors, the mechanisms are probably conserved between family members. It is likely in fact, the main differences in signalling between receptor isoforms resides in the strength of the kinase activity, and not in the recruited effector molecules, nor in the activated downstream signalling cascades (Raffioni et al., 1999).

Receptor activation follows the general paradigm for RTK function described in section 1.2. One important feature is a requirement for HSPGs. These molecules interact with and stabilise Fgf, trapping the growth factor on the cell surface ensuring a paracrine mechanism of activation. Furthermore, they interact directly with the receptor to form a ternary Fgf/HSPG/FgfR complex (Kan et al., 1993). This requirement for HSPGs in FgfR activation has been demonstrated both in vitro (Yayon et al., 1991), and in an in vivo Drosophila model (Lin et al., 1999, reviewed in Omitz, 2000).

Although receptor dimerisation is thought to principally involve like receptors, an additional layer of complexity through receptor heterodimerisation may be an important consideration (Bellot et al., 1991). Receptor activation leads to the recruitment of a number of SH2 domain-containing proteins, some of which are also recruited the Met receptor (see Figure 1.6, reviewed in Klint and Claesson-Welsh, 1999; Powers et al., 2000). Direct recruitment and activation of PLC-γ leads to the hydrolysis of phosphatidyl-4,5-bisphosphate to inositol triphosphate and diacylglycerol, resulting in the release of calcium stores from the endoplasmic reticulum and activation of protein kinase-C family members. This pathway has been shown to be important for mitogenesis and motogenesis in other RTK families, but its role in FgfR signalling is less clear as mutation of the PLC-γ binding site does not affect cell migration and has only limited effects on mitogenesis (Klint and Claesson-Welsh, 1999). Recruitment of the Src kinase may also play a role in the mito- and motogenic response, although this has yet to be shown conclusively. The adaptor protein Crk binds to a juxtamembrane tyrosine upon receptor activation and may be essential for Fgf-mediated mitogenesis through an interaction with Shc (Larsson et al., 1999). Fgf receptor substrate 2 (FRS2) is another adaptor molecule that plays a major role in Fgf-mediated signal transduction by binding directly to FgfRs. Following phosphorylation, FRS2 functions to recruit Grb-2 to the activated receptor, providing an essential link to the Grb-2/Sos/Ras/MEK/MAPK pathway.
Figure 1.6 - Fgf receptor splicing and signalling

A) Alternate splicing leads to the generation of two distinct isoforms of FgfR-2 with different tissue locations and ligand-binding specificities. Depicted is the exon structure of the third Ig-like (Ig-III) and transmembrane domains of FgfR-2, the dotted lines show the alternate splicing events that create the IIlb or IIIc isoforms (colour coded). B) FgfR signalling: ligand presentation leads to activation of FRS2 (which associates with the receptor in the absence of phosphorylation), Crk and PLC-γ and their associated signal transduction cascades. Src activation may also be involved. For diagrammatic purposes, the sub-cellular locations of effector molecules are not depicted accurately.
Introduction

(Fgfrs lack consensus binding motifs for Grb-2). This pathway has been suggested to be important for proliferation and certain differentiative responses to Fgf signalling (Kouhara et al., 1997).

1.2.2c Roles in development

The expression patterns of Fgfs and their receptors, together with the broad range of biological effects they exert, suggests they have fundamental roles in development (reviewed in Powers et al., 2000; Wilkie et al., 1995). Indeed, Fgfs have been implicated in angiogenesis, wound healing, and organogenesis (especially of the lungs, limbs and nervous system), as well as early embryonic patterning events such as gastrulation. The phenotypes of genetically manipulated mice containing null mutations in the various Fgfs/Fgfrs are testament to this (reviewed in Ornitz and Itoh, 2001; Powers et al., 2000). Accordingly, disregulation of the Fgf/Fgfr axis is observed in tumourigenesis (where the pro-angiogenic properties of Fgf signalling can promote tumour progression) and skeletal disorders, such as dwarfism and craniosynostosis syndromes.

The role of Fgfr-2 has been investigated by targeted gene disruption. The functional consequences of this ablation are observed very early on in embryogenesis, and indicate that Fgfr-2 signalling is required at the post-implantation/pre-gastrulation stage of development (Arman et al., 1998). By performing tetraploid rescue techniques to overcome this early lethality or by using an alternative allele of Fgfr-2, additional roles were identified in lung and limb development (Arman et al., 1999; Xu et al., 1998). It is not clear which of these knock-out models represents the true phenotype of an Fgfr-2 null mouse, as the Arman model may have additional dominant negative effects, and the Xu model may contain a hypomorphic allele of Fgfr-2. Although informative, these studies abrogated signalling through Fgfr-2 and did not distinguish between the IIIb and IIIc isoforms of the receptor. In one study a dominant negative Fgfr-2IIIb expressed in the lung bud epithelium of transgenic mice, resulted in a lack of branching and epithelial differentiation in the developing lung implicating the IIIb isoform in lung development (Peters et al., 1994).

By specifically targeting the IIIb exon of Fgfr-2, mice were developed that retained normal function of Fgfr-2IIIc, but did not possess a functional Fgfr-2IIIb receptor (De Moerlooze et al., 2000; Revest et al., 2001a). Consequently, these mice showed a less severe phenotype than mice lacking both isoforms of the receptor, and in addition to uncovering novel roles for Fgfr-2IIIb in mouse development, assigned
some of the phenotypes previously observed in the tetraploid rescue of the full receptor knock-out to ablation of the IIIb isoform of FgfR-2. These mice are viable until birth, but show multiple abnormalities consistent with an interruption of instructive mesenchymal-epithelial signalling. Most strikingly, development of the limbs, lungs and anterior pituitary gland were all severely impaired and further defects were apparent in the salivary glands, inner ear, teeth, skull and skin. FgfR-2IIIb is also essential for proliferation and cellular differentiation of thymic epithelial cells, and mice lacking this receptor possess a severely hypoplastic thymus (Revest et al., 2001b). A general finding from these studies is that: i) organ induction occurs normally in the absence of FgfR-2IIIb, but then extensive apoptosis leads to a failure in organogenesis (e.g. in the limbs), or, ii) the absence of FgfR-2IIIb results in organ dysgenesis characterised by a defect in proliferation (as observed in the thymus) (De Moerlooze et al., 2000; Revest et al., 2001b). Some of these observations were also observed in a transgenic mouse model overexpressing a non-tissue specific soluble dominant negative FgfR-2IIIb receptor driven by the metallothionein promoter (Celli et al., 1998). Interestingly, mice containing a functional germ-line deletion of Fgf-10 exhibit very similar defects to mice deficient for FgfR-2IIIb. Given that Fgf-10 binds with high-affinity to FgfR-2IIIb (Igarashi et al., 1998), it is likely that Fgf-10 is the major ligand for FgfR-2IIIb controlling organogenesis (Ohuchi et al., 2000; Sekine et al., 1999).

Mammary development

The original discovery of Fgf-3, -4, and -8 as proto-oncogenes that had been transcriptionally activated by nearby insertion of MMTV proviral DNA suggests Fgf signalling plays a role in normal mammary gland development (see Powers et al., 2000). Analysis of mammary gland gene transcription has identified several members of the Fgf family and their receptors that are expressed in a developmentally regulated manner (Coleman-Krnacik and Rosen, 1994; Pedchenko and Imagawa, 2000). FgfR-2IIIb is expressed in the mammary epithelium of both the embryonic and postnatal mammary gland (section 1.1.1b, Pedchenko and Imagawa, 2000). In the postnatal gland expression is maximal in the mature virgin, declines during pregnancy and lactation, before rising again after weaning. Consistent with this, FgfR-2IIIb transcripts are more readily detected in isolated ductal epithelia than alveolar cells. The major ligands for FgfR-2IIIb, Fgf-7 and Fgf-10, are expressed in the mammary stroma and follow a similar intensity of expression to the receptor. However, as expression levels of these genes remain constant in cleared, epithelium-free fat pads during pregnancy, the observed decline is probably due to the dilution of mammary stroma concurrent with
epithelial proliferation (Pedchenko and Imagawa, 2000). Fgf-7 is also expressed in the embryonic mammary mesenchyme (section 1.1.1b).

The expression patterns of Fgfr-2Iib and its ligands, together with the branching defect observed in the lungs of mice containing a functional deletion of this gene, point to the possibility of a role in mammary ductal growth and branching. Surprisingly however only a lobulo-alveolar developmental retardation (apparent from the 14\textsuperscript{th} day of gestation) was observed in mice overexpressing a dominant negative Fgfr-2Iib receptor (Jackson et al., 1997).

1.2.3 The ErbB-2 Receptor

ErbB-2 is the second member of a group of four epidermal growth factor receptors (EGFRs) that together comprise the Type I RTK family: ErbB-1 (also called HER-1), ErbB-2 (HER-2), ErbB-3 (HER-3), and ErbB-4 (HER-4). The ErbB nomenclature refers to the viral oncogene \textit{v-ErbB}, which was identified as a transforming gene product in the avian erythroblastosis virus (Vennstrom and Bishop, 1982). It encodes a mutated EGFR lacking the extracellular domain that is involved in activity-regulating ligand binding, and as such is constitutively active and oncogenic. The HER nomenclature refers exclusively to the Type I RTK family in humans (human epidermal growth factor receptors).

ErbB-2 was originally isolated as an activated oncogene in chemically-induced rat neuroglioblastomas that was able to transform NIH-3T3 fibroblasts in cell cultures (Shih et al., 1981). Biochemical analyses determined the oncogene, designated \textit{Neu} in rats, to be a 185kD phospho-protein related to ErbB-1 (Padhy et al., 1982; Schechter et al., 1984). Comparison of oncogenic \textit{Neu} cDNA with the endogenous gene later demonstrated that a single point mutation (T\rightarrow A, causing a valine\rightarrow glutamic acid substitution) in the transmembrane domain of the receptor was sufficient to cause constitutive activation, and thus provide its transforming potential. Subsequent cloning from human cDNA libraries eventually led of the identification of the human homologue of \textit{Neu}, \textit{ErbB-2/HER-2} (Coussens et al., 1985; King et al., 1985; Semba et al., 1985; Yamamoto et al., 1986).

A family of ligands, the EGF-related peptide growth factors, bind the extracellular domain of ErbB RTKs, leading to the formation of receptor dimers (reviewed in Gullick, 2001; Hynes et al., 2001; Riese and Stern, 1998; Yarden and Sliwkowski, 2001). These include EGF, amphiregulin, and transforming growth factor-\textalpha, which bind to ErbB-1; betacellulin, heparin-binding EGF, and epieregulin, which exhibit
dual specificity for ErbB-1 and ErbB-4; neuregulins-1 and -2, which bind to ErbB-3 and ErbB-4; and finally, neuregulins-3 and -4, which bind just ErbB-4. All of these ligands contain a common EGF-like motif, typified by 6 highly conserved cysteine residues that interact covalently to form three loops involved in receptor binding and activation. Despite the abundance of activating ligands, no direct high-affinity ligand for ErbB-2 has been discovered so far, and increasing evidence suggests that the primary function of ErbB-2 is as a co-receptor. Indeed, neuregulin-1 is bivalent, containing two binding sites for ErbB receptors: a high-affinity site for ErbB-3 and ErbB-4, and a low-affinity binding site that preferentially binds ErbB-2 (Tzahar et al., 1997). This may be a common feature of EGF-like ligands, and would suggest that ErbB-2 developed as a low-affinity receptor for many ligands (Yarden, 2001).

The existence of multiple ligands and receptors in mammals with overlapping specificity has led to a vast signalling network from this receptor family, whose routes can be traced back through evolution. In Caenorhabditis elegans for instance, just one ErbB receptor (Let-23) and one ligand (Lin-3) exists (see section 1.3.2c), whereas in Drosophila melanogaster one receptor (DER) controls signalling from four EGF-like ligands (Vein, Spitz, Gurken, and Argos – a negative regulator of DER signalling) (Wasserman and Freeman, 1997).

1.2.3a ErbB-2 structure

The domain structure of ErbB-2 is typical of RTKs. The extracellular domain comprises two cysteine-rich repeats which together mediate ligand binding, a transmembrane domain, an intracellular tyrosine kinase domain and a regulatory carboxy-terminal segment. The main structural differences between ErbB-2 and the Met receptor are evident in the extracellular domain where Met contains two chains, linked by a disulphide bridge, and within the intracellular regulatory domain which is multi-functional (i.e. recruits a broad spectrum of signalling molecules). By contrast, ErbB-2 consists of a single chain and contains multiple tyrosine residues that recruit a relatively specific array of signalling molecules (see Figure 1.4 and Figure 1.7). The other members of the ErbB family exhibit a similar domain structure, however, ErbB-3 contains a defective tyrosine kinase domain and as a result is devoid of intrinsic kinase activity (Guy et al., 1994).

1.2.3b ErbB signalling by hetero- and homodimerisation

Ligand binding to the extracellular domain of the ErbB receptors leads to their activation. This occurs by a similar mechanism as described for the Met receptor, involving dimerisation, intrinsic kinase activity and phosphorylation of key
tyrosine residues that act as docking sites for adaptor proteins and enzymes (see section 1.2.1). Dimerisation can be homodimeric or more commonly heterodimeric, and this activation mechanism provides a horizontal network of differential interactions between the receptors. This, coupled with the relatively non-specific and bivalent nature of the ligands (Tzahar et al., 1997), generates an extremely complex and diverse signalling system that is capable of eliciting different cellular responses. As previously mentioned, ErbB-2 seems not to have a direct high-affinity ligand (Klapper et al., 1999) and ErbB-3 is devoid of kinase activity (Guy et al., 1994), thus in isolation, neither ErbB-2 nor ErbB-3 can support linear signalling by homodimerisation. By contrast, generation of ErbB-2/ErbB-3 heterodimers upon ligand binding allows a complimentary signalling dimer where ErbB-3 is competent to bind high-affinity ligand, and ErbB-2 gives the necessary kinase activity for receptor activation (see Yarden, 2001; Yarden and Sliwkowski, 2001). Moreover, the ErbB-2/ErbB-3 heterodimer is the most mitogenic (Pinkas-Kramarski et al., 1996), and is transforming (Alimandi et al., 1995). Interestingly, heterodimerisation between the Met receptor and Ron has also been reported in vitro (Follenzi et al., 2000), although the physiological relevance of this interaction, if any, has yet to be determined in vivo.

In addition to preferentially forming heterodimers with ErbB-3, ErbB-2 can heterodimerise with ErbB-1 and ErbB-4. Furthermore, the formation of these heterodimers is favoured over homodimeric or other heterodimeric configurations (Graus-Porta et al., 1997; Tzahar et al., 1996). Thus, a signalling hierarchy exists in the ErbB family that is determined by the availability of ligand and the cell-specific ErbB receptor profile. In the presence of ErbB-2, oligomerisation is shifted towards the formation of ErbB-2 containing heterodimers and consequently stronger signalling activity.

As a consequence of the multi-levelled complexity inherent to ErbB signalling, it has been more difficult to link specific signalling cascades originating from receptor dimers with cellular outcomes. This is in contrast to the Met receptor where receptor signalling occurs in a more linear fashion (see section 1.2.1b). Instead, ErbB signalling can be thought of as a network consisting of three layers that transduce signals (Yarden and Sliwkowski, 2001). Firstly, the 'Input Layer' comprises the ligands and their receptors; secondly, the 'Signal-Processing Layer' contains the adaptor proteins and their associated signalling partners that bind to the ErbB carboxy-terminal docking sites; and finally, the 'Output Layer' represents the cellular responses resulting from receptor stimulation. As can be seen in the diagrammatic representation of this model (Figure 1.7), the signalling pathways
that are initiated by ErbB receptor activation are similar to those described for the Met receptor (Figure 1.5). For instance, the Shc/Grb-2/Sos/Ras/MEK/MAPK pathway is an invariable target of ErbB receptor activation; similarly, PI3K activation (either by direct interaction with ErbB receptors, or by indirect coupling to Gab-1) is a common phenomenon amongst this family (Figure 1.7, Alroy and Yarden, 1997; Prenzel et al., 2001; Yarden and Sliwkowski, 2001).

ErbB signalling is further complicated by ligand-independent transactivation of the receptors, leading to tyrosine phosphorylation and recruitment of signalling adaptors. Such transactivation has been reported as a cellular response to many agents including ultraviolet and gamma radiation, in addition to substances that affect the osmotic state of cells. Furthermore, G-protein coupled receptors and growth hormone can result in phosphorylated ErbB-1, leading to the activation of downstream signalling cascades (reviewed in Hackel et al., 1999).

1.2.3c Roles in development

Northern blot and immunohistochemical analysis of ErbB-2 in foetal and adult tissues demonstrated a relatively broad expression pattern. At mid-gestation in the rat, ErbB-2 was detected in a variety of tissues including the nervous system, connective tissue, and secretory epithelium. In the adult rat, secretory epithelial tissues and basal cells of the skin express ErbB-2 (Kokai et al., 1987). In a similar manner to HGF/Met signalling, ErbB-2, its heterodimeric partners, and its ligands act as paracrine signalling molecules and are involved in several developmental processes.

Development of the embryonic cardiac system is the best characterised of these, and clearly demonstrates the principles of ErbB signalling described above (reviewed in Burden and Yarden, 1997; Garratt et al., 2000; Olayioye et al., 2000; Yarden and Sliwkowski, 2001). Mice that contain a homozygous null mutation for either neuregulin-1, ErbB-4 or ErbB-2 exhibit a similar cardiac phenotype and die in utero at E10 - 11 (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995). Lethality in these mice is due specifically to the arrest of trabeculae formation – finger-like extensions of muscle cells that form ridges in the ventricular wall of the heart. The defective embryonic heart is characterised by an irregular beat, enlarged common ventricle and reduced blood flow. Trabeculae formation therefore requires an inductive signalling interaction between neuregulin-1 (expressed in the endocardium) and the two receptors, ErbB-2 and ErbB-4, (expressed in the adjacent myocytes). Moreover, the non-redundant nature of the cardiac defect in these three
Model of ErbB receptor signalling. The 'Input Layer': EGF-like ligands (HB = heparin binding, NRG = neuregulin) can bind to a number of ErbB family members resulting in homo- or heterodimerisation of the receptors. Different receptor pairs have the potential to activate a variety of intracellular signalling effectors within the 'Signal-Processing Layer'. A number of cellular responses are observed after ErbB receptor activation - the 'Output Layer'. Adapted from Alroy and Yarden, 1997 and Yarden and Sliwkowski, 2001.
mice strongly suggests that the functional complex that mediates neuregulin-1 signalling in the heart is an ErbB-2 / ErbB-4 heterodimer. In the case of ErbB-3 deficient mice, the other family member that potentially responds to neuregulin signalling by heterodimerisation with ErbB-2, trabeculation proceeds normally, but these mice show defects in heart valve formation and peripheral nervous system (PNS) development (Erickson et al., 1997; Riethmacher et al., 1997). That these defects represent an inability to form ErbB-2 / ErbB-3 heterodimers in response to neuregulin-1 is strongly implied by the studies of Morris et al. Rescue of the cardiac defect in ErbB-2 null mice, by transgenic expression of ErbB-2 under the control of the heart-specific α-myosin heavy chain promoter, caused a PNS phenotype overlapping with that of mice lacking functional ErbB-3 (Morris et al., 1999). In summary, the phenotypes of ErbB-2 and neuregulin-1 deficient mice appear to be identical, and represent the sum of the phenotypes observed in ErbB-3 and ErbB-4 deficient mice.

Mammary development

The early embryonic lethality observed in ErbB-2 deficient mouse embryos obscures potential roles for the gene in late embryonic and adult development. However, a postnatal function for ErbB-2 has been delineated in the mammary gland. All four ErbB receptors are expressed in a developmentally regulated pattern in the mammary gland (Darcy et al., 2000; Schroeder and Lee, 1998). Whereas stromally expressed ErbB-1 appears to have a role in mammary ductal growth (see section 1.1.2b, Wiesen et al., 1999), ErbB-2 has been implicated in the later stages of mammary development. Mice expressing of a dominant negative ErbB-2 receptor in mammary epithelia exhibit defective lobulo-alveolar development and impaired lactation (Jones and Stern, 1999). This finding is consistent with organ culture and transplantation studies that have identified a role for neuregulin and ErbB-2 in the morphogenic programs that lead to lobulo-alveolar development (Niemann et al., 1998; Yang et al., 1995). Interestingly, the dominant negative expression of ErbB-2 in the studies by Jones et al. was driven by the mouse mammary tumour virus long terminal repeat (MMTV-LTR) promoter, which targets expression to a predominantly epithelial mammary tissue (Jones and Stern, 1999). Given that a convincing stromal requirement for ErbB-1 involvement in ductal growth has been defined (Wiesen et al., 1999), and that ErbB-2 is the preferred dimerisation partner for other family members, a potential requirement for stromal ErbB-2 in ductal development, through heterodimerisation with ErbB-1, may have been missed in these analyses.
1.3 Epithelial Cell Polarity

Critical to the development and survival of an organism is the ability of cells to group areas of cytoplasm and plasma membrane into spatially and functionally distinct sub-domains. Compartmentalising the cell in this way facilitates diverse cellular processes, such as directional membrane growth, nutrient uptake, vectorial transport and cell migration, and allows cells to adopt different morphological and functional states to perform specialised tasks. This cellular asymmetry or polarity is a fundamental property of virtually all eukaryotic cells and is inextricably linked to development and organ function in more complex organisms. Moreover, the generation of cellular polarity allows asymmetric cell division to be achieved; this crucial process occurs in certain specialised cells and permits specific cell types to be placed at defined locations during embryogenesis (reviewed in Knoblich, 2001).

While most, if not all cell types exhibit some degree of polarisation, the most widely studied are neurons and epithelial cells. Although morphologically and functionally distinct from each other, studies have shown that the mechanisms which initiate, regulate, and maintain the polarised phenotype of these two cell lineages are similar (see Colman, 1999 for review), and probably represent a conserved mechanism.

1.3.1 The Polarised Epithelial Cell Phenotype

Epithelium is one of the principal tissue types of the body. In the developing embryo, the morphogenesis of a sheet of polarised epithelial cells (the trophectoderm) is the first overt sign of cellular differentiation. In the adult, epithelial cells line all of the body cavities and tissues, providing a barrier function that separates the external luminal environment from the internal extracellular milieu. Furthermore, they perform a vectorial function in a variety of organs, for example in the kidney, regulating the local ionic environment by carrying out specialised transport activities such as absorption, secretion and transcytosis. As described in section 1.1, polarised epithelial cells form the majority of cell mass in the mammary gland, mediate most of the developmental changes during pregnancy, and synthesise and secrete milk during lactation.

1.3.1a Epithelial cell structure

Epithelia come in many different organ-specific forms, but can be classified into three basic types: (1) the simple epithelium, in which a single layer of cells sits on a
basement membrane; (2) stratified epithelium where layers of epithelia are stacked on top of each other, with only the lowest layer resting on the basement membrane; and (3) pseudo-stratified epithelium where the cells pile up, but still retain some contact with the basement membrane. Stratified and pseudo-stratified epithelia develop from the simple form, and it is this class of epithelia that has been studied most thoroughly, principally through the use of cells isolated from the lining of the kidney and intestine. Simple epithelia are exposed to different environments on each of side of the cell and as a consequence exhibit a cell surface polarity that consists of at least three domains. The apical domain usually faces the organ lumen and is often rich in microvilli or cilia. By contrast, the basal domain is attached to the basement membrane, and the lateral domain is in contact with neighbouring epithelial cells through desmosomal and adherens junctions. The basal and lateral domains are often classified as a single 'basal-lateral' or 'basolateral' domain. The apical and basolateral domains are separated by tight junctions which form a barrier to the intercellular diffusion of ions and macromolecules (see Figure 1.8). The various junctional complexes and the cytoskeletal networks that connect them, coupled with the asymmetric distribution of protein and lipid components, gives epithelial cells the strength and selective permeability required for their primary function.

1.3.1b Mechanisms involved in the establishment of epithelial cell polarity

Essential to the generation of epithelial cell polarity is the detection of spatial cues (reviewed in Wodarz, 2002). Evidence that these cues involve cellular adhesion events came from studies using isolated epithelial cells (reviewed in Yeaman et al., 1999). When epithelial cells were grown in suspension, and therefore lacked cell-cell or cell-substratum contact, they exhibited a non-polarised distribution of marker proteins of the apical and basolateral membrane domains, indicating that the apico-basolateral axis, essential to epithelial polarity, was not yet established (Rodriguez-Boulan et al., 1983; Wang et al., 1990). However, upon cell-cell or cell-substratum contact an initial level of cell polarity was achieved, as evidenced by the segregation of membrane marker proteins into contacting and non-contacting membrane surfaces.

Adhesion between two cells in culture is sufficient to cause an endogenous apical protein (gp135) to locate to the free, non-contacting cell surface. Conversely, the basolateral proteins Na^+-K^+-ATPase and E-cadherin become localised to the contacting cell surface (Wang et al., 1990), perhaps indicating at this early stage of epithelial morphogenesis, that the contacting surface will form the basolateral
Figure 1.8 - The polarised epithelial phenotype

Representation of a polarised epithelial cell. Left cell: depiction of sub-cellular domains and junctional complexes as indicated. Right cell: proteins destined for the cell surface can be transported in specialised endosomes to the apical membrane (blue ovals), or to the basolateral membranes (green ovals).
domain and the free surface the apical domain. The whole process seems to be mediated by the onset of adhesion, and it is E-cadherin, a member of the calcium dependent cadherin superfamily of adhesion receptors, that is the principal component of cell-cell adhesion junctions. That E-cadherin is central to the development of the epithelial phenotype was demonstrated most noticeably by McNeill et al, who showed that ectopic expression of E-cadherin was sufficient to induce the segregated distribution of endogenous proteins in L-fibroblasts that lacked the domain characteristics of epithelial cells (McNeill et al., 1990).

Cell-substratum adhesion is driven by members of the integrin superfamily. These transmembrane signalling molecules link elements of the extracellular matrix (ECM) with cytoskeletal components within the cell and appear to be important in generating membrane asymmetry. Upon attachment to the ECM, several apical marker proteins become localised to the free non-contacting membrane (Ojakian and Schwimmer, 1988; Vega-Salas et al., 1987); furthermore, the free surface forms microvilli reminiscent of a true apical membrane. However, in contrast to cell-cell mediated polarisation, cell-substratum contact is not sufficient to cause a polarised distribution of basolateral proteins (Nelson and Veshnock, 1986).

The formation of tight junctions at the apico-lateral border is another important step in the morphogenesis of a polarised epithelium and signifies the generation of an apico-basal axis. By analysing the distribution of the protein zona occludens-1 (ZO-1), an integral part of the tight junction complex, it was demonstrated that cell-substratum adhesion was required for the formation of tight junctions. In the presence of cell-cell contacts, ZO-1 was co-localised with E-cadherin along the contacting surface and did not form a junctional complex (Wang et al., 1990). Thus the apico-basal axis of polarity is established only after integrin-mediated adherence to the ECM.

It seems likely therefore that a combination of cell-cell and cell-ECM adhesion is required to provide the spatial cues for the initial steps in the morphogenesis of a polarised epithelium. Once the initial cue has been established, a localised assembly of signalling and cytoskeletal networks is formed, leading to an enhancement of membrane asymmetry (see Davies and Garrod, 1997; Drubin and Nelson, 1996; Knust, 2000; Rodriguez-Boulan and Nelson, 1989; Yeaman et al., 1999 for review; see Michaux et al., 2001 for review focusing on in vivo epithelial biology). Membrane structures that specify delivery and retention of newly synthesised membrane proteins are produced and this reinforces membrane asymmetry. Once fully established, the epithelial phenotype is maintained by the
selective targeting of protein and lipid components to their correct sub-cellular domains.

1.3.2 Protein Sorting and Traffic in Polarised Epithelial Cells

The turnover of proteins at the cell surface necessitates the continuous synthesis and correct delivery of new proteins in order to maintain epithelial polarity. Newly synthesised proteins are sorted in the trans-Golgi network (TGN), from there they are delivered in vesicles to either the apical or basolateral membrane domains. The fidelity of vectorial delivery is facilitated by the organisation of the secretion apparatus and cytoskeleton generated during the morphogenesis of a polarised epithelium. However, accurate targeting of membrane proteins also requires the presence of signals that specify the delivery fate of the targeted protein. In addition, a recognition event must also take place to interpret this signal and direct the protein to its correct location.

That polarised protein targeting is critical for epithelial integrity and function was recently highlighted by Koivisto et al. By analysing mutations found in familial hypercholesterolaemia they demonstrated that mutation of a critical glycine at position 823 of the LDL receptor caused the protein to be mis-localised in kidney epithelial and hepatic cells (Koivisto et al., 2001). The consequence of this mis-localisation was an inability of the receptor to mediate cholesterol clearance in vivo. This observation is the first to directly link pathogenesis of a human disease to defects in protein sorting. Defects in polarised protein sorting also underlie the clinical disorder autosomal polycystic kidney disease, characterised by a general deregulation of vesicle trafficking. However the mechanisms that underlie this defect are yet to be elucidated.

1.3.2a Post-Golgi biosynthetic trafficking

Direct and indirect pathways exist for a newly synthesised protein to reach the cell surface (Matter, 2000; Zegers and Hoekstra, 1998). In the direct route, protein sorting occurs in the TGN where proteins are packaged into vesicles which are then transported to either the apical or basolateral membrane domains. In contrast, proteins taking the indirect route to the cell surface are packaged into vesicles at the TGN destined for the basolateral membrane domain. From there, apical proteins are endocytosed and reach the apical membrane by a transcytosis event. Another less common mechanism involves a process known as selective stabilisation. In this pathway, proteins are randomly sorted to the cell membrane, but are only stabilised
Introduction

on one particular membrane, usually the basolateral membrane. Those proteins that have reached the ‘wrong’ cell surface are then either degraded or transcytosed to the ‘correct’ cell surface. The relative importance of each pathway is dependent upon both the cell type and the protein. For instance, Madin-Darby canine kidney (MDCK) cells utilise mainly the direct route from the TGN to the apical or basolateral membranes, whereas CACO-2 cells and hepatocytes depend heavily on indirect sorting.

Sorting from the TGN to the cell surface is clearly an important process for generating and maintaining epithelial polarity. However, only 5% of total cell surface proteins are newly synthesised per hour. In contrast, approximately 50% of cell surface proteins are endocytosed per hour. This discrepancy is corrected by efficient recycling of endocytosed products back to the cell surface; thus correct polarised sorting in the endocytic pathway is also crucial for the maintenance of epithelial polarity (Mostov et al., 2000). In many proteins, for example the LDL receptor (Matter et al., 1993), the signals that control TGN to cell surface sorting also regulate sorting in the endocytic compartment, indicating that sorting and biosynthetic pathways may utilise similar mechanisms.

1.3.2b Sorting of proteins to the apical membrane domain

Apical sorting signals

The mechanisms regulating protein sorting to the apical membrane domain are still relatively unclear, although evidence suggests that signals contained within the membrane anchor, luminal (extracellular) and/or transmembrane domain of the protein are important. Unlike the hydrophobic signal sequences that specify protein translocation across the rough endoplasmic reticulum membrane, apical sorting signals appear to be cryptic and may rely more on protein structure or post-translational modifications than linear peptide based sorting signals which have not been identified to date.

There are multiple types of signals that are thought to specify an apical location. One of the original hypotheses suggested the use of glycosylphosphatidylinositol (GPI) anchors as apical sorting signals. GPI anchors allow proteins to associate with glycosphingolipid-cholesterol rafts (lipid microenvironments within a plasma membrane that can incorporate proteins) destined for the apical membrane. Initially this notion gained credence as endogenous proteins containing a GPI anchor are found apically in epithelial cells; moreover, if cellular cholesterol is depleted with β-methyl-cyclodextrin then apical delivery of GPI-anchored proteins
is inhibited (Keller and Simons, 1998). Furthermore, addition of a GPI-anchor onto the vesicular stomatitis viral glycoprotein reversed the polarity of this protein, causing it to localise to the apical membrane in epithelial cells (Brown et al., 1989). However, it was recently discovered that a GPI anchor is not always sufficient to direct a raft-associated protein to the apical surface, suggesting other signals are required (Benting et al., 1999). Moreover, Fisher rat thyroid epithelial cells sort most of their GPI-anchored proteins to the basolateral membrane (Zurzolo et al., 1993).

Oligosaccharides, both N- and O-linked, have also been suggested to act as apical sorting signals (Breuza et al., 2002; Gut et al., 1998; Kitagawa et al., 1994; Yeaman et al., 1997). Gut et al. convincingly demonstrated that addition of N-linked carbohydrates to occludin and chimeric ERGIC-53 or Fc receptors induced apical targeting (Gut et al., 1998). These proteins accumulated in the Golgi in the absence of carbohydrate moieties, however, there are numerous exceptions, suggesting again that oligosaccharides are only one of multiple mechanisms for apical targeting. It is not clear whether oligosaccharides act directly as an apical targeting signal, or whether the 'signal' is indirect, affecting the structural configuration of the protein (reviewed in Rodriguez-Boulan and Gonzalez, 1999). Interestingly, most GPI anchored proteins are also N-glycosylated in their extracellular domain, creating the possibility that glycosylation and not the GPI anchor specifies apical localisation. This seems to be the case in an apically localised modified rat growth hormone (Benting et al., 1999), although the validity of this study is questionable as a modified protein containing N-glycosylation sites and a GPI membrane anchor was used, rather than an endogenous apical protein containing both of these putative targeting determinants. Table 1.2 categorises the proposed sorting signals of a number of apical proteins.

Recognition of apical sorting signals

The cellular machinery that recognises apical sorting signals have yet to be identified, and are likely to be as diverse as the signals themselves. One potential mechanism involves the recognition of carbohydrate moieties by transmembrane lectins, but although compelling, the evidence for this hypothesis is weak.

Thus, while some proteins may interact directly with rafts to reach the apical surface, others might use their carbohydrate chains to interact with unidentified apical sorting machinery, and still others might use a combination of both, or alternative mechanisms.
Introduction

Table 1.2 - Apical sorting signals

<table>
<thead>
<tr>
<th>Apical Protein</th>
<th>Sorting Signal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placental alkaline phosphatase</td>
<td>GPI anchor</td>
<td>Brown et al., 1989</td>
</tr>
<tr>
<td>Decay accelerating factor</td>
<td>GPI anchor</td>
<td>Lisanti et al., 1989</td>
</tr>
<tr>
<td>Thy-1</td>
<td>GPI anchor</td>
<td>Wilson et al., 1990</td>
</tr>
<tr>
<td><strong>Extracellular carbohydrate-dependent sorting signals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P75 neurotrophin receptor</td>
<td>O-glycosylation</td>
<td>Breuza et al., 2002; Yeaman et al., 1997</td>
</tr>
<tr>
<td>gp-80</td>
<td>N-glycosylation</td>
<td>Urban et al., 1987</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>N-glycosylation</td>
<td>Kitagawa et al., 1994</td>
</tr>
<tr>
<td><strong>Transmembrane sorting signals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influenza virus neuraminidase</td>
<td>Transmembrane domain</td>
<td>Kundu et al., 1996</td>
</tr>
<tr>
<td>Influenza virus haemagglutinin</td>
<td>Transmembrane domain</td>
<td>Lin et al., 1998</td>
</tr>
</tbody>
</table>

Apical sorting signals are very diverse in nature, but can be divided into three disparate groups - membrane anchored, extracellular, and transmembrane. Listed are a number of apically localised proteins whose apical sorting signals have been characterised.

1.3.2c Targeting of basolateral proteins

**Basolateral sorting signals**

Targeting determinants that specify basolateral sorting in the TGN and endosomes were first identified in the polymeric immunoglobulin receptor (pIgR) (Casanova et al., 1991). It was established that deletion of the entire cytoplasmic domain of the receptor (except for two amino acids proximal to the transmembrane domain) caused a mis-localisation of pIgR to the apical domain. Subsequent cytoplasmic domain truncations combined with internal deletions of the pIgR revealed a 14 amino acid signal that was necessary and sufficient for basolateral localisation. The signal was shown to be autonomous as it could redirect a heterologous apical protein (placental alkaline phosphatase) to the basolateral membrane. Sequential alanine substitution mutations later revealed that three amino acids (His-656, Arg-657 and Val-660) within this basolateral signal were key to its function (Aroeti et al., 1993). Before these discoveries it had been assumed that delivery of a protein to the basolateral membrane was the default pathway for newly synthesised membrane proteins. The creation of mutated proteins, together with cell surface imaging and labelling techniques, have identified the basolateral sorting sequences of a multitude of basolateral membrane associated proteins. It is now well...
established that transport of proteins to the basolateral surface can be mediated by
distinct amino acid sequences in the cytoplasmic domain of membrane proteins
(usually in a juxtamembrane position). Most basolateral signals identified are
generally dominant over apical signals.

Although these signals are somewhat variable, they can nevertheless be categorised
into three groups. A first set of signals rely on an essential tyrosine residue in close
proximity to a bulky hydrophobic residue. The second type consists of a
di-hydrophobic pair of amino acids. These two groups of basolateral sorting signal
are at least superficially related to, and are often found to be co-linear with,
endocytosis signals. Growing evidence suggests they are recognised by a similar
set of proteins as discussed below. Finally, an expanding group of basolateral
sorting signals bear no resemblance to endocytosis signals and are not tyrosine- or
di-hydrophobic based. Table 1.3 gives a comprehensive list of known basolateral
sorting signals.

Tyrosine-based basolateral sorting motifs normally conform to the sequence NPXY
or YXXΦ (where X is any amino acid and Φ is a bulky hydrophobic residue). In
several cases these sequences have been proposed to form a tight β-turn that is
essential for their function in endocytosis (Collawn et al., 1990; Eberle et al., 1991).
Di-hydrophobic based basolateral sorting signals are normally comprised of a
di-leucine motif, but can also be leucine-isoleucine or leucine-valine (see Table 1.3).
It is unclear whether di-hydrophobic motifs can form tight β-turns, however a
number of basolateral sorting signals unrelated to endocytosis signals have been
proposed to form a tight β-turn suggesting the same cellular machinery may
interact with these signals (Aroeti et al., 1993; Le Gall et al., 1997). In addition to
these defined peptide sequences, many basolateral sorting signals are coupled with
acidic-rich clusters of amino acids in close proximity, the significance of which
remains to be determined (Madrid et al., 2001; Matter et al., 1992; Simmen et al.,
1999).

PDZ domain-containing proteins - protein sorting and stabilisation

Both selective targeting and stabilisation involve PDZ domain-containing proteins.
These proteins can act in trans to correctly target or to retain membrane proteins at
the apical or basolateral domain of epithelial cells, as well as at the pre- or
postsynaptic membranes of neurons. In addition it is hypothesised that PDZ
domain-containing proteins organise protein modules at the cell surface creating
polarised signalling complexes (reviewed in Fanning and Anderson, 1999; Kim,
1997).
Table 1.3 - Basolateral sorting signals

<table>
<thead>
<tr>
<th>Basolateral Protein</th>
<th>Sorting Signal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL receptor</td>
<td>NPVY EDE, GYSY EDD</td>
<td>Matter et al., 1992</td>
</tr>
<tr>
<td>Vesicular stomatitis virus glycoprotein</td>
<td>Y + surrounding sequences</td>
<td>Thomas et al., 1993</td>
</tr>
<tr>
<td>HIV-1 envelope glycoprotein</td>
<td>YSPL</td>
<td>Lodge et al., 1997</td>
</tr>
<tr>
<td>Aquaporin 4</td>
<td>YMEV ETEDLIL</td>
<td>Madrid et al., 2001</td>
</tr>
<tr>
<td>IL-6R (gp80)</td>
<td>YSLG and PLI</td>
<td>Martens et al., 2000</td>
</tr>
<tr>
<td>Di-hydrophobic sorting signals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG Fc receptor</td>
<td>LL</td>
<td>Hunziker and Fumey, 1994</td>
</tr>
<tr>
<td>Furin</td>
<td>FI EEDE</td>
<td>Simmen et al., 1999</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>LL</td>
<td>Miranda et al., 2001</td>
</tr>
<tr>
<td>CD44</td>
<td>LV</td>
<td>Sheikh and Isacke, 1996</td>
</tr>
<tr>
<td>NPP1</td>
<td>LL</td>
<td>Bello et al., 2001</td>
</tr>
<tr>
<td>MHC class I chain-related MICA</td>
<td>LV</td>
<td>Suemizu et al., 2002</td>
</tr>
<tr>
<td>Other sorting signals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ErbB-1</td>
<td>KRTLRRLLGERELVEPLTPSGEA</td>
<td>Hobert and Carlin, 1995; Hober et al., 1997</td>
</tr>
<tr>
<td>NCAM</td>
<td>AFSKDESEPKPEVREETPN-HDDGGKHTEPNETTPLTE</td>
<td>Le Gall et al., 1997</td>
</tr>
<tr>
<td>Transferrin receptor</td>
<td>VDGDNSSHVEMLA</td>
<td>Odorizzi and Trowbridge, 1997</td>
</tr>
<tr>
<td>plgR</td>
<td>RHRRN/DRVSIGSYR</td>
<td>Casanova et al., 1991</td>
</tr>
</tbody>
</table>

Basolateral sorting signals can be divided into three distinct types: i) tyrosine-based, ii) di-hydrophobic based (both related to clathrin-mediated endocytosis signals), and iii) other signals unrelated to endocytosis signals. Listed are a number of proteins whose basolateral sorting signals have been identified.

The PDZ domain was originally characterised as a 90 amino acid motif found in three proteins of the membrane-associated guanylate kinases (MAGUKs): PSD-95, a synaptic protein; DLG-1, a Drosophila protein found at cell junctions; and ZO-1, a component of tight junctions as described above. The PDZ domain is a protein-protein binding domain that usually interacts with a short amino acid sequence at the extreme carboxy-terminus of its binding partners. The binding specificities of PDZ domains are dictated by i) S/T-X-V/I/L motif (class I PDZ), ii) Ψ-X-Ψ motif
(where \( \Psi \) is a hydrophobic residue, class II PDZ), and iii) D-X-V motif (class III PDZ) (Borg et al., 2000; Songyang et al., 1997).

An in vivo role for PDZ domain-containing protein involvement in polarised localisation has been delineated by analysing Let-23 function in *Caenorhabditis elegans* (reviewed in Kim, 1997; Rongo, 2001). Let-23, the orthologue of mammalian epidermal growth factor receptor, was shown to be crucial for vulval induction through an activating interaction with the EGF-like ligand Lin-3. Localisation studies demonstrated that Let-23 is present at the basolateral membrane of vulval precursor cells, where it lies in close proximity to the anchor cells that locally secrete Lin-3. When Let-23 signalling was abrogated through inactivating mutations of *Let-23* or *Lin-3*, a vulvaless phenotype was observed; by screening for this phenotype in mutant worms, several vulval induction genes were isolated—including *Lin-2*, *Lin-7* and *Lin-10*. The gene products were characterised and shown to contain PDZ domains which, when mutated, led to a mis-localisation of Let-23 to the apical membrane (Simske et al., 1996; Whitfield et al., 1999). By using a yeast two-hybrid screen, in vitro binding, as well as co-immunoprecipitation techniques, it was shown that Lin-10 can bind to Lin-2, which in turn can bind Lin-7, which subsequently binds a PDZ binding signal present at the carboxy-terminus of Let-23 (Kaech et al., 1998). As Let-23 overexpression can suppress the vulvaless phenotype in *Lin-2*, *Lin-7* or *Lin-10* negative worms, it is likely that the PDZ proteins do not have additional signalling functions downstream of Let-23, but act instead to localise Let-23 to the basolateral membrane (Simske et al., 1996). It is of interest to note that the mammalian homologues of these proteins also form tripartite complexes when isolated from brain lysates, although it has yet to be established if the mechanism of localisation is conserved between worms and mammals (reviewed in Rongo, 2001).

The formation of PDZ domain-containing protein complexes is crucial for the localisation of a number of proteins. For example, in mammalian cells, ErbB-2 contains a PDZ binding motif at its carboxy-terminus (GLDVPV) that interacts with a PDZ domain-containing protein called ERBIN. ErbB-2 is localised to the basolateral membrane of epithelial cells when bound to ERBIN, however, if this interaction is abrogated by deleting the ERBIN binding site from ErbB-2, then the receptor becomes apically localised (Borg et al., 2000). Moreover, when the ERBIN binding site is fused to a heterologous apical protein, it redirects the protein to the basolateral membrane, suggesting that an interaction with ERBIN is sufficient for basolateral localisation (Jaulin-Bastard et al., 2001).
PDZ domain-containing proteins also play a role in protein stabilisation at the membrane by preventing internalisation. Much controversy exists as to the role of PDZ domain-containing proteins with regard to epithelial polarity, as many reports fail to distinguish between a role in selective targeting of a membrane protein and selective stabilisation, or retention of the protein at the plasma membrane. At least for the GABA transporter, which is localised to the basolateral membrane in MDCK cells, interactions with PDZ domain-containing proteins cause GABA retention at the cell surface and do not participate in the active targeting of the protein (Perego et al., 1999).

Regulation of basolateral sorting

As described above, one striking feature of many basolateral sorting signals is their resemblance to signals involved in clathrin-mediated endocytosis and lysosomal trafficking. The molecular basis of endocytic signal recognition is well understood and involves direct interactions with clathrin adaptor complexes at the plasma membrane. It was therefore postulated that basolateral sorting might also involve interactions with similar, or identical adaptor complexes. Preliminary support for this hypothesis came from the finding that pIgR could be immunoprecipitated with the adaptor complex AP1 (Orzech et al., 1999).

Four adaptor protein complexes have been discovered to date in mammals (AP1 - 4). All four adaptor complexes are heterotetrameric and consist of two large subunits (β, and a more divergent subunit, either γ, α, δ or ε), a medium (μ) and a small (σ) subunit. The adaptor complexes have distinct cellular locations, AP1 is found in clathrin-coated buds in the TGN and in endosomes; AP2 is generally found at the plasma membrane and is involved in endocytosis; AP3 is located at the TGN and a post-endosomal compartment; and AP4 co-localises with markers of the TGN (reviewed in Heilker et al., 1999; Robinson and Bonifacino, 2001). Although all four complexes have been shown to bind basolateral/endocytic signals, only AP1 and AP4 have been implicated directly in sorting of basolateral proteins. In the case of AP1, interaction with tyrosine based determinants is mediated by the μ subunit, and di-hydrophobic determinants interact with the β subunit (Ohno et al., 1995; Rapoport et al., 1998). It is unclear whether basolateral sorting motifs that do not resemble clathrin-mediated endocytosis signals interact with AP1, however the basolateral sorting signal of the transferrin receptor can apparently interact with AP4, at least in vitro (Simmen et al., 2002).

Despite strong circumstantial evidence for a role in basolateral sorting, only recently, with the discovery of a novel AP1 subunit called μ1B, has a specific
adaptor complex been implicated in this process (Folsch et al., 1999; Ohno et al., 1999). \(\mu 1B\) shows 79% homology to the previously identified medium subunit of AP1 (now termed \(\mu 1A\)). \(\mu 1A\) and \(\mu 1B\) complex with AP1 specific subunits (\(\gamma\), \(\beta 1\), and \(\sigma 1\)) to form two TGN-associated adaptor complexes AP1A and AP1B. Unlike AP1A which is ubiquitously expressed, AP1B appears to be present only in polarised epithelial cells (including CACO-2 and MDCK cells which are routinely used for studying epithelial polarity), suggesting that it may be important for polarised trafficking. That \(\mu 1B/\text{AP1B}\) is required for basolateral targeting was demonstrated by Fölsch et al. using LLC-PK1 cells, a porcine derived epithelial cell line originating from renal proximal tubules (Folsch et al., 1999; Folsch et al., 2001). Unlike most epithelial cells, LLC-PK1 cells do not express the \(\mu 1B\) subunit and, as a consequence, mis-sort basolateral proteins containing a tyrosine based basolateral determinant to the apical membrane. For instance, the LDL receptor, human poliovirus receptor \(\alpha\), and the H,K-ATPase\(\beta\) are all apically localised in LLC-PK1 cells (Folsch et al., 1999; Ohka et al., 2001; Roush et al., 1998). By contrast, sorting of apical and basolateral proteins containing a di-hydrophobic motif, such as E-cadherin or FcRII-B2, are unaffected by the absence of \(\mu 1B\) (Miranda et al., 2001; Roush et al., 1998). By exogenously expressing the \(\mu 1B\) subunit in LLC-PK1 cells it was possible to rescue this 'defect' in protein sorting, restoring a basolateral localisation of the mis-sorted LDL receptor (Folsch et al., 1999). Interestingly, the mis-localisation of the transferrin receptor could also be rescued by exogenous \(\mu 1B\) expression, indicating a possible interaction with non-tyrosine based sorting motifs. Exogenous \(\mu 1B\) expression also enhanced the overall polarised phenotype of LLC-PK1 cells, presumably by correctly localising a number of basolateral membrane proteins contributing to the establishment and maintenance of polarity. Hence, AP1B interaction is required for correct membrane targeting of a subset of basolateral proteins, and at least in the case of the human poliovirus receptor \(\alpha\), this interaction is direct (Ohka et al., 2001).

Although the functional importance of AP1B in epithelial cells is clear, so too is the requirement for other proteins that mediate basolateral sorting, such as AP4. In MDCK cells where levels of the medium subunit of AP4 (\(\mu 4\)) have been depleted using an antisense \(\mu 4\) cDNA construct, a number of basolateral proteins are mis-localised. Further, surface plasmon resonance binding experiments using fractionated AP4 and synthetic peptides revealed that AP4 could bind to a variety of basolateral targeting signals (Simmen et al., 2002). Interestingly, by indirectly analysing endogenous LDL receptor localisation by measuring the cellular uptake of LDL, the authors showed a requirement for AP4 in basolateral LDL receptor
localisation, further to the requirement of AP1B demonstrated by Fölsch et al. It appears therefore the LDL receptor uses at least two independent pathways to reach the cell surface, which are not functionally redundant. However, it cannot be discounted that overexpression of μ1B affects the fidelity of AP4, and/or antisense μ4 indirectly interrupts AP1B complex formation/function. As defects in basolateral targeting of proteins containing di-hydrophobic targeting signals have rarely been described, it would be interesting to determine whether the trafficking of these proteins are affected by depletion, or mutation of the di-hydrophobic motif binding site of the β subunit of AP1A and AP4.

Hence, AP1B and AP4 can mediate basolateral sorting in kidney cells in vitro. However, given that defects in basolateral sorting are incomplete in these models, coupled with the fact that other polarised cells, such as neurons and hepatocytes do not express μ1B (yet utilise the same targeting determinants to localise proteins to the somatodendritic and basolateral domains respectively), there must be other mechanisms involved in basolateral signal recognition and sorting. Other candidate sorters include AP3 which can bind to some di-hydrophobic signals and the GGA proteins (Honing et al., 1998). The GGAs (Golgi-localised γ ear-containing, ARF binding proteins) consist of three monomeric family members in humans, and are characterised by a four domain structure (reviewed in Black and Pelham, 2001; Boman, 2001; Robinson and Bonifacino, 2001). Recent evidence suggests that the GGAs act in combination with the adaptor protein complexes to sort proteins from the TGN to the endosomal/lysosomal compartments, and that they can recognise di-leucine sorting signals (Puertollano et al., 2001). The importance of GGA-mediated transport in basolateral sorting has yet to be determined.

Recent studies also indicate an unexpected role for the small GTPase cdc42 in basolateral trafficking. In MDCK cells depleted of cdc42, basolateral transport is impaired whereas apical trafficking remains unaffected. This phenotype does not reflect a major alteration of the actin cytoskeleton, but rather results from the selective inhibition of membrane traffic in both the endocytic and secretory pathways (Kroschewski et al., 1999). cdc42 is likely to be involved in the generation of different populations of transport vesicles, and also in controlling the exit of these vesicles from the TGN through minor modulation of the actin cytoskeleton (Musch et al., 2001).
Aims

The main aims of this thesis concern the analysis of RTKs function in the mammary gland. A dual approach was taken to achieve this: i) to determine the functional consequence of RTK signalling abrogation on the morphogenesis of the mammary gland, and ii) to examine the signals that are involved in intracellular RTK targeting in polarised epithelial cells.

Specifically, the following areas were examined:

- The identification and characterisation of basolateral targeting signals that control intracellular trafficking of the ErbB-2 RTK in epithelial cells
- The role of the Met RTK in the development of postnatal mammary gland development
- The requirement for FgfR-2IIIb RTK signalling in embryonic and postnatal mammary gland development
Chapter 2 | Materials and Methods

Materials

2.1 Chemicals/Reagents

With the exceptions of those listed below, all chemicals/reagents were obtained from Sigma-Aldrich or BDH/Merck.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% acrylamide mix</td>
<td>Anachem</td>
</tr>
<tr>
<td>4-Nitro blue tetrazolium chloride</td>
<td>Roche</td>
</tr>
<tr>
<td>Agarose</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>Blasticidin S HCl</td>
<td>Invitrogen Life Technologies</td>
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<td>Bromophenol Blue</td>
<td>BIO-RAD</td>
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<td>D-19 developer</td>
<td>Kodak</td>
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<td>Digoxigenin-NTP mix</td>
<td>Roche</td>
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<td>dNTPs</td>
<td>Amersham Biosciences</td>
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<td>Ethidium bromide</td>
<td>BIO-RAD</td>
</tr>
<tr>
<td>EZ-link™ Sulfo-NHS-biotin</td>
<td>Pierce Biotech</td>
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<td>Ficoll™ PM400</td>
<td>Amersham Biosciences</td>
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<td>GELCODE® Blue Stain Reagent</td>
<td>Pierce Biotech</td>
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<tr>
<td>Human epidermal growth factor</td>
<td>PeproTech EC Ltd</td>
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<td>Human hepatocyte growth factor</td>
<td>R&amp;D Systems</td>
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<td>Immobilised streptavidin beads</td>
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<tr>
<td>K5 photographic emulsion</td>
<td>Ilford</td>
</tr>
<tr>
<td>Mowiol®</td>
<td>CALBIOCHEM®</td>
</tr>
<tr>
<td>Phenylmethyl-sulfonylfluorid</td>
<td>Roche</td>
</tr>
<tr>
<td>Protein G sepharose beads</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>RNasin</td>
<td>Promega</td>
</tr>
<tr>
<td>TEMED</td>
<td>BIO-RAD</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>TRizol®</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>X-phosphate/5-bromo-4-chloro-3indolyl-phosphate</td>
<td>Roche</td>
</tr>
<tr>
<td>Xylene cyanol</td>
<td>BIO-RAD</td>
</tr>
<tr>
<td>Zeocin™</td>
<td>Invitrogen Life Technologies</td>
</tr>
</tbody>
</table>
### Materials and Methods

#### 2.2 Buffers

All buffers were made as described below. ’RT’ indicates room temperature, ‘Filter’ indicates sterilisation using a 0.2μm filter.

<table>
<thead>
<tr>
<th>Buffer Description</th>
<th>Ingredients</th>
<th>Storage</th>
<th>Sterilisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x MOPS buffer</td>
<td>200mM MOPS (pH7), 50mM sodium acetate, 10mM EDTA; adjusted to pH7</td>
<td>4°C, Dark</td>
<td>Filter</td>
</tr>
<tr>
<td>10x RNA loading buffer</td>
<td>50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol</td>
<td>RT</td>
<td>Autoclave</td>
</tr>
<tr>
<td>20x SSC</td>
<td>3M NaCl, 0.3M tri-sodium citrate (pH5 or 7)</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>2x Laemmli buffer</td>
<td>125mM Tris-HCl (pH6.8), 20% glycerol, 4% SDS, 0.2% bromophenol blue, 2.5% β-mercaptoethanol (added fresh)</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>5x Transcription Buffer</td>
<td>200mM Tris-HCl (pH7.9), 30mM MgCl₂, 10mM spermidine, 50mM NaCl</td>
<td>-20°C</td>
<td>-</td>
</tr>
<tr>
<td>6X DNA loading buffer (DNA)</td>
<td>15% Ficoll™ PM400, 0.25% bromophenol blue, 0.25% xylene cyanol</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>Church buffer (DNA)</td>
<td>200mM sodium phosphate buffer (pH7.2), 1mM EDTA, 1% BSA, 7% SDS, 15% formamide</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>Church buffer (RNA)</td>
<td>200mM sodium phosphate buffer (pH7.2), 1mM EDTA, 1% BSA, 7% SDS, 15% formamide</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>Church wash buffer</td>
<td>40mM sodium phosphate buffer (pH7.2), 1mM EDTA, 1% SDS</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>EB</td>
<td>10mM Tris-HCl (pH8.5)</td>
<td>RT</td>
<td>Autoclave</td>
</tr>
<tr>
<td>L-agar</td>
<td>1% bacto-tryptone, 0.5% yeast extract, 170mM NaCl, 15% agar</td>
<td>RT</td>
<td>Autoclave</td>
</tr>
<tr>
<td>L-broth</td>
<td>1% bacto-tryptone, 0.5% yeast extract, 170mM NaCl</td>
<td>RT</td>
<td>Autoclave</td>
</tr>
<tr>
<td>MAB-T</td>
<td>100mM Maleic acid, 150mM NaCl, 1% Tween-20</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>NET buffer</td>
<td>50mM Tris-HCl (pH7.5), 150mM NaCl, 1mM EDTA, 1% Triton X-100</td>
<td>4°C</td>
<td>-</td>
</tr>
<tr>
<td>NTE</td>
<td>500mM NaCl, 10mM Tris-HCl (pH8.0), 5mM EDTA</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>NTM-T</td>
<td>100mM NaCl, 100mM Tris-HCl (pH9.5), 50mM MgCl₂, 0.1% Tween-20, 2mM Levamisole (added fresh)</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>PBSA</td>
<td>140mM NaCl, 2.5mM KCl, 10mM Na₂HPO₄ (pH7.2), 1.5mM KH₂PO₄</td>
<td>RT</td>
<td>Autoclave</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBSA + 0.1% Tween-20</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>Semi-dry transfer buffer</td>
<td>48mM Tris base, 39mM glycine, 0.04% SDS, 20% methanol</td>
<td>RT</td>
<td>-</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Ingredients</th>
<th>Storage</th>
<th>Sterilisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAE</td>
<td>40mM Tris base, 40mM acetic acid, 1mM EDTA</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>Tail lysis buffer</td>
<td>100mM Tris-HCl (pH8.5), 5mM EDTA, 0.2% SDS, 200mM NaCl</td>
<td>RT</td>
<td>Filter</td>
</tr>
<tr>
<td>TBE</td>
<td>89mM Tris base, 89mM boric acid, 2mM EDTA</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>TBS-T</td>
<td>137mM NaCl, 2.7mM KCl, 25mM Tris-HCl (pH7.5), 0.1% Tween-20, 2mM Levamisole (added fresh)</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>TE</td>
<td>10mM Tris-HCl (pH8.0), 1mM EDTA</td>
<td>RT</td>
<td>Filter</td>
</tr>
<tr>
<td>Transgenic injection buffer</td>
<td>10mM Tris-HCl (pH7.4), 0.1mM EDTA</td>
<td>-20°C</td>
<td>Filter</td>
</tr>
<tr>
<td>Tris-glycine electrophoresis buffer</td>
<td>25mM Tris base, 250mM glycine, 0.1% SDS</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>Wet transfer buffer</td>
<td>48mM Tris base, 390mM glycine, 0.1% SDS, 20% methanol</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>WM hybridisation buffer</td>
<td>50% formamide, 6X SSC (pH5.0), 5mM EDTA (pH8.0), 50µg/ml Yeast RNA, 0.2% Tween-20, 0.5% CHAPS, 100µg/ml heparin</td>
<td>-20°C</td>
<td>-</td>
</tr>
<tr>
<td>X-gal staining buffer</td>
<td>PBSA + 5mM K$_4$Fe(CN)$_6$, 5mM K$_3$Fe(CN)$_6$, 2mM MgCl$_2$, 0.01% sodium deoxycholate, 0.02% NP-40</td>
<td>4°C</td>
<td>-</td>
</tr>
</tbody>
</table>

### 2.3 Radioisotopes

All radioisotopes were obtained from Amersham Biosciences.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>[$\alpha$-$^{35}$S] UTP</td>
<td>800 Ci/mmol</td>
</tr>
<tr>
<td>Redivue™ [$\alpha$-$^{32}$P] dCTP</td>
<td>3,000 Ci/mmol</td>
</tr>
<tr>
<td>Redivue™ L-$^{35}$S] methionine</td>
<td>1000Ci/mmol</td>
</tr>
</tbody>
</table>

### 2.4 Enzymes

All enzymes were obtained from New England Biolabs with the following exceptions.
Materials and Methods

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf intestinal alkaline phosphatase (CIP)</td>
<td>Roche</td>
</tr>
<tr>
<td>DNase I, RNase free</td>
<td>Roche</td>
</tr>
<tr>
<td>Eco47III</td>
<td>Roche</td>
</tr>
<tr>
<td>Pfu Turbo®</td>
<td>Stratagene</td>
</tr>
<tr>
<td>Pic Taq</td>
<td>Cancer Research UK</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>Sigma</td>
</tr>
<tr>
<td>RNA polymerases (T3, T7, Sp6)</td>
<td>Promega</td>
</tr>
<tr>
<td>RNase A</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

2.5 Immunoreagents

All antibodies were used at the concentrations indicated below. Where an antibody was used for more than one application, the correct concentration for each application is given. IF = immunofluorescence, IB = immunoblot, IP = immunoprecipitation, ISH = in situ hybridisation.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type (isotype)</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>9E10</td>
<td>Mouse (IgG1)</td>
<td>IP - 5μg/reaction</td>
<td>Cancer Research UK</td>
</tr>
<tr>
<td>9G6.10 anti-ErbB2</td>
<td>Mouse (IgG1)</td>
<td>IF - 1:200</td>
<td>NeoMarkers</td>
</tr>
<tr>
<td>Anti-β-Catenin</td>
<td>Mouse (IgG1)</td>
<td>IF - 0.5μg/ml</td>
<td>Transduction Laboratories</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IB - 1:2,000</td>
<td></td>
</tr>
<tr>
<td>Anti-digoxigenin AP-conjugated</td>
<td>Sheep (Fab fragments)</td>
<td>ISH - 1:2,000</td>
<td>Roche</td>
</tr>
<tr>
<td>Anti-ErbB-1</td>
<td>Mouse (IgG2b)</td>
<td>IF - 4μg/ml</td>
<td>Cancer Research UK</td>
</tr>
<tr>
<td>B325 anti-transferrin receptor</td>
<td>Mouse (IgG1)</td>
<td>IF - 1:100</td>
<td>Gift from Dr. Colin Hopkins</td>
</tr>
<tr>
<td>Clone 42 anti-ErbB2</td>
<td>Mouse (IgG1)</td>
<td>IB - 1,2500</td>
<td>Transduction Laboratories</td>
</tr>
<tr>
<td>c-Met (p-Met)</td>
<td>Rabbit (IgG)</td>
<td>IB - 1:1,000</td>
<td>Biosource International</td>
</tr>
<tr>
<td>pYpYpY1230/1234/1235</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-21 anti-Met</td>
<td>Mouse (IgG1k)</td>
<td>IB - 1:5,000</td>
<td>Upstate Biotechnology</td>
</tr>
<tr>
<td>DO-24 anti-Met</td>
<td>Mouse (IgG2ae)</td>
<td>IF - 1:250</td>
<td>Upstate Biotechnology</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>Mouse (IgG1)</td>
<td>IF - 0.5μg/ml</td>
<td>Transduction Laboratories</td>
</tr>
</tbody>
</table>
### Materials and Methods

#### Antibody Type (isotype) Application Source

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type (isotype)</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC and TRITC conjugated secondary antibodies</td>
<td>Goat (IgG)</td>
<td>IF - 1:100</td>
<td>Jackson ImmunoResearch Laboratories, Inc.</td>
</tr>
<tr>
<td>HRP-conjugated goat anti-mouse</td>
<td>Goat (IgG)</td>
<td>IB - 1:5,000</td>
<td>DAKO</td>
</tr>
<tr>
<td>HRP-conjugated goat anti-rabbit</td>
<td>Goat (IgG)</td>
<td>IB - 1:5,000</td>
<td>DAKO</td>
</tr>
<tr>
<td>IRDye™ 800-goat anti-mouse</td>
<td>Goat (IgG)</td>
<td>IB - 1:5,000</td>
<td>LI-COR Biosciences</td>
</tr>
<tr>
<td>M2 anti-FLAG</td>
<td>Mouse (IgG₁)</td>
<td>IB - 1:500</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>MAB1520 anti-ZO-1</td>
<td>Rat (IgG₁)</td>
<td>IF - 1:1,000</td>
<td>Chemicon International</td>
</tr>
<tr>
<td>MAPK-YT anti-diphosphorylated ERK1&amp;2</td>
<td>Mouse (IgG₁)</td>
<td>IB - 1:10,000</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>ME20.4 anti-P75NTR</td>
<td>Mouse (IgG₁)</td>
<td>IF - 1:500</td>
<td>Upstate Biotechnology</td>
</tr>
<tr>
<td>PY99 anti-phosphotyrosine</td>
<td>Mouse (IgG₂B)</td>
<td>IB - 1:1000</td>
<td>Santa-Cruz Biotechnology, Inc.</td>
</tr>
</tbody>
</table>

#### 2.6 Miscellaneous

The following table lists the remaining products that were used.

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Culture</td>
<td></td>
</tr>
<tr>
<td>Foetal calf serum</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>LipofectAMINE™</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>Naigene Cryo 1C Container</td>
<td>Nalgene®</td>
</tr>
<tr>
<td>Newborn calf serum</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>Opti-MEM® I reduced serum medium</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>Tissue culture plastics</td>
<td>Beckton Dickinson or Corning</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Molecular Biology Kits</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ApopTag® Plus In Situ Apoptosis Detection Kit</td>
<td>Intergen Company</td>
</tr>
<tr>
<td>Enhanced Chemiluminescence kit (ECL™)</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>QIAGEN® Plasmid Maxi Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>QIAprep® Spin Miniprep Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>QIAquick PCR Purification Kit</td>
<td>Qiagen</td>
</tr>
</tbody>
</table>
### Materials and Methods

**Product**

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>QuikChange™ XL Site-Directed Mutagenesis Kit</td>
<td>Stratagene</td>
</tr>
<tr>
<td>Ready-To-Go™ DNA labelling beads dCTP</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>TNT® Quick Coupled Transcription/Translation System</td>
<td>Promega</td>
</tr>
</tbody>
</table>

**Membranes & Films**

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>3MM chromatography paper</td>
<td>Whatman®</td>
</tr>
<tr>
<td>Nytran® N membrane</td>
<td>Schleicher &amp; Schuell</td>
</tr>
<tr>
<td>Nytran® N SuPerCharge membrane</td>
<td>Schleicher &amp; Schuell</td>
</tr>
<tr>
<td>Parafilm®</td>
<td>American National Can™</td>
</tr>
<tr>
<td>Protran® nitrocellulose membrane</td>
<td>Schleicher &amp; Schuell</td>
</tr>
<tr>
<td>Saran Wrap</td>
<td>DOW</td>
</tr>
<tr>
<td>SuperRX film</td>
<td>Fuji</td>
</tr>
<tr>
<td>X-OMAT™ AR film</td>
<td>Kodak</td>
</tr>
</tbody>
</table>

**Markers**

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1kb DNA ladder</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>Prestained protein markers</td>
<td>New England Biolabs</td>
</tr>
</tbody>
</table>

**Other**

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chroma Spin-30 DEPC-H2O Columns</td>
<td>Clontech</td>
</tr>
<tr>
<td>Micolance™ sterile needles</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>Microspin™ S-200 HR columns</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>Millex®-GP Filters</td>
<td>Millipore</td>
</tr>
<tr>
<td>Plastipak® Syringes</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>Stericup™ Filtration System</td>
<td>Millipore</td>
</tr>
<tr>
<td>ULTRA GOLD™ Scintillation fluid</td>
<td>Packard BioScience</td>
</tr>
</tbody>
</table>

### 2.7 List of Suppliers/Distributors

**Company**

<table>
<thead>
<tr>
<th>Company</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>American National Can™</td>
<td>Illinois, US</td>
</tr>
<tr>
<td>Amersham Biosciences</td>
<td>Little Chalfont, UK</td>
</tr>
<tr>
<td>Anachem</td>
<td>Luton, UK</td>
</tr>
<tr>
<td>BDH/Mercck</td>
<td>Dorset, UK</td>
</tr>
<tr>
<td>Beckton Dickinson</td>
<td>California, US</td>
</tr>
<tr>
<td>Biosource International</td>
<td>California, US</td>
</tr>
<tr>
<td>BIO-RAD</td>
<td>Hemel Hempstead, UK</td>
</tr>
</tbody>
</table>

88
<table>
<thead>
<tr>
<th>Company</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>CALBIOCHEM®</td>
<td>California, US</td>
</tr>
<tr>
<td>Chemicon International</td>
<td>Chandlers Ford, UK</td>
</tr>
<tr>
<td>Clontech</td>
<td>California, US</td>
</tr>
<tr>
<td>Corning</td>
<td>New York, US</td>
</tr>
<tr>
<td>DAKO</td>
<td>Cambridgeshire, UK</td>
</tr>
<tr>
<td>Dow Chemical Co. (GRI Ltd.)</td>
<td>Braintree, UK</td>
</tr>
<tr>
<td>Fuji (GRI Ltd.)</td>
<td>Braintree, UK</td>
</tr>
<tr>
<td>Ilford</td>
<td>Knutsford, UK</td>
</tr>
<tr>
<td>Intergen Company</td>
<td>Georgia, US</td>
</tr>
<tr>
<td>Invitrogen Life Technologies</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Jackson ImmunoResearch Laboratories, Inc</td>
<td>Pennsylvania, US</td>
</tr>
<tr>
<td>Kodak (Sigma-Aldrich)</td>
<td>Dorset, UK</td>
</tr>
<tr>
<td>Millipore</td>
<td>Watford, UK</td>
</tr>
<tr>
<td>Nalgene</td>
<td>New York, US</td>
</tr>
<tr>
<td>NeoMarkers</td>
<td>Newmarket, UK</td>
</tr>
<tr>
<td>New England Biolabs</td>
<td>Hitchin, UK</td>
</tr>
<tr>
<td>Novagen</td>
<td>Wisconsin, US</td>
</tr>
<tr>
<td>Packard BioScience</td>
<td>Connecticut, US</td>
</tr>
<tr>
<td>PeproTech EC Ltd</td>
<td>London, UK</td>
</tr>
<tr>
<td>Pierce Biotech</td>
<td>Tattenhall, UK</td>
</tr>
<tr>
<td>Promega</td>
<td>Southampton, UK</td>
</tr>
<tr>
<td>Qiagen</td>
<td>Crawley, UK</td>
</tr>
<tr>
<td>R&amp;D Systems</td>
<td>Minneapolis, US</td>
</tr>
<tr>
<td>Roche</td>
<td>Basel, Switzerland</td>
</tr>
<tr>
<td>Santa-Cruz Biotechnology, Inc.</td>
<td>California, US</td>
</tr>
<tr>
<td>Schleicher &amp; Schuell</td>
<td>Dassel, Germany</td>
</tr>
<tr>
<td>Sigma-Aldrich</td>
<td>Dorset, UK</td>
</tr>
<tr>
<td>Stratagene</td>
<td>Amsterdam, Netherlands</td>
</tr>
<tr>
<td>Transduction Laboratories</td>
<td>California, US</td>
</tr>
<tr>
<td>Upstate Biotechnology</td>
<td>Virginia, US</td>
</tr>
<tr>
<td>Whatman®</td>
<td>Maidstone, UK</td>
</tr>
</tbody>
</table>
Methods

2.8 Preparation, Manipulation and Analysis of Plasmid DNA

2.8.1 Transformation of Competent DH5α Bacterial Cells

2-10 μl of plasmid DNA at 1ng/μl or 10μl of ligation mixture was added to 50μl of competent DH5α cells on ice and left for 30 minutes. The bacteria were heat shocked at 42°C for 1 minute before being placed back on ice for 2 minutes. 500μl of L-broth was then added to the bacteria which were subsequently cultured for 45 minutes at 37°C with shaking at 150rpm. 150-400μl of bacterial culture was spread out using sterile techniques on to L-agar plates containing 50μg/ml of ampicillin.

2.8.2 Small Scale (Mini) Preparation of Plasmid DNA

Single colonies of antibiotic resistant bacteria were picked from L-agar plates using sterile yellow pipette tips. The bacteria were used to inoculate 5ml of L-broth containing 50μg/ml of ampicillin. Cultures were grown for a minimum of 6 hours or overnight at 37°C with shaking at 250rpm. 2ml of the culture was transferred to a 2ml eppendorf tube and centrifuged on a benchtop centrifuge at 14,000rpm for one minute. The supernatant was discarded and DNA isolated from the bacterial pellet using a QIAPrep® Spin Miniprep Kit according to the manufacturers instructions. DNA was eluted in TE or EB buffer and stored at -20°C.

2.8.3 Large Scale (Maxi) Preparation of Plasmid DNA

500μl of small scale bacterial culture was used to inoculate 200ml of L-broth supplemented with 50μg/ml of ampicillin. The culture was grown overnight at 37°C with shaking at 250rpm. Plasmid DNA was isolated from the culture using a QIAGEN® Plasmid Maxi Kit according to the manufacturers instructions. DNA was dissolved in TE or EB buffer to a concentration of 1mg/ml and stored at -20°C.

2.8.4 Restriction Endonuclease Digestion of DNA

DNA was digested in a total volume of 20-100μl depending on the amount of DNA present and the activity of the enzyme. Digestions were performed in a 1x restriction endonuclease buffer recommended by the manufacturer and 1x BSA was
added if required. 2-50 units of enzyme were added and digestions were performed for between 15 minutes to overnight at the recommended temperature. If a double digest was required, then an appropriate buffer was chosen according to the manufacturers recommendations. If a double digest was not possible due to incompatibility of the enzymes, a sequential digest was performed and the DNA extracted between digests.

2.8.5   Extraction/Precipitation of DNA

DNA extraction was performed by the phenol:chloroform method for larger quantities of DNA or the QIAquick® PCR Purification Kit for smaller quantities of linear DNA used for cloning.

2.8.5a   Phenol:chloroform extraction

1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA sample. The mixture was vortexed and then centrifuged on a benchtop centrifuge at 14,000rpm for 1 minute. The upper aqueous phase was carefully removed and placed in a new tube containing an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The mixture was then vortexed, re-centrifuged and the upper aqueous phase was then either subjected to another extraction step or transferred to a new tube containing an equal volume of chloroform:isoamyl alcohol (24:1) and then vortexed and centrifuged as above. Once extraction was complete, the upper aqueous phase was ethanol precipitated.

2.8.5b   Ethanol precipitation

DNA was precipitated by addition of 2.5 volumes of ethanol and 0.1 volumes of 3M sodium acetate (pH5.2). The sample was placed on ice for 1 hour or dry ice for 15 minutes and then centrifuged for 10 minutes at 14,000rpm on a benchtop centrifuge. The DNA pellet was washed by addition of 3 volumes (original sample volume) of 70% ethanol, vortexed and left at room temperature for 5 minutes before centrifugation as described above for 5 minutes. After removing the supernatant, the pellet was allowed to air dry for 5 minutes before being dissolved in TE or EB buffer.

2.8.5c   QIAquick® PCR Purification Kit

DNA was purified according to the manufacturers instructions.
2.8.6 Agarose Gel Electrophoresis of DNA

DNA was resolved in agarose gels ranging in concentration from 0.8% - 2% (w/v). Agarose was melted in either a 1x TBE or 1x TAE buffer by heating in a microwave oven, 1 µl ethidium bromide (from a 5mg/ml stock) per 100ml agarose solution was then added, and the gel allowed to set in a gel deck containing a comb. After the gel had set, the comb was removed and the gel deck was placed in a gel tank (Life Technologies, Inc.). 1x TBE or 1x TAE buffer was added to cover the gel by approximately 2-3mm. DNA samples were added to 6x loading buffer in a 5:1 sample:buffer ratio and then loaded into the wells. Samples were resolved against a 1kb DNA ladder by applying a voltage of 30-110V and were then visualised and photographed under ultraviolet (UV) illumination.

2.8.7 DNA Extraction From Agarose Gels

DNA fragments were cut out of an agarose gel using a sterile scalpel, and extracted using the QIAquick® Gel Extraction Kit according to the manufacturers instructions.

2.8.8 Creating Blunt-Ended DNA From 5’ Sticky-Ends

Extracted DNA was resuspended in 1x EcoPol buffer (New England Biolabs) supplemented with 33mM of each dNTP. 1 unit of DNA polymerase I, large fragment (Klenow) was added per µg of DNA. The reaction was incubated for 15 minutes at room temperature and then terminated by addition of 10mM EDTA and heating the sample to 75°C for 10 minutes.

2.8.9 Generation and Precipitation of Oligonucleotides

All oligonucleotides were synthesised by the Oligonucleotide Synthesis Service (Cancer Research UK, Clare Hall Laboratories) and supplied as a dried pellet. If the oligonucleotides were supplied desalted then the pellets were dissolved in TE or EB buffer, if however the side products of synthesis were still present, then the oligonucleotides were precipitated. Precipitation was performed in 600µl cold ethanol, 200µl 0.3M sodium acetate and 10mM magnesium chloride by placing the sample on dry ice for 15 minutes. The oligonucleotides were pelleted by centrifugation at 14,000rpm on a benchtop centrifuge for 20 minutes, and washed by removing the supernatant and adding 800µl cold 80% ethanol before vortexing briefly. After 5 minutes the sample was centrifuged as before for 5 minutes, the supernatant carefully removed and the pellet allowed to air dry for 5 minutes.
before dissolving in TE or EB buffer. All oligonucleotides were diluted to a final concentration of 100μM. See Appendix A6.1 a list of oligonucleotides used.

2.8.10 Preparation of Polylinker DNA

Polylinkers were generated by addition of 5' phosphate groups to, and annealing of, complementary oligonucleotides. 1μg of each oligonucleotide was phosphorylated using T4 polynucleotide kinase in the manufacturers recommended buffer supplemented with 1mM ATP. Reactions were performed for 30 minutes at 37°C in a total volume of 10μl. Reactions were boiled for 2 minutes and then slowly cooled to room temperature to allow complementary oligonucleotide annealing. Polylinker DNA was then used for vector ligation as described in section 2.8.14.

2.8.11 Polymerase Chain Reaction (PCR) – Generation of cDNA for Cloning

Amplification of DNA products for cloning was performed using Stratgene's *Pfu* Turbo PCR kit. Reactions were carried out according to the manufacturers instructions.

2.8.12 Site-Directed Mutagenesis

Site-directed mutations were created using Stratagene's QuikChange™ XL Site-Directed Mutagenesis Kit. Reactions were performed according to the manufacturers recommendation, except that transformation of mutated DNA was achieved using competent DH5α bacterial cells.

2.8.13 Dephosphorylation of 5' Phosphate Groups From DNA

To prevent the self-ligation of vector DNA during vector-insert ligations, the 5' phosphate groups were removed from all linearised vectors used for cloning. Dephosphorylation was carried out in a total volume of 100μl by addition of 2μl calf intestinal alkaline phosphatase. The reaction mixture was buffered either with 1x dephosphorylation buffer (Roche), or using any of the standard restriction endonuclease buffers (New England Biolabs) if dephosphorylation was performed immediately following restriction endonuclease digestion. The mixture was incubated at 37°C for 15-30 minutes and then inactivated by heating to 60°C in the
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presence of 10mM EDTA. Alternatively the reaction was inactivated by gel extraction or using the QIAquick® PCR Purification Kit.

2.8.14 Ligation of Plasmid Vector DNA and Insert DNA

The concentration of vector and insert DNA were estimated by comparing band intensities with a *HindIII/EcoRI* digested lambda DNA ladder of known concentration. Ligations were performed with an estimated vector:insert molar ratio of 1:3 in 1x T4 DNA ligase buffer supplemented with 2μl T4 DNA ligase in a total volume of 20μl. Ligations were left at room temperature for a minimum of 10 minutes for 'sticky-ended' ligations or 3 hours for 'blunt-ended' ligations.

2.8.15 Sequencing of DNA

All DNA sequencing was performed by the DNA Sequencing Service (Cancer Research UK) using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturers instructions. Briefly, 0.5μg template DNA was added to 3.2pmol sequencing primer and 8μl Big-Dye™ Terminator mix in a total volume of 20μl. Polymerase chain reactions were carried out as recommended by the manufacturer on an MJ Research thermal cycler and DNA products were ethanol precipitated at room temperature for 15 minutes by addition of 300μl 95% ethanol, 5μl 3M sodium acetate (pH4.5) and 80μl dH2O. After centrifugation on a benchtop centrifuge at 14,000rpm for 20 minutes, the pellet was washed with 500μl 70% ethanol, vortexed briefly, centrifuged as described above for 5 minutes, air dried and then stored at -20°C until sequenced.

2.8.16 Preparation of Plasmid DNA for Generation of Transgenic Mice

30μg plasmid DNA was digested with *SmaI* and separated on a 0.8% (w/v) TBE gel. The desired fragment was then gel extracted using the QIAquick® Gel Extraction Kit and eluted in sterile transgenic injection buffer. The concentration of the extracted fragment was then estimated on a 0.8% (w/v) agarose gel by loading 1, 2 and 5μl against a *HindIII/EcoRI* digested lambda ladder of known concentration. The DNA solution was then diluted to 5ng/μl in transgenic injection buffer, divided into 6x30μl aliquots, and then sent for injection into F1 fertilised eggs (Transgenic Services, Cancer Research UK).
2.9 Maintenance and Manipulation of Mammalian Cells in Culture

2.9.1 Cell Types and Medium Requirements

Cells were maintained in a humidified incubator at 37°C (10% carbon dioxide) and routinely grown as monolayers on plastic dishes. All cell culture work was performed in a BioMAT II microbiological safety cabinet using pre-warmed medium. Growth medium, versene (200mg/l in PBSA) and trypsin (0.25% (w/v) in versene) were obtained from Cancer Research UK Cell Services.

The following cell lines were used, and grown in the medium indicated.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin / Type</th>
<th>Growth Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>Human lung carcinoma / epithelial</td>
<td>E4 + 10% foetal calf serum</td>
</tr>
<tr>
<td>CACO-2</td>
<td>Human colon carcinoma / epithelial</td>
<td>E4 + 10% foetal calf serum</td>
</tr>
<tr>
<td>HC11</td>
<td>Mouse mammary / epithelial</td>
<td>RPMI 1640 3.7% + 10% foetal calf serum, 5μg/ml insulin, 10ng/ml EGF</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>Porcine kidney / epithelial</td>
<td>E4 + 10% foetal calf serum</td>
</tr>
<tr>
<td>MDCK</td>
<td>Canine kidney / epithelial</td>
<td>E4+ 10% foetal calf serum</td>
</tr>
</tbody>
</table>

2.9.2 Passaging of Cells

Cells were routinely passaged to maintain them at sub-confluent levels. Cells were rinsed once in pre-warmed (37°C) versene and then incubated in versene at 37°C for 3 minutes. Versene was then replaced with a pre-warmed (37°C) 1:4 trypsin:versene mixture, and incubated at 37°C. Once cells were detached from the plastic dish they were resuspended in complete growth medium and then subcultured at a ratio of 1:10 –1:20 in a new petri dish.

2.9.3 Cryo-Preservation and Recovery of Cell Stocks

Cells were grown to 70-80% confluency, detached from the dish as described above, and then prepared for cryo-preservation. After resuspension, the cell were placed in a 15ml Falcon tube and centrifuged in a Harrier 15/80 benchtop centrifuge at 1,200rpm for 2 minutes. The growth medium was aspirated and the cell pellet

\(^1\) E4 is equivalent to Dulbecco's Modified Eagle Medium
resuspended in 7ml of a 9:1 solution of complete growth medium:DMSO. The cell suspension was then split into freezing vials as 1ml aliquots. The vials were transferred to a Nalgene Cryo 1C container containing iso-amyl alcohol and placed at -70°C overnight before being transferred to liquid nitrogen for long-term storage.

Cells were recovered by warming frozen vials of cells in a 37°C water bath until thawed, and then transferring the cell suspension to a 10cm plastic dish containing 10ml of the appropriate complete growth medium. After the cells had adhered to the plastic dish, the medium was replaced with fresh complete growth medium.

2.9.4 Plasmid DNA Transfection of Mammalian Cells

All transfections were performed using LipofectAMINE™ reagent according to the manufacturers instructions.

2.9.4a Transient transfections

1.8x10^5 cells were seeded on a 3.5cm dish in 2ml complete growth medium and allowed to recover overnight. Alternatively, 2.5x10^5 cells were seeded on to a Falcon® polyethylene tetrathalate (PET) membrane insert placed inside a well of a 6 well plate. Lipofection preparation was carried out in sterile 5ml polystyrene tubes. In one tube 2.5μg - 3μg DNA was mixed with 100μl Opti-MEM® I reduced serum medium; in a separate tube, 100μl Opti-MEM® I reduced serum medium was mixed with 10μl LipofectAMINE™ reagent. The two mixtures were combined into one tube and left for 45 minutes at room temperature to allow DNA:lipid complexes to form. 800μl Opti-MEM® I reduced serum medium was added to the mixture and then the entire contents of the tube were gently placed onto cells which had been washed once with 2ml of pre-warmed (37°C) Opti-MEM® I reduced serum medium. The transfection mixture was left on the cells for 5 hours, after which time it was replaced with the appropriate complete growth medium.

Cells were assayed 24-48 hours post-transfection.

2.9.4b Generation of stable cell lines

Transfections were carried out as described for transient transfections. 48 hours post-transfection, one-tenth (for single transfections) or one-half (for cotransfections) of the cells were sub-cultured onto a 10cm dish containing growth medium + antibiotic(s). Fresh growth medium containing the antibiotic(s) were added to the cells every 2-3 days. When stable cell clones were approximately
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4-5mm in diameter they were picked using a sterile pipette tip and transferred to a 24 well plate. These clones were cultured in selective medium and later assayed for gene expression. The correct antibiotic concentration was determined for each cell line by titration and is shown in the table below.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Zeocin™</th>
<th>Blasticidin S HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC11</td>
<td>150µg/ml</td>
<td>-</td>
</tr>
<tr>
<td>MDCK</td>
<td>400µg/ml</td>
<td>5µg/ml</td>
</tr>
</tbody>
</table>

2.9.5 Immunofluorescence

Immunofluorescence was performed on cells grown on Falcon® PET cell culture inserts placed in a 6 well dish. Growth medium was removed and the cells were washed twice in PBSA before fixation in cold methanol (-20°C). Fixation was performed for 5 minutes at -20°C and then the cells were washed twice in PBSA before blocking in a 0.2% (v/v) fish gelatin solution in PBSA for 1 hour. Alternatively, fixation was performed in a 4% (w/v) paraformaldehyde solution in PBSA for 10 minutes at room temperature, permeabilised in a 0.2% (v/v) Triton X-100 solution in PBSA for 2 minutes, and then blocked in a 3% (w/v) BSA, 0.2% (v/v) Triton X-100 solution in PBSA for 1 hour. Incubation with the primary antibody was performed in blocking solution by placing 70µl of antibody solution on a piece of Parafilm and then placing a small cut square (approximately 1cm²) of membrane cell-side down on to the solution (see section 2.5 for antibody concentrations). Primary antibody incubations were performed at room temperature in a humid box for 1 hour. 5 x 5 minute PBSA washes were then carried out to remove unbound antibody. Incubations with fluorophore-conjugated secondary antibodies were performed as described for primary antibody incubation. All secondary antibodies were diluted 1:100 in blocking solution and incubations were performed in the dark. Cells were then washed as described above and rinsed in distilled water before being mounted on to a glass microscope slide using Mowiol solution. Mounted slides were left to set overnight before being viewed with either a Zeiss Axiophot fluorescence microscope or a Zeiss Laser Scanning Microscope 510.

Mowiol mounting solution was prepared by stirring 2.4g Mowiol 4-88 into 6g glycerol. 6ml distilled water was added and left for 4 hours at room temperature. 12ml 200mM Tris-HCl (pH8.5) was added and the mixture heated to 50°C for 20
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minutes with occasional stirring. After the Mowiol had dissolved, the solution was clarified in a Sorvall RC5 centrifuge at 5,000g for 10 minutes. 1,4-diazabicyclo-[2,2,2]-octane (DABCO) was added to 2.5% (w/v) to decrease photo-bleaching during microscopy. The Mowiol solution was aliquotted and then stored at -20°C.

2.10 Analysis of Genomic DNA

2.10.1 Isolation of Genomic DNA From Mouse Tissue

2.10.1a Tail snips

Tail snips were stored at -20°C until ready for processing. 600μl tail lysis buffer (including freshly added proteinase K at 100μg/ml) was added to the tail snip. Digestion was allowed to proceed at 55°C in a rotating hybridisation oven overnight, or for a minimum of 4 hours. Digested samples were spun on a benchtop centrifuge at 14,000rpm after which the supernatant was decanted into a fresh 1.5ml eppendorf tube containing 600μl isopropanol. The samples were shaken briefly to aid precipitation of the DNA, centrifuged as described above, the isopropanol mixture decanted, and the pellet allowed to air dry for 5 minutes. DNA was resuspended in TE or EB buffer. Resuspension volume varied between 100μl and 500μl depending on the size of the pellet and the application for which the DNA was being used.

2.10.1b Yolk sacs

Yolk sac DNA was isolated as described for tail snips.

2.10.2 Restriction Enzyme Digestion of Mouse Genomic DNA

Genomic DNA was digested as described in section 2.8.4 for plasmid DNA except that the following components were added to the digestion mixture: 100μg/ml BSA, 2.5μg/ml RNase A and 1mM spermidine. Digestions were allowed to proceed overnight; a humidified chamber was used if digestions were performed in a 96 well plate.

2.10.3 Genotyping Mouse Genomic DNA (PCR)

Pic Taq polymerase (Cancer Research UK) was used for PCR based genotyping of mouse genomic DNA. A typical reaction and cycling programme is described in
the tables below. Reactions were prepared in a 500µl eppendorf tube on ice and the ingredients added in the order that they appear. All reactions were performed with a hot-start on a Techne Genius or Gene E thermo-cycler.

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Temperature (°C)</th>
<th>Time (Mins)</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Template</td>
<td>1.0</td>
<td>94</td>
<td>2</td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>3.2</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td>dNTPs (10mM of each)</td>
<td>0.8</td>
<td>Annealing Temp.</td>
<td>1</td>
</tr>
<tr>
<td>Primer A (1µM)</td>
<td>10.0</td>
<td>72</td>
<td>1</td>
</tr>
<tr>
<td>Primer B (1µM)</td>
<td>10.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formamide</td>
<td>0.4</td>
<td>72</td>
<td>1</td>
</tr>
<tr>
<td>Pic Taq (0.2U/µl)</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled Water²</td>
<td>10.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Volume</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.10.4 Southern Blotting and Hybridisation

Digested DNA was separated on a 0.8% (w/v) agarose TBE gel and transferred on to Nytran® N SuPerCharge membrane. Blotting was performed as follows. A tray was filled with 400mM NaOH and a glass plate resting on an inverted plastic tub placed in the middle of the tray. Three pieces of 3MM paper soaked in 400mM NaOH were then placed on the glass and their ends allowed to dip into the sodium hydroxide, thereby acting as a wick. The agarose gel was placed onto the 3MM paper and the Nytran® N SuPerCharge membrane was placed on top of the gel. Three more pieces of 3MM paper, the same size as the gel, were placed on top of the membrane and Saran wrap was positioned around the edges of the blot apparatus to isolate the wick. A stack of tissues and then a glass plate were placed on top of the 3MM paper, and finally a flask containing 500ml of water was placed on top of the glass plate to weigh down the tissues. DNA blotting was allowed to proceed for a minimum of 4 hours or overnight. Once blotting was complete, the apparatus was carefully dismantled and the membrane washed in 2x SSC. During construction of the blot apparatus, air bubbles were removed by rolling with a sterile pipette.

² Substituted with 10µl of 1µM primer C if a third primer was required for genotyping
2.10.4a Random oligo-primed synthesis – labelling of DNA probes

Probe DNA was excised from plasmid DNA by restriction endonuclease digest followed by gel extraction. Labelling of DNA probes was performed using Ready-To-Go™ DNA labelling beads according to the manufacturers instructions. Unincorporated nucleotides were removed from the probe mixture by passing the whole reaction through a Microspin™ S-200HR column.

2.10.4b Hybridisation of Southern blots

Hybridisation of DNA probes to membranes was performed in a Hybaid rotisserie oven that had been pre-heated to 65°C. The membrane to be probed was placed in 2X SSC solution and then rolled up in a clean gauze before being placed in a rotisserie bottle. 20ml DNA Church buffer was added to the bottle which was then placed inside the oven to pre-hybridise with rotation. Pre-hybridisation was performed for a minimum of 30 minutes. The radio-labelled DNA probe was denatured by boiling for 2 minutes in a 1.5ml eppendorf tube and placed on ice for 1 minute before being added directly to the pre-hybridisation buffer. Hybridisations were performed overnight at 65°C.

Membranes were washed in several changes of pre-heated Church wash buffer at 68°C. Once the majority of unbound probe had been washed away, the membrane was wrapped in Saran wrap and exposed to Kodak X-OMAT™ AR film at -70°C.

2.11 Analysis of Total RNA

2.11.1 Isolation of Total RNA

2.11.1a Cells in culture

TRIzol® reagent was used to isolate total RNA. The manufacturers instructions were followed. Total RNA pellets were resuspended in distilled water and stored at -20°C.

2.11.1b Mouse tissue

Dissected tissue was immediately snap-frozen in liquid nitrogen and the total RNA was isolated as for cells, after tissue homogenisation using a Jencons polytron.
2.11.2 Denaturing RNA Gel Electrophoresis

A 1.2% (w/v) denaturing agarose gel was used for electrophoretic separation of total RNA. 2.4g agarose was added to 160ml distilled water and then heated in a microwave oven until all of the agarose had melted. After the temperature of the agarose solution had dropped to 60°C, 20ml 10X MOPS buffer and 20ml 37% formaldehyde were added. The gel was cast with a comb in a BIO-RAD DNA SUB Cell™ electrophoresis tank and allowed to set. Before the RNA samples were added, the gel was transferred to the gel tank and equilibrated by immersing in 1x MOPS buffer for 1 hour and then applying a voltage of 120V for 15 minutes.

The total RNA samples were prepared by adding 10-30µg RNA to 3µl of 10x MOPS buffer, 5.25 µl formaldehyde and 15µl deionised formamide in a total volume of 30µl. Samples were heated to 65°C for 15 minutes and then loaded on to the gel after addition of 3 µl RNA loading buffer. Electrophoresis was performed at 120V for approximately 2 hours in 1x MOPS buffer.

2.11.3 Northern Blotting and Hybridisation

Northern blotting and hybridisation was performed as described for DNA in section 2.10.4 with the following exceptions: Nytran® N membrane was used instead of Nytran® N SuPerCharge membrane and 20X SSC solution replaced 400mM NaOH as the blotting solution. Once blotting was complete, the membrane was allowed to semi-dry and the RNA was cross-linked to the membrane using a Stratech UV Stratalinker 2400. Pre-hybridisation and hybridisations were performed using RNA Church buffer at 60°C. Washes were performed at 65°C.

2.12 Analysis of Proteins

2.12.1 Isolation of Protein Extracts from Mammalian Cells

2.12.1a Direct lysis in Laemmli buffer

Cell monolayers were washed twice in PBSA. 2x Laemmli buffer was then added directly onto the cell culture plate (100µl for a 35mm dish or 600µl for a 10cm dish). Cells were scraped off the dish into a 1.5ml eppendorf tube using a policeman, sonicated using a Kontex micro ultrasonic cell disrupter, and then boiled for 4 minutes. Samples were stored at -20°C.
2.12.1b RIPA buffer lysis

Cell monolayers were washed twice in cold PBSA. RIPA buffer was added directly onto the cell culture plate (100μl for a 35mm dish or 600μl for a 10cm dish), and left on ice with occasional rocking for 30 minutes. Cells were scraped off the dish into a 1.5ml eppendorf tube using a policeman. The cell lysate was then passed through a 23-gauge needle using a 1ml syringe 20 times. Lysates were cleared by centrifugation at 16,000g for 10 minutes. Samples were prepared by mixing lysate with an equal volume of 2x Laemmli buffer and boiled for 4 minutes. Lysates and samples were stored at -20°C. Protease and phosphatase inhibitors were added if required.

2.12.1c NET buffer lysis

Washing and lysis as described for RIPA lysis, except RIPA buffer was replaced by NET buffer. After cells had been left on ice for 30 minutes, the NET buffer was collected and transferred to a 1.5ml eppendorf tube. Samples were prepared by mixing lysate with an equal volume of 2x Laemmli buffer and then boiled for 4 minutes. Lysates and samples were stored at -20°C. Protease and phosphatase inhibitors were added to the lysis buffer if required.

2.12.2 SDS-Polyacrylamide Gel Electrophoresis

Protein extracts were separated by electrophoresis through 6-12% SDS-containing polyacrylamide gels using an Atto Corporation vertical gel electrophoresis system. A resolving gel was poured between two clean glass plates separated by a rubber seal to prevent leakage, and overlayed with water-saturated iso-butanol to allow polymerisation to occur throughout the gel. Once set, the iso-butanol was washed away with distilled water and a stacking gel poured onto the resolving gel, finally a comb was placed at the top of the glass plates into the stacking gel. After polymerisation, the rubber seal and comb were removed and the gel was placed in the gel tank. Tris-glycine electrophoresis buffer was added to the two chambers in the tank. Protein samples were loaded into the wells and separated by molecular weight next to a prestained protein marker by applying a voltage of 120V for protein migration through the stacking gel, and 30-220V for migration through the resolving gel.

Resolving gels were created by adding the following components in the order that they appear: 6-12% (v/v) acrylamide mix (from a 40% stock solution), 375mM Tris-HCl (pH8.8), 0.1% (w/v) SDS, 0.1% (w/v) APS and 0.04-0.08% (v/v) TEMED.
Stacking gels were created by adding the following components in the order that they appear: 5% (v/v) acrylamide mix (from a 40% stock solution), 125mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.1% (w/v) APS and 0.1% (v/v) TEMED.

2.12.3 Immunoprecipitations

Cells grown in 6-well cell culture dishes were lysed in 200µl cold NET buffer containing 1mM sodium vanadate, and a cocktail of protease inhibitors (1mM Phenylmethyl-sulfonylfluorid (PMSF), 1µg/ml aprotinin, and 1µg/ml leupeptin). Lysis was performed on ice with gentle rocking for 30 minutes. Lysates were then cleared by centrifugation at 4°C for 10 minutes at 10,000g in a Sorvall RC5 centrifuge. Supernatants were removed to a fresh tube and then diluted down to 500µl with cold NET buffer. 5µl 9E10 anti-myc antibody (1µg/µl) was then added to the lysate which was rocked gently at 4°C for 2 hours to bind myc-tagged proteins. 50µl protein G sepharose beads (packed slurry) was added to the tube to capture immuno-complexes over 1 hour at 4°C with gentle rocking. Samples were then washed in cold NET buffer, briefly centrifuged using a benchtop centrifuge, and washed a further two times following aspiration of the supernatant. Samples were then centrifuged, the supernatant removed, and the immuno-complexes released from the protein G sepharose beads by boiling in 70µl Laemmli buffer.

2.12.4 Western Blotting and Immuno-Detection of Proteins

2.12.4a Blotting of proteins to nitrocellulose membranes

Semi-dry and wet blotting techniques were used to transfer proteins from SDS-polyacrylamide gels on to Protran® Nitrocellulose membranes. The stacking gel was removed and discarded and the resolving gel was transferred to semi-dry or wet transfer buffer for 5 minutes with gentle rotation. A piece of nitrocellulose membrane, cut to the size of the gel, was equilibrated in distilled water for 5 minutes and then placed in transfer buffer for a further 5 minutes.

A sandwich was created using 3 pieces of Whatman® 3MM paper which were cut to the size of the gel and soaked in transfer buffer. The nitrocellulose membrane was then placed on top of the 3MM paper, the sandwich was completed by the poly-acrylamide gel and a further 3 pieces of gel-sized, transfer buffer-soaked 3MM paper. All air bubbles were removed by rolling the sandwich with a glass tube as each layer was added.
Protein transfer was allowed to proceed at 100-300mA using a Bio-Rad Trans-blot transfer cell, with the nitrocellulose membrane placed nearest to the positive electrode. Proteins were transferred for 30-60 minutes (300mA, semi-dry transfer) or overnight (100mA, wet transfer) depending on the size and density of the gel. After blotting, the sandwich was dismantled and the transfer efficiency was checked by staining the membrane with Ponceau S solution for 1 minute. Excess stain was removed by washing with PBSA.

2.12.4b Immuno-detection of proteins on nitrocellulose membranes

The nitrocellulose membrane was blocked in a solution of 5% (w/v) non-fat dried skimmed milk in PBSA supplemented with 0.1% (v/v) Tween-20 (PBS-T) to prevent non-specific binding of immunoglobulins to the membrane. Alternatively, the membrane was blocked in 3% BSA (w/v) in TBS supplemented with 0.1% (v/v) Tween-20 (TBS-T). Blocking was performed with agitation for 1 hour at room temperature or overnight at 4°C. The membrane was next incubated with the primary antibody diluted in blocking solution with agitation for 1 hour at room temperature or overnight at 4°C (see section 2.5 for antibody dilutions). The membrane was then washed by rinsing twice in PBS-T or TBS-T, followed by three 5 minute washes in a large volume of PBS-T or TBS-T. The secondary antibody was diluted in blocking solution and was chosen according to the species of origin of the primary antibody host, in most cases horseradish peroxidase-conjugated goat anti-mouse immunoglobulins were used. Incubations were performed with agitation for 1 hour at room temperature. The membrane was then washed as described above. Detected antigens were revealed using the Enhanced Chemiluminescence (ECL™) kit according to manufacturers instructions, followed by exposure to Fuji SuperRX film.

2.12.5 Cell Surface Labelling and Quantification of Precipitated ErbB-2

Stable clones of MDCK cells were grown to confluence on polyethylene tetrathalate (PET) membranes placed in 6 well cell culture dishes. Following overnight induction of gene-expression with 1µg/ml tetracycline, cells were washed on ice with PBSA supplemented with 0.1mM CaCl₂ and 1mM MgCl₂ (PBS++). Cell surface labelling was achieved by addition of 0.5mg/ml EZ-link™ sulfo-NHS biotin dissolved in PBS++ for 20 minutes added to either the basolateral or apical chamber of the well/membrane. Cell surface labelling was performed on ice and repeated for a further 20 minutes with fresh biotin solution. Labelling
reactions were quenched with 50mM NH$_4$Cl in PBSA. Cells were subsequently washed in PBSA and lysed in NET buffer containing 0.1% (w/v) BSA and a cocktail of protease inhibitors (PMSF, aprotinin, leupeptin). After clearing by centrifugation in a Sorvall RC5 centrifuge at 16,000g the lysate was incubated with immobilised streptavidin beads at 4°C with gentle mixing to ensure efficient streptavidin-biotin complex formation. Beads were washed three times in NET buffer and then prepared for SDS-PAGE as described in section 2.12.1. Immuno-detection and quantification were performed using clone 42 anti-ErbB2, IR-goat anti mouse immunoglobulins and an Odyssey scanner (LI COR Biosciences).

2.12.6 Transcription and Translation in vitro

The Promega TNT® Quick Coupled Transcription/Translation System was used for in vitro transcription and translation experiments. The manufacturers instructions were followed using Redivue™ L-[³⁵S] methionine. Reaction products incorporating radioactive methionine were separated by size using an appropriate SDS-polyacrylamide gel. The gel was then soaked in 7% acetic acid, 7% methanol, 1% glycerol for 5 minutes to prevent it from cracking, and then dried using a conventional vacuum gel dryer at 80°C. Dried gels were visualised by exposure to Kodak X-OMAT film.

2.13 Histology, Wholemount, and In Situ Hybridisation Analyses

2.13.1 Histology

Mouse tissues were fixed overnight in neutral buffered formalin (NBF) at 4°C and then stored in 70% ethanol at 4°C. Samples were paraffin embedded and sectioned at 5µm by the Histopathology Department, Cancer Research UK. Histological staining with haematoxylin and eosin or nuclear fast red was performed by the Histopathology Department.

2.13.2 Carmine Staining of Wholemount Mouse Mammary Glands

Number 4 inguinal mammary glands were carefully dissected from mice, and flattened out on a glass microscope slide using fine forceps. The slide was
transferred to a 50ml falcon containing carnoys fixative (6:3:1 ethanol:chloroform:glacial acetic acid) to fix and defat the gland. Following fixation, the glands were gradually rehydrated through 70% ethanol, 30% ethanol and distilled water before being stained overnight with carmine stain. Carmine Stain was made by adding 1g carmine and 2.5g potassium aluminium sulphate to 500ml distilled water, boiling for 20 minutes, filtering, and then restoring the volume to 500ml with distilled water; the stain was supplemented with 100mg thymol as a preservative and stored at 4°C. Glands were stained overnight at room temperature, rinsed with distilled water and then stored at room temperature in 70% ethanol.

2.13.3 Detection of β-galactosidase Activity in Mammary Glands Using X-Gal

Mammary glands were dissected as described in section 2.13.2 and fixed for 2 hours in PBSA containing 2% (w/v) paraformaldehyde, 0.25% (v/v) glutaraldehyde and 0.01% (v/v) NP-40. After rinsing in PBSA, glands were removed from the glass slide and stained overnight in X-gal staining buffer supplemented with 1mg/ml X-gal (diluted from a stock solution of 40mg/ml). X-gal staining was carried out at 30°C in the dark. Glands were post fixed using NBF for 1 hour, dehydrated through graded ethanols to 100% ethanol and then cleared overnight in xylene. Glands were stored in 70% ethanol at room temperature. Some glands were sectioned and counter-stained with nuclear fast red (see section 2.13.1).

2.13.4 Detection of Apoptotic Cells In Situ (TUNEL Assay)

Experiments were performed on paraffin embedded tissue sections obtained from NBF fixed embryos (see section 2.13.1). The ApopTag® Plus In Situ Apoptosis Detection Kit was used to detect apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) of free 3'OH ends of fragmented DNA as recommended by the manufacturer. Sections were counterstained using a 0.5% (w/v) methyl green in a 0.1M sodium acetate (pH 4.0) solution.
2.13.5 Analysis of Gene Expression by *In Situ* Hybridisation

2.13.5a Wholemount mouse embryos – using digoxigenin-labelled probes

Embryos were dissected in cold PBSA and fixed in 4% (w/v) paraformaldehyde in PBSA overnight at 4°C with gentle agitation. Post-fixed embryos were dehydrated through 5 minute changes in the following solutions: PBS-T (twice), 30% methanol/PBS-T, 50% methanol/PBS-T (twice), 80% methanol/PBS-T, 100% methanol (twice). Dehydration was carried out at room temperature with gentle agitation and embryos were stored in 100% methanol at -20°C. Digoxigenin-labelled riboprobes were generated by adding 5μl 5x transcription buffer, 1μl 0.75M DTT, 2.5μl 10x DIG-NTP mix, 1μg linearised DNA template, 1μl RNasin (40U/μl) and 1.5μl RNA polymerase (T3, T7 or SP6 at 17U/μl) in a total volume of 25μl. The reaction was allowed to proceed at 37°C for 2 hours; the probe was then precipitated by adding 20.5μl distilled water, 2μl 0.5M EDTA (pH8.0), 2.5μl 8M LiCl and 150μl 100% ethanol. After 15 minutes at -70°C the sample was centrifuged in a Sorvall RC5 centrifuge at 16,000g at 4°C, the supernatant removed and then the pellet was washed in 70% ethanol. After another centrifugation step (10 minutes), the ethanol was decanted, the pellet dried and then resuspended in 100μl 10mM EDTA (pH8.0) and stored at -20°C.

Riboprobe hybridisation was achieved as follows. Embryos were rehydrated in PBS-T through subsequent 5 minute changes in 75%, 50% and 25% methanol/PBS-T solutions, digested in 10μg/ml proteinase K in PBS-T for 30-50 minutes depending on the embryonic stage (E11.5 – E14.5). Proteinase K digestion was quenched in 2mg/ml glycine in PBS-T for 10 minutes and the embryos were subsequently washed twice in PBS-T before a 20 minute post-fixation step at room temperature in 4% (w/v) paraformaldehyde + 0.1% (v/v) glutaraldehyde in PBS-T. Embryos were rinsed in PBS-T and then again in a 1:1 PBS-T:WM hybridisation buffer mixture. Pre-hybridisation was for at least one hour at 70°C using pre-warmed WM hybridisation buffer in a Hybaid hybridisation oven. Hybridisation was performed overnight under the same conditions using WM hybridisation buffer containing 100ng/ml of antisense riboprobe.

Post-hybridisation washes were performed initially at 70°C (WM hybridisation buffer (twice), 2x SSC/50% formamide (twice), 1:1 2x SSC/50% formamide:TBS-T) and then at room temperature (TBS-T (twice), MAB-T (twice)). Embryos were then blocked in MAB-T containing 10% heat-treated sheep serum for 1 hour and
incubated overnight at 4°C in blocking solution containing an anti-digoxigenin antibody (1:2,000).

Unbound antibody was removed by washing with MAB-T on a roller: six times for 1 hour at room temperature and then overnight at 4°C. Embryos were developed in the dark by first washing in NTM-T and then by incubation with NTM-T containing 4-Nitro blue tetrazolium chloride (NBT) (9µl/ml) and X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (7µl/ml). Developing reactions were quenched by washing the embryos in three changes of PBS-T. Embryos were stored at 4°C in PBS-T.

2.13.5b  Histological sections – using radio-labelled probes

All distilled water and PBSA was supplemented with 0.1% (v/v) Diethyl pyrocarbonate (DEPC) and autoclaved. Embryos were fixed overnight in PBSA containing 4% (w/v) paraformaldehyde at 4°C with gentle rotation, embedded in paraffin wax, and then sectioned at 5µm intervals as described (section 2.13.1). Histological sections were dewaxed in xylene and rehydrated in PBSA through subsequent changes in 100%, 95%, 70%, 50%, 30% ethanol/PBSA solutions. Sections were digested in a 50mM Tris-HCl (pH8.0), 5mM EDTA, 7µg/ml Proteinase K solution for 10 minutes at 37°C. Digestions were quenched by rinsing in PBSA containing 0.2% (w/v) glycine, and the sections were then post-fixed in 4% (w/v) paraformaldehyde in PBSA for 20 minutes and washed in distilled water. Sections were then acetylated with 100mM triethanolamine, acetic anhydride (0.25% v/v) for 10 minutes, washed in PBSA and then dehydrated by reversing the rehydration process described above.

Radio-labelled riboprobes were generated by combining 2.5µl 5x Transcription Buffer, 0.5µl Rnasin (40U/µl), 0.7µl 100mM DTT, 2µl 6.25mM ATP/GTP/CTP mixture, 1.4µl 1mg/ml linearised DNA template, and 3.5µl [α-35S]-UTP, 0.4µl RNA polymerase (T3, T7 or SP6). The reaction was allowed to proceed for 1 hour at 37°C after which time the DNA template was destroyed by addition of 1µl RNase-free DNase I (10U/µl). The reaction was then diluted with 25µl 10mM DTT + 1.5µl rRNA (10mg/ml) and passed through an equilibrated Chroma Spin-30 column. 4µl 100mM DTT was then added to the eluate and the probe was stored at -20°C. Radioactive [α-35S]-UTP incorporation was estimated by adding 1µl of riboprobe to 3ml of scintillation fluid, and then counting on a Beckman Coulter scintillation counter before and after filtration through the column.
Hybridisation of riboprobes was carried out in a solution containing (300mM NaCl, 10mM Na₂HPO₄, 10mM Tris-HCl, 50mM EDTA, pH6.8), 50% (v/v) de-ionised formamide, 300μg/ml rRNA, 10% (w/v) dextran sulphate, 1x Denhardt’s solution and 10mM DTT. Approximately 1x10⁶ cpm of radio-labelled probe was left to hybridise to each section overnight at 51°C in a humid sealed box.

After hybridisation, sections were washed in 5x SSC containing 10mM DTT at 50°C, then with 50% formamide, 2x SSC + 20mM DTT at 65°C for 30 minutes each. Sections were next washed three times for 10 minutes in NTE buffer at 37°C followed by a further 30 minutes at the same temperature with NTE buffer containing 20μg/ml RNase A, then once more again with NTE buffer. After another wash with 50% formamide, 2x SSC + 20mM DTT at 65°C for 30 minutes, sections were washed at room temperature in 2x SSC and then with 0.1x SSC for 15 minutes before dehydration through 30%, 60%, 80%, 95% ethanol solutions containing 300mM ammonium acetate. Dehydration was completed in 100% ethanol and the slides were allowed to dry in a dust-free environment. Slides were dipped, under dark room conditions, into Ilford K5 photographic emulsion, dried and then left to expose the emulsion for up to 2 weeks in the dark at 4°C. Slides were developed using Kodak D-19 developer, stopped in a 1% (v/v) acetic acid solution, light-fixed in 30% (w/v) sodium thiosulphate, washed and then counterstained with Giemsa stain (Histopathology Department, Cancer Research UK) and mounted in DPX mounting medium under glass cover slips.
Chapter 3  |  Intracellular Targeting of the ErbB-2 Receptor Tyrosine Kinase

3.1 Introduction

The formation of many organs and tissues involves a complex series of reciprocal interactions between the epithelia and underlying mesenchyme (reviewed in Hogan, 1999). The ErbB family of receptor tyrosine kinases have been implicated in the signalling between these two cell lineages, and play crucial roles in animal development and the process of tumourigenesis (section 1.2.3, reviewed in Alroy and Yarden, 1997). In epithelia, ErbB-1 and ErbB-2 are predominantly localised to the basolateral membrane where they are able to mediate signals between the epithelium and the adjacent mesenchyme (Borg et al., 2000; Darcy et al., 2000; Robert and Carlin, 1995). Although structurally homologous to other members in the family, ErbB-2 is unique as ligand-mediated homodimers have not been identified, suggesting that normal signal transduction occurs through the formation of heterodimers (Klapper et al., 1999). Moreover, ErbB-2 seems to be the preferred heterodimerisation partner for other family members, and therefore plays a central role in ErbB family mediated signalling events (Graus-Porta et al., 1997).

Despite a high degree of homology between ErbB family members, the mechanisms that govern receptor localisation appear to differ. Whereas ErbB-1 has been shown to contain an autonomous basolateral sorting signal in the cytoplasmic juxtamembrane region (Hobert and Carlin, 1995; Hobert et al., 1997), a trans-acting mechanism of localisation through an interaction at the carboxy-terminus with the PDZ domain-containing protein ERBIN appears to control ErbB-2 localisation (Borg et al., 2000). Recently, a putative basolateral targeting signal mapping within the intracellular juxtamembrane region of the molecule has been identified in ErbB-2, (unpublished results, Creer, 2000). An extensive mutagenic analysis of ErbB-2 was undertaken to fully characterise this targeting signal; in addition, the targeting properties of the juxtamembrane and carboxy-terminal ErbB-2 basolateral targeting signals were compared. These studies principally relied on the introduction of mutated ErbB-2 proteins into polarised epithelial cells, and the subsequent assessment of the polarised distribution of these proteins by confocal microscopy.
3.2 MDCK Cells: a System for Studying Epithelial Cell Polarity

A simple and reproducible model of epithelial cell polarity was required to examine the polarised trafficking of ErbB-2. By combining cell culture and transfection techniques with immunofluorescence and confocal microscopy, a system was generated that allowed a large number of mutated proteins to be assayed.

3.2.1 MDCK Cells Form a Polarised Epithelial Monolayer

MDCK cells were used to study the signals involved in the basolateral targeting of human ErbB-2. This cell line has been used extensively to study many different aspects of cell polarity as they form tight polarised monolayers when grown under appropriate conditions, and they benefit from a simple protein trafficking system.

In order to confirm that the particular clone of MDCK cells obtained for these studies could form polarised monolayers, they were grown on PET membranes, and the localisation of a variety of epithelial polarity markers were assessed in order to confirm the establishment of a polarised epithelial monolayer. Two days after the cells reached confluency, membranes on which MDCK cells were grown were fixed in cold methanol and the distribution of E-cadherin, β-catenin, and the tight junction component protein ZO-1 was analysed by indirect immunofluorescence and confocal microscopy.

Immunofluorescence was visualised using a Zeiss LSM 510 upright confocal microscope. Images were captured through the XZ plane at 0.2μm intervals to permit the apical and basolateral membrane domains to be visualised on the same image, thereby allowing the intensity of staining of any single protein between these two domains to be compared directly. In addition, an XY confocal slice was captured through the level of the tight junctions (as judged by ZO-1 staining) to allow a further distinction to be made between apical and basolateral fluorescent signals. These techniques were used to analyse the distribution of all expressed proteins presented in this chapter.

As expected in a polarised monolayer of epithelial cells, E-cadherin and β-catenin (both key components of the adherens junctions) were localised to the lateral membrane of these MDCK cells (see Figure 3.1). ZO-1 was localised exclusively at the interface between the basolateral and apical membrane domains, consistent with its integration into tight junctions. The tight junction protein ZO-3 showed an identical localisation pattern to ZO-1 (data not shown). MDCK cells grew in tight
MDCK cells were seeded onto PET membranes and allowed to reach confluency. The distribution of marker proteins of epithelial polarity were assessed by indirect immunofluorescence. The figure shows that both β-catenin and E-cadherin (green) are localised to the lateral domain in MDCK cells, and that the tight junction protein ZO-1 (red) is correctly localised to the interface between the apical and lateral domains.

The top panels show an XY confocal slice through the level of the tight junctions and the bottom panels represent an XZ confocal slice from the apical domain (top), through the lateral domains to the basal domain (bottom), as depicted schematically in Figure 1.8. Scale bar represents 10μm.
islands with an epithelial morphology, and were able to colonise a dish when expanded from a single cell, suggesting that stable clones of MDCK cells could be isolated after transfection with an expression plasmid. Having displayed a suitable polarised epithelial phenotype, these clones of MDCK cells were subsequently used for all of the experiments described below unless otherwise stated.

3.2.2 Tetracycline-On Inducible Gene Expression System

In order to examine the signals involved in the control of human ErbB-2 localisation, stable clones of MDCK cells expressing full length and mutated human ErbB-2 proteins needed to be generated. Producing stable clones expressing these proteins has unique advantages over analysing protein localisation using transient transfection assays. For example, in stable cell populations the gene of interest is expressed at similar levels in every cell making the result easier to interpret both visually by immunofluorescent staining, and biochemically by cell surface labelling experiments. This homogeneity also meant that the localisation studies were highly reproducible, thereby decreasing the number of replicate experiments that needed to be performed. Also, expression levels tend to be lower in stably expressing cells, reducing the risk of saturating the endogenous trafficking machinery which could lead to protein mis-localisation.

A tetracycline-on inducible gene expression system was used to produce stable MDCK cell clones expressing wild type and mutant proteins (Figure 3.2). An inducible gene expression system was employed as previous attempts to isolate stable cell clones constitutively expressing ErbB-2 proteins had failed. Briefly, the system relies upon the stable integration of two plasmids, i) a repression plasmid (pcDNA6/TR, Invitrogen Life Technologies), and ii) an expression plasmid (pcDNA4/TO, Invitrogen Life Technologies) into which the gene of interest has been cloned. pcDNA6/TR constitutively expresses the tetracycline-repressor protein which binds to tetracycline-operator elements present in a modified cytomegalovirus (CMV) promoter that drives gene expression from pcDNA4/TO. This interaction effectively blocks the CMV promoter activity of the expression plasmid, resulting in a tight repression of gene expression. In the presence of small amounts of tetracycline, the tetracycline-repressor protein preferentially binds to this antibiotic, thus releasing the block on gene transcription. It is therefore possible, using low levels of antibiotic, to switch from undetectable basal levels of gene expression, to high levels of induced gene expression in a short time frame. This system therefore allowed gene expression of ErbB-2 whilst minimising the risk of cellular transformation that could indirectly affect cell polarisation (Di Fiore et
ErbB-2 Basolateral Targeting

al., 1987). Furthermore, gene expression could be induced after the cells had become confluent, avoiding possible non-polarised localisation of protein that might occur prior to the establishment of a polarised monolayer, leading to an inaccurate evaluation of protein distribution.

**Figure 3.2 – Tetracycline-on inducible gene expression system**

Tetracycline repressor protein (green) expressed from pcDNA6/TR binds to tetracycline-operator elements (red) within the CMV promoter of pcDNA4/TO based plasmids causing a repression of gene expression. Tetracycline addition releases the transcriptional block on the gene of interest (yellow) by binding with high affinity to the repressor protein.

Stable MDCK cell lines were generated by co-transfection of pcDNA6/TR and pcDNA4/TO based plasmids as described in section 2.9.4. DNA was digested with FspI prior to transfection to linearise the vectors in a region that was not required for gene expression. A total of 3μg linearised DNA was used in all co-transfections using a repression:expression plasmid ratio of 5:1 (2.5μg pcDNA6/TR:0.5μg pcDNA4/TO) to increase the relative amounts of tetracycline repressor protein within the system, thereby decreasing the basal levels of ErbB-2 expression.

Stable integration of pcDNA6/TR and pcDNA4/TO based plasmids was selected for by growing transfected cells in the presence of the antibiotics blasticidin S HCl and Zeocin™ respectively. Complete medium supplemented with 5μg/ml blasticidin S HCl and 400μg/ml Zeocin™ was established (by titration) to be optimal for killing untransfected MDCK cells, whilst remaining essentially non-
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toxic to transfected cells. This combination was subsequently used for the selection and maintenance of all stable MDCK clones.

3.2.3 ErbB-2 is Targeted to the Basolateral Membrane of Epithelial Cells

To establish whether human ErbB-2 is localised to the basolateral membrane of polarised MDCK cells when expressed from the inducible gene expression system, stable MDCK clones were generated containing cDNAs encoding the full length ErbB-2 receptor. It was essential to confirm that ErbB-2 was targeted to the basolateral membrane domain using this system, as further experiments aimed to decipher the signals that control its localisation.

3.2.3a Generation of stable MDCK cell clones expressing full length ErbB-2

A ~4 kb fragment of cDNA encoding the entire human ErbB-2 sequence was excised from the vector pSV2ERBB-2 (kind gift from Helen Hurst; Yamamoto et al., 1986) by digestion with HindIII. The fragment was subsequently cloned into the HindIII site of pcDNA4/TO to form the vector pc4-B2 (see appendix A6.2.6). pcDNA6/TR and pc4-B2 were co-transfected into MDCK cells, and individual clones of MDCK cells incorporating both plasmids were isolated after their selection with blasticidin S HCl and Zeocin™.

3.2.3b Expression of ErbB-2 is induced by tetracycline in stable MDCK cell clones

Individual stable MDCK clones were tested for exogenous ErbB-2 expression by immunoblot analysis. 2.5 x 10^5 cells were seeded on 35mm cell culture dishes in duplicate, and the medium replaced every two days with fresh complete medium. After the cells had reached confluency, 1μg/ml tetracycline was added to one of the dishes to induce gene expression. The following day, cell lysates from both samples were prepared in Laemmli buffer for SDS-PAGE and immunoblotting as described in section 2.12. The primary antibody used to detect the presence of exogenous ErbB-2 protein was Clone 42 which is directed against the extracellular domain of human ErbB-2. Approximately 50% of the stable clones tested expressed a protein of the predicted size when induced with tetracycline. Most clones did not express detectable levels of ErbB-2 in the absence of tetracycline, indicating that the inducible system was effective in MDCK cells, and providing confirmation that clone 42 anti-ErbB-2 did not cross-react with endogenous MDCK proteins (see
Figure 3.3A). Stable cell clones demonstrating i) undetectable basal levels, and high levels of induced ErbB-2 expression, and ii) a morphological resemblance to the parental cell line, were cryo-preserved and used for subsequent experiments. All stable cell lines described in this chapter were generated and selected for analysis using these same techniques.

3.2.3c Exogenous ErbB-2 is targeted to the basolateral membrane in MDCK cells

Three independent clones of stable MDCK cells expressing full length ErbB-2 were seeded (2.5 x 10^5 cells) in duplicate on to PET membranes which were individually placed in the wells of a 6 well cell culture dish, thus forming a two-chamber system. Fresh complete medium containing blasticidin S HCl and Zeocin™ was added every two days to each chamber of the filter/well bathing both the apical and basolateral sides of the cultured cells. Two days after the cells had reached confluent levels, 1μg/ml tetracycline was added to one of the samples to induce ErbB-2 expression. The following morning, cells were fixed in cold methanol and the sub-cellular distribution of ZO-1 and exogenously expressed ErbB-2 were analysed by indirect-immunofluorescent staining and confocal microscopy. Cells were incubated with primary antibodies 9G6.10 (a mouse monoclonal antibody reactive against the extracellular domain of human ErbB-2) and anti ZO-1. Immunofluorescent staining was achieved using FITC-conjugated goat anti-mouse and TRITC-conjugated goat anti-rat antibodies as described in section 2.9.5. In agreement with the published findings (Borg et al., 2000), ErbB-2 was localised predominantly to the basolateral membrane domain in all three stable clones of MDCK cells tested. No ErbB-2 was detected in the uninduced samples providing confirmation of the specificity of the antibodies. Furthermore, no expression of ErbB-2 could be detected by immunoblot or immunofluorescence analyses in MDCK cells containing stably integrated pcDNA6/TR and empty pcDNA4/TO vectors (data not shown). Figure 3.3B shows the induction of ErbB-2 protein synthesis, and basolateral localisation of ErbB-2 in one of the stable clones examined.

To ensure that individual MDCK stable cell clones maintained their epithelial polarity after i) their selection and maintenance in antibiotic supplemented growth medium, and ii) tetracycline-induced expression of ErbB-2, the distribution of marker proteins was assessed. Stable MDCK cells expressing full length ErbB-2 were grown on PET membranes to form a polarised epithelial monolayer as previously described (section 3.2.1). ErbB-2 expression was induced overnight by
Stable MDCK cells expressing full length human ErbB-2 (FL ErbB-2) were isolated and the sub-cellular distribution of exogenous ErbB-2 assessed.

A) An immunoblot representative of a typical MDCK stable cell clone expressing full length ErbB-2. Tetracycline was shown to induce the expression of ErbB-2. A431 cells which strongly express ErbB-2 were used as a control for ErbB-2 expression.

B) Indirect immunofluorescence showing basolateral localisation of full length human ErbB-2 (green) after induction with tetracycline. The tight junction protein ZO-1 is stained in red and acts as a marker for the visual distinction between the apical and basolateral domains. XY and XZ confocal images are shown. Scale bar represents 10µm.
addition of 1μg/ml tetracycline into the growth medium. The following day the
distribution of epithelial polarity markers were assessed by immunofluorescence
and confocal microscopy (see section 3.2.1). No discernible differences could be
detected in the levels, and distribution of E-cadherin, β-catenin and ZO-1 when
compared to the parental cells (data not shown). This demonstrates the polarised
epithelial phenotype was not significantly compromised in this system thereby
validating its use to analyse the signals controlling receptor localisation.

3.3 The Juxtamembrane Domain of
ErbB-2 Contains a Basolateral
Targeting Signal

Recent studies using carboxy-terminal truncated forms of ErbB-2 transiently
expressed in polarised MDCK cells identified the presence of a basolateral targeting
signal within the juxtamembrane domain of ErbB-2 (Creer, 2000). To confirm the
presence of the basolateral targeting signal, and to establish whether these mutated
ErbB-2 proteins were localised to the same membrane domains when
homogenously expressed in a polarised epithelial monolayer, stable MDCK cell
lines expressing the truncated ErbB-2 proteins were generated.

3.3.1 Generation of Stable MDCK Cell Clones Expressing
Carboxy-Terminally Truncated ErbB-2 Proteins

Figure 3.4 shows the sequence of the cytoplasmic juxtamembrane region of ErbB-2,
and the position of the introduced stop codon in each truncated receptor. cDNAs
encoding four different mutated forms of ErbB-2 proteins consisting of the full
extracellular and transmembrane domains, but truncated within the intracellular
domain at amino acid positions 690, 695, 701 and 706 (numbering based on
Coussens et al., 1985) were excised from plasmids pcDNA3MB2del1 – del4 (Creer,
2000) using HindIII and XbaI. cDNA fragments were gel extracted and cloned into
HindIII/XbaI digested pcDNA4/TO to produce the plasmids pc4-A690, pc4-A695,
pc4-A701, and pc4-A706. These plasmids contain an in-frame stop codon
immediately 3' to the coding sequence and 5' of the XbaI site. Each plasmids was
linearised with FspI and separately co-transfected with pcDNA6/TR as described in
section 3.2.3a to generate tetracycline inducible stable clones of MDCK cells
expressing truncated ErbB-2 proteins. At least three independent stable cell clones
were analysed for each truncated ErbB-2 receptor.
3.3.2 Expression and Sub-Cellular Localisation of Truncated ErbB-2 Proteins

Inducible expression of mutated ErbB-2 proteins was analysed as described previously in section 3.2.3b. All stable clones expressing mutated ErbB-2 receptors produced a protein of the predicted size following induction overnight with 1μg/ml tetracycline (see Figure 3.5A). Mutated ErbB-2 receptors were not detected in uninduced stable clones.

The sub-cellular distribution of truncated ErbB-2 receptors was assessed by indirect immunofluorescence and confocal microscopy as described in section 3.2.3c (see Figure 3.5B). Whereas full length ErbB-2 had a predominantly basolateral localisation, an ErbB-2 receptor truncated at position 690 (Δ690) was localised mainly to the apical membrane (compare Figure 3.3 and Figure 3.5B). A basolateral localisation was slightly restored in the mutated receptor Δ695 which displayed a shared apical/basolateral distribution. This suggests that the amino acid residues between position 691-695 (LQETE) constitute an important part of a basolateral...
targeting signal. The polarised distribution of receptors Δ701 and Δ706 were indistinguishable from that of the full length receptor, with the vast majority of protein being detected at the basolateral membrane domain. This indicates that amino acids 696-701 (LVEPLT) also form part of the targeting signal. All stable clones of MDCK cells examined gave indistinguishable results. A similar localisation of full length and truncated receptors was obtained when expressed transiently from the pcDNA4/TO based vectors in MDCK cells (data not shown). Taken together, these data indicate the presence of a basolateral targeting signal in ErbB-2 between amino acids 690-701 and agree with previous findings (Creer, 2000). This signal is sufficient to target ErbB-2 to the basolateral membrane domain of MDCK cells in the absence of other influencing factors within the remaining intracellular domain of ErbB-2. Furthermore, in the absence of this signal, ErbB-2 is targeted to the apical domain of MDCK cells indicating that either, i) apical localisation is the default pathway in these clones of MDCK cells, or ii) the extracellular or transmembrane domain of ErbB-2 contains active apical targeting information. In either case (which are not mutually exclusive) the juxtamembrane basolateral targeting signal is able to redirect a truncated ErbB-2 receptor from the apical membrane domain to the basolateral membrane domain in MDCK cells.

To determine whether these findings apply to trafficking of ErbB-2 in other polarised epithelia, full length and truncated ErbB-2 proteins were expressed transiently in CACO-2 cells. CACO-2 cells are derived from a primary human colonic tumour and, similar to MDCK cells, form a polarised epithelial monolayer when grown on PET membranes and are a commonly used tool for analysing protein trafficking. 2.5x10^6 CACO-2 cells were seeded onto PET membranes and left overnight to recover. The following day cells were transiently transfected with pc4-B2, pc4-Δ690, or pc4-Δ706 and left for two days to form a confluent polarised monolayer. Cells were fixed in 4% (w/v) paraformaldehyde and ErbB-2 proteins were revealed by immunofluorescence using 9G6.10 and FITC-conjugated goat anti mouse immunoglobulins (see Materials and Methods section 2.9.5). The tight junction proteins ZO-1 and ZO-3 could not be detected in CACO-2 cells with our antibodies as they were raised against canine immunogens. An assessment of protein distribution revealed results similar to those obtained with MDCK cells which are shown in Figure 3.5C.

### 3.3.3 Quantification of Cell Surface ErbB-2

To obtain a more quantitative measure of the localisation of ErbB-2 proteins, the steady-state cell surface distribution of full length and truncated ErbB-2 proteins
Figure 3.5 - Expression and localisation of truncated ErbB-2 receptors

A) Immunoblot of stable MDCK cell clones showing tetracycline inducible gene expression of mutated ErbB-2 proteins indicated.

B) Confocal images showing sub-cellular localisation of truncated ErbB-2 proteins after their induction with tetracycline (1µg/ml). ErbB-2 proteins are stained in green, red indicates the presence of ZO-1.

C) ErbB-2 truncated proteins show a similar location in CACO-2 cells. ErbB-2 proteins as indicated were transiently expressed in polarised CACO-2 cells and then revealed by immunofluorescence (green). Scale bar represents 10µm.
was assessed. Stable MDCK cell clones expressing full length or the truncated ErbB-2 proteins Δ690, Δ695, Δ701 or Δ706 were seeded onto PET membranes in complete growth medium containing Zeocin™ and blasticidin S HCl as described in section 3.2.2. Two days after the cells had reached confluency they were induced overnight to express ErbB-2 by addition of 1μg/ml tetracycline into the growth medium. Cells were then labelled from the apical or basolateral chamber of the PET membrane with a non-membrane permeating derivative of biotin. Labelled cell surface proteins were captured on immobilised streptavidin beads, separated by SDS-PAGE and detected by immunoblot analysis using clone 42 anti-ErbB-2 followed by an infrared goat anti-mouse secondary antibody (see Materials and Methods section 2.12.5). The relative proportion of receptor on the apical and basolateral membranes was calculated as a percentage of the total population of cell surface receptors:

\[
\% \text{ apical ErbB-2} = \frac{a}{a + b} \\
\% \text{ basolateral ErbB-2} = \frac{b}{a + b}
\]

where \( a \) = intensity of apical ErbB-2, and \( b \) = intensity of basolateral ErbB-2. The intensity was determined by measuring the strength of the infrared signal emitted from the secondary antibody (800 channel).

Full length ErbB-2 (FL ErbB-2) was predominantly found on the basolateral membrane (88.5% basolateral, 11.5% apical) and Δ690 was found predominantly on the apical membrane (7.8% basolateral, 92.2% apical) in agreement with the immunofluorescence data (Figure 3.6). Stable MDCK clones expressing Δ701 or Δ706 localised a similar proportion of truncated ErbB-2 as the full length receptor to the basolateral membrane (72.4% basolateral, 27.6% apical and 76.6% basolateral, 23.4% apical respectively). In the stable clone expressing Δ695 the receptor distribution was more concentrated on the apical than basolateral membrane (28.7% basolateral, 71.3% apical), consistent with a basolateral targeting signal being compromised in this protein.

Taken together with the immunofluorescence data (sections 3.2.3c and 3.3.2) these results confirm the presence of a basolateral targeting signal in the juxtamembrane region of ErbB-2. This signal is sufficient to reverse the polarised distribution of the majority of exogenously expressed ErbB-2 when retained in the truncated receptor (compare localisation of Δ690 and Δ706). However, MDCK cells localise a higher proportion of full length ErbB-2 receptor to the basolateral membrane compared to the truncated version of the receptor containing the signal (Δ706). The reasons for
Figure 3.6 – Quantification of cell surface ErbB-2

A quantitative comparison of cell surface distribution of full length and truncated ErbB-2 receptors. Stable clones expressing the indicated receptors were grown to confluence on PET membranes and then labelled with biotin. After precipitating labelled cell surface proteins with streptavidin, samples were separated by SDS-PAGE and quantified on an IR LI COR Biosciences scanner. The proportion of receptors on the apical (Ap) and basolateral (BL) surface are shown. The standard errors (SEs) of three independent experiments are shown.
this difference are unclear, but it is possible full length and truncated receptors have different properties which influence their steady-state cell surface distribution, such as changes in protein stability, or recycling efficiency. Alternatively it might indicate that there are other factors within the carboxy-terminal intracellular region of ErbB-2 that are involved in the basolateral targeting, or membrane retention of ErbB-2.

3.4 The Juxtamembrane Targeting Signal is Required for Basolateral Delivery of ErbB-2

The juxtamembrane basolateral targeting signal of ErbB-2 was shown to be active when large portions of the intracellular domain of ErbB-2 were truncated. However, it is possible other signals present within the intracellular domain of ErbB-2 may influence the efficacy of this signal. To assess this, small internal deletion mutations were previously generated in and around the region of the juxtamembrane basolateral targeting signal, using the full length ErbB-2 receptor as a template (Creer, 2000). These receptor cDNAs were transferred to the tetracycline-on inducible gene expression system and stably introduced into MDCK cells. Stable cell clones were induced to express mutated receptor proteins and their localisation assessed.

3.4.1 Generation of Stable MDCK Cell Clones Expressing Internally Deleted Forms of ErbB-2

cDNAs encoding ErbB-2 receptors lacking amino acids 684-690, 684-695, 684-706, 696-701 and 702-706 were excised from plasmids pcDNA3MB2-M1, -M2, -M4, -M6, or -M7 (Creer, 2000) using HindIII. All receptors possessed the natural stop codon immediately 5’ of the 3’ HindIII site. Fragments were gel extracted and cloned into the HindIII site of pcDNA4/TO to generate the plasmids pc4-Δ684-690, pc4-Δ684-695, pc4-Δ684-706, pc4-Δ696-701, and pc4-Δ702-706. The correct orientation of the coding insert was confirmed by restriction endonuclease digestion. Figure 3.7 shows a schematic depiction of these mutated ErbB-2 receptors.

Stable clones of MDCK cells expressing the internally deleted forms of ErbB-2 were generated by co-transfection of the appropriate expression plasmid with
ErbB-2 Basolateral Targeting

pcDNA6/TR (see section 3.2.3a). At least three independent expressing clones were analysed for each mutated ErbB-2 receptor; all clones produced a protein of the predicted size as determined by immunoblot analysis using the primary antibody clone 42 anti-ErbB-2 (data not shown).

Figure 3.7 – Mutated ErbB-2 receptors containing internal deletions

Diagram detailing the domain structure of ErbB-2 (TM = transmembrane) and the amino acid sequence of the intracellular juxtamembrane domain (red). Internal deletion mutations were previously generated (bottom portion) to delete the amino acid residues indicated (inclusive). These mutated receptors possessed a full extracellular and transmembrane domain, and a full intracellular domain excluding the depicted deletion. (-) indicates deleted amino acids.

3.4.2 Sub-Cellular Localisation of Mutated ErbB-2 Receptors Containing Internal Deletions

Individual clones of MDCK cells containing stable mutant cDNAs were grown on PET membranes to confluence, left for a further 24 hours, and then induced overnight to express internally deleted ErbB-2 proteins. Full length receptor proteins containing deletions close to the proposed signal (receptors $\Delta 684-690$ and $\Delta 702-706$) were shown to localise to the basolateral membrane similar to the full-length receptor (compare Figure 3.8 and Figure 3.3). This indicates that neither the
Figure 3.8 - Localisation of mutated ErbB-2 receptors containing internal deletions

MDCK cells were transfected with pcDNA6/TR and pc4-Δ684-690, pc4-Δ684-695, pc4-Δ684-706, pc4-Δ696-701, or pc4-Δ702-706. Stable clones expressing the proteins indicated were selected and seeded onto PET membranes. 2 days after cells reached confluence ErbB-2 proteins were induced overnight with tetracycline and the distribution of mutant receptors analysed by indirect immunofluorescence (green = ErbB-2 proteins, red = ZO-1). XY and XZ confocal images are shown. Scale bar represents 10μm.
KYTMRRL nor PSGAM motif constitutes an important part of the basolateral targeting signal.

By contrast, deletion of amino acids 684-695 or 684-706 (receptors A684-695 and A684-706 respectively) resulted in an apical localisation, although the larger deletion also showed a very significant punctate distribution in the cytoplasm suggesting a perturbation of endosomal trafficking. A deletion encompassing amino acids 696-701 (protein A696-701) resulted in a shared apical/basolateral localisation. These results, together with the localisation studies of truncated ErbB-2 receptors, establish the presence of a basolateral targeting signal between amino acids 690-701 (LLQETELVEPLT) of ErbB-2. Moreover, they demonstrate that the juxtamembrane signal of ErbB-2 is required for basolateral targeting, even in the presence of the intracellular carboxy-terminal domain of ErbB-2.

3.5 The ErbB-2 PDZ Binding Domain is Not Required for Basolateral Localisation

The presence of the ErbB-2 juxtamembrane targeting signal presents an interesting conundrum concerning the role of the carboxy-terminus of ErbB-2 in its basolateral targeting. In previous experiments (Borg et al., 2000), either loss of the six carboxy-terminal amino acids, or a valine to alanine carboxy-terminal substitution was sufficient to redirect a chimeric receptor composed of ErbB-1/ErbB-2 (ErbB-2 intracellular domain) from the basolateral to the apical membrane of polarised MDCK cells. These mutations correlated with the loss of binding for a PDZ domain-containing protein designated ERBIN, suggesting that ERBIN is responsible for the basolateral targeting of ErbB-2. In the experiments described in section 3.3, the last 549 or 554 carboxy-terminal amino acids could be deleted without affecting the basolateral localisation of ErbB-2. These mutated ErbB-2 proteins (Δ706 and Δ701) presumably do not interact with ERBIN yet they retain basolateral localisation, suggesting that the interaction with ERBIN is not required for basolateral localisation. In an attempt to resolve this paradox, the mutations in the ErbB-1/ErbB-2 chimera generated by Borg and colleagues that abolished the ERBIN binding site were created within the full length ErbB-2 cDNA. The localisation of the resulting proteins were analysed in MDCK cells.
3.5.1 Generation of Stable MDCK Clones Expressing ERBIN Binding Mutants of ErbB-2

Receptor V1255A and Δ1249 contain a valine to alanine substitution at position 1255 and a deletion of the last 6 carboxy-terminal amino acids of ErbB-2 respectively (see Figure 3.9). Mutations were generated by PCR using a forward primer annealing to sequences 5' of the Eco47III site in ErbB-2 (primer: B2E47) and a reverse primer containing the appropriate mutation (primer: VPAmut or D6mut), followed by a 3' stop codon and an XbaI site. PCR products were digested with Eco47III and XbaI, gel extracted, and then cloned into pc4-B2 to replace the unmutated carboxy-terminus of ErbB-2 using Eco47III and XbaI. This formed the inducible expression plasmids pc4-V1255A and pc4-Δ1249 whose sequences were verified by DNA sequencing. Stable clones of MDCK cells were generated by co-transfection with pcDNA6/TR as previously described. At least three independent expressing clones were isolated for each mutated receptor and were shown to express a protein of the predicted size as determined after immunoblotting (data not shown).

![Diagram showing the carboxy-terminal domain (green) amino acid sequence of full length ErbB-2 encompassing the ERBIN binding domain (GLDVPV), TM = transmembrane domain. The bottom portion of the figure shows the location of the introduced changes in two ERBIN binding mutants of ErbB-2.](image)

---

**Figure 3.9 – Mutations in the ERBIN binding domain of ErbB-2**

Full Length ErbB-2

<table>
<thead>
<tr>
<th>Extracellular</th>
<th>TM</th>
<th>Intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
<td>C</td>
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</table>

1240 - TPTAENPEYLGDLVPV* - 1256

ErbB-2 ERBIN Binding Mutations

<table>
<thead>
<tr>
<th>Extracellular</th>
<th>TM</th>
<th>Intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
<td>C</td>
</tr>
</tbody>
</table>

V1255A 1240 - TPTAENPEYLGDLVPA* - 1256

Δ1249 1240 - TPTAENPEYL* - 1251

Diagram showing the carboxy-terminal domain (green) amino acid sequence of full length ErbB-2 encompassing the ERBIN binding domain (GLDVPV). TM = transmembrane domain. The bottom portion of the figure shows the location of the introduced changes in two ERBIN binding mutants of ErbB-2.
3.5.2 Localisation of ERBIN Binding Mutants of ErbB-2

Contrary to the findings reported by Borg and co-workers (Borg et al., 2000), the ErbB-2 mutated proteins, either lacking the last six carboxy-terminal amino acids (Δ1249) or containing a carboxy-terminal V to A substitution at position 1255 (V1255A) showed a basolateral localisation that was indistinguishable from the full length ErbB-2 (compare Figure 3.10 and Figure 3.3). These results suggest, at least in this experimental system, ERBIN is not necessary for targeting ErbB-2 to the basolateral membrane of polarised MDCK cells. It is also likely ERBIN binding is not required for ErbB-2 localisation in CACO-2 cells given that the Δ706 receptor localises to the basolateral domain when expressed in these cells (see Figure 3.5C).

In attempt to finally clarify these contradictory results, the expression constructs used in the Borg study containing ErbB-1/ErbB-2 chimeric receptors either containing the full carboxy-terminus of ErbB-2 (RK5-HER1/2) or the carboxy-terminus lacking the last six amino acids (RK5-HER1/2A6) were obtained (kind gift from Dr. J.P. Borg, INSERM, Marseille, Borg et al., 2000). Using an antibody directed against the extracellular domain of ErbB-1, the distribution of HER1/2 and HER1/2A6 was assessed. When transiently transfected into polarised MDCK cells both of the expressed proteins localised to the basolateral domain. This result is in contrast to the published findings (Borg et al., 2000), and in agreement with the data presented here.

3.6 A Novel Bipartite Motif Targets ErbB-2 to the Basolateral Membrane in MDCK Cells

Results obtained using truncated or internally deleted forms of ErbB-2 revealed a basolateral targeting signal located between amino acids 690-701. To characterise the key residues that constitute the basolateral sorting motif, site-directed mutagenesis was used to create a series of alanine substitution mutations of the full length receptor within the candidate region (690-LLQETELVEPLT-701). All mutated proteins were expressed in MDCK cells by transient transfection with the appropriate vector, and the location of the encoded protein assessed by indirect immunofluorescence and confocal microscopy as described in sections 2.9.4a and 3.2.3c. At least 20 transfected cells for each mutated receptor were analysed to determine the distribution of each alanine substitution mutation of ErbB-2.
Immunofluorescence analysis of ErbB-2 proteins containing ERBIN binding mutations (green = ErbB-2 proteins, red = ZO-1). Representative XY and XZ confocal images are shown for each mutated receptor.

A) Stable MDCK cells expressing the mutated proteins indicated in a polarised monolayer after induction with tetracycline.

B) MDCK cells were transiently transfected with the expression constructs used in the Borg study (Borg et al. 2000). The figures show the localisation of a chimeric receptor composed of ErbB-1/ErbB-2 with (HER1/2), or without (HER1/2Δ6) the ERBIN binding domain. Scale bar represents 10μm.
### Table 3.1 – Creation of alanine substitution mutants of ErbB-2

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<th>Plasmid Name</th>
<th>Primer Pairs</th>
<th>Mutated Basolateral Targeting Signal</th>
<th>DNA Template</th>
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<td>LLQETELVEPLA</td>
<td>pc4-B2</td>
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<td>pc4-B2</td>
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</table>

**Table listing the different alanine substitution mutations of ErbB-2.** The plasmid name (column 1), primer pairs (column 2), and DNA template (column 4) used to generate the mutation within the basolateral targeting motif (column 3) are given. The designated name given to each of these mutated receptors is derived from the plasmid name (i.e. pc4-QETE(A) expresses the receptor QETE(A)).
3.6.1 Generation of Site-Directed Mutations in ErbB-2

Site-directed mutagenesis was performed as described in section 2.8.12. A combination of single and multiple alanine substitutions were created within the candidate region and are listed in the table below. All reactions used a pcDNA4/TO based plasmid as a DNA template (pc4-B2 or derivatives of), and so directly created a plasmid that was ready to transfect and express in mammalian cells. The primers and DNA templates used to generate each alanine mutation are listed. If alanine substitutions could not be made in a single reaction, then sequential reactions were performed as indicated, using previously mutated plasmid templates. Mutations created within the candidate basolateral targeting signal (LLQETELVEPLT) are shown (underlined) and were verified by DNA sequencing. These mutations were generated within the full length ErbB-2 receptor and therefore retained the ERB1N binding site at the carboxy-terminus.

3.6.2 Expression and Localisation of Mutated ErbB-2 Proteins Containing Alanine Substitutions

Expression of mutated ErbB-2 proteins was determined by immunoblot analysis using clone 42 anti-ErbB-2 as a primary antibody. All plasmids containing alanine mutations of ErbB-2 produced a product of the predicted size (indistinguishable from the full length ErbB-2 receptor) when lysates prepared from transiently transfected MDCK cells were immunoblotted (data not shown).

Surprisingly, all the ErbB-2 proteins containing single mis-sense point mutations within the candidate region were correctly localised to the basolateral membrane as determined by indirect immunofluorescence and confocal microscopy (Figure 3.11), suggesting a functional redundancy or plasticity of the targeting motif.

As several membrane receptors have been shown to contain basolateral targeting signals with essential di-hydrophobic residues (see Table 1.3), the two pairs of hydrophobic residues at position 690/691 (LL) and 696/697 (LV) were separately substituted with alanines. Following expression, the two mutated proteins (LL(A)) and (LV(A)) were both shown to locate to the basolateral membrane suggesting that neither of these di-hydrophobic residues are essential for basolateral sorting (Figure 3.12). To determine whether these di-hydrophobic residues might be acting as a redundant basolateral targeting signal, an ErbB-2 receptor with all four hydrophobic residues substituted with alanines (LL/LV(A)) was expressed in MDCK cells. This mutated protein was also located basolaterally (Figure 3.12) with
ErbB-2 Basolateral Targeting

a distribution that was indistinguishable from the full length receptor (compare with Figure 3.3), suggesting that neither pair of hydrophobic residues are essential to the targeting signal.

The cluster 692-QETE-695 was next substituted with alanine residues (QETE(A)). Expression of this mutated receptor resulted in a shared apical and basolateral localisation, suggesting an impairment of the basolateral targeting signal (Figure 3.12).

That the di-hydrophobic residues 690/691 (LL) and 696/697 (LV) do not form part of the basolateral targeting signal was further demonstrated by the introduction of additional alanine substitutions within the QETE(A) receptor of the downstream LV (QETELV(A)), and the upstream LL (LLQETELV(A)). These mutations did not significantly enhance the level of apical staining observed in the mutated QETE(A) receptor (Figure 3.12). However, alanine substitution of the next four carboxy-terminal residues (EPLT(A)) also resulted in an apical and basolateral distribution of ErbB-2 identifying a second element that contributes to basolateral targeting activity. Interestingly, a mutated ErbB-2 with just the EP substituted with alanines (EP(A)) was found in a basolateral distribution, suggesting that the LT might constitute the more important residues within this motif.

When all 12 amino acids within the candidate region were substituted for alanines (12(A)) an essentially apical distribution was obtained as expected (Figure 3.12). A similar result was obtained if the two di-hydrophobic pairs within the candidate region were left intact (QETE/EPLT(A)). To determine whether the ERBIN binding domain might be contributing to basolateral localisation when the mutated proteins showed a partial apical relocation (e.g. QETE(A) and EPLT(A)), these mutated receptors were generated with an additional deletion of the last 6 carboxy-terminal amino acids to remove the ERBIN binding site. These receptors were generated as previously described (see section 3.5.1), except that pc4-QETE(A) and pc4-EPLT(A) were used instead of pc4-B2 as a cloning vector. No significant changes in apical or basolateral membrane distribution of mutated proteins compared to the analogous constructs containing an intact PDZ binding domain were detected (data not shown). Hence, these results indicate a bipartite basolateral targeting signal composed of QETE and EPLT and further demonstrates that the carboxy-terminal PDZ binding domain of ErbB-2 is not required for ErbB-2 basolateral targeting in MDCK cells.
2.5x10^5 MDCK cells were seeded onto PET membranes and then separately transfected the following day with plasmids to express the single alanine substitution mutants of ErbB-2 indicated. Two days post-transfection, the location of mutated proteins were assayed by indirect immunofluorescence. Mutated ErbB-2 proteins are shown in green and the tight junction protein ZO-1 is stained red. Figures represent a typical positive cell and are shown as XZ confocal images. Scale bar represents 10µm.
The subcellular localisation of the multiple alanine substitution mutated forms of ErbB-2 listed in Table 3.1 were assessed in MDCK cells as described in Figure 3.11. ErbB-2 proteins are stained in green and ZO-1 (red) marks the position of the tight junctions. Representative XY and XZ confocal images are shown. Scale bar represents 10μm.
3.7 The Basolateral Targeting Signal of ErbB-2 is Autonomous

To determine whether the basolateral targeting signal of ErbB-2 could function as an autonomous or dominant signal, its ability to direct a heterologous protein to the basolateral membrane domain of MDCK cells was tested. The human P75 neurotrophin receptor (P75NTR) was used to create chimeric receptors containing the extracellular and transmembrane domains of the P75NTR fused to intracellular sequences of ErbB-2. P75NTR is normally directed to the apical membrane when expressed in MDCK cells due to the presence of critical O-glycosylation sites which act as apical targeting signals within the stalk domain of the receptor (Breuza et al., 2002; Yeaman et al., 1997). Using the techniques described in the previous sections, stable clones of MDCK cells expressing chimeric receptors were generated and their distribution assessed by indirect immunofluorescence and confocal microscopy.

3.7.1 Generation of Stable Clones of MDCK Cells Expressing P75NTR:ErbB-2 Chimeric Receptors

A truncated form of the P75NTR was amplified by PCR using a P75NTR expression plasmid as a DNA template (a kind gift from Senthil Muthuswamy, Cold Spring Harbor Laboratories). This cDNA fragment was generated using a forward primer that annealed to the initial coding sequences of P75NTR and also contained a 5' HindIII site prior (5') to the P75NTR coding sequences (primer: p75-FoH3). The reverse primer was designed to anneal up to complementary position 930 of the P75NTR cDNA at the 5' end of the oligonucleotide (numbering based on Johnson et al., 1986) and contained a 5' EcoRV site to produce an EcoRV linker at the 3' end of the truncated P75NTR cDNA (primer: P75-ReRV). A similar cDNA was created using an alternate reverse primer annealing to the same sequences of P75NTR but containing an in frame double stop codon 5' to the EcoRV site (primer: P75St-ReRV). These cDNAs encoded a truncated P75NTR comprising the extracellular domain, the transmembrane domain, and the first two amino acids of the intracellular domain. Products were restriction digested using HindIII and EcoRV and cloned into the HindIII/EcoRV sites of pcDNA4/TO to produce the plasmids pc4-P75-TrPu (lacking the stop codons) and pc4-P75-Tr (containing the stop codons). The following oligonucleotides were used to generate three polylinker cDNA fragments (see section 2.8.10) encoding ErbB-2 amino acids 680-690 (primers: B2-SeD1 and B2ASeD1), 680-706 (primers: B2-SeD4 and B2ASeD4), and 1241-1255 (primers:
B2SeC15 and B2ASeC15) – all polylinkers contained a double in-frame stop codon 3’ of the ErbB-2 sequences. These polylinkers, when hybridised together, produced ends that allowed the reconstitution of EcoRV (5’ of ErbB-2 sequences) and NotI (3’ of stop codons) sites after ligation into EcoRI/NotI digested DNA vectors. EcoRV/NotI digestion of pP75-TrFu then allowed the ligation of these fragments to generate the plasmids i) pc4-P75B2(680-690), ii) pc4-P75B2(680-706), and iii) pc4-P75B2(1241-1255). A further plasmid, pc4-P75B2(680-1255), was generated by ligating an EcoRV/NotI digested cDNA fragment corresponding to ErbB-2 amino acids 680-1255 to pc4-P75-TrFu. This fragment was created by PCR amplification of pc4-B2 using the primers B2-FoR5 and B2-ReNI to contain a double in-frame stop codon 3’ of the ErbB-2 sequences. Figure 3.13 graphically depicts the domain structure of the receptors expressed from these constructs.

**Figure 3.13 – Chimeric P75NTR/ErbB-2 receptors**

Diagrammatic representation of the chimeric P75NTR:ErbB-2 receptors described in the text. Receptors comprise the extracellular and transmembrane domains of P75NTR and intracellular sequences of ErbB-2. (-) represents amino acid residues between the indicated residues that are not shown, and the underlined alanine residues indicate the location of an introduced missense mutation. (*) marks the position of introduced in-frame double stop codons.
Individual stable clones of MDCK cells expressing inducible truncated P75NTR (P75-Tr) and chimeric P75NTR:ErbB-2 receptors were isolated after co-transfection of pcDNA6/TR with pP75-Tr or each of the four chimeric receptor expression constructs described above. Clones were induced to express the chimeric proteins and then analysed by immunoblotting cell lysates with ME20.4, an antibody that recognises the human P75NTR. All expressing clones revealed a product of the predicted size after overnight induction with 1μg/ml tetracycline (data not shown). The distribution of each receptor was then assessed in at least three independent stable clones by indirect immunofluorescence and confocal microscopy. The experiments were performed as described in section 3.2.3, except ME20.4 was used instead of 9G6.10 as the primary antibody.

3.7.2 Distribution of Truncated P75NTR and P75NTR:ErbB-2 Chimeric Receptors

To test whether the basolateral targeting signal of ErbB-2 was autonomous it was necessary to demonstrate that the truncated form of the receptor (P75-Tr), which forms the extracellular and transmembrane domains of the chimeric receptors, was localised to the apical domain. As can be seen in Figure 3.14A, the majority of P75-Tr was localised to the apical membrane in MDCK cells. No staining was detected when the same stable clones were grown in the absence of tetracycline, indicating that ME20.4 anti-P75NTR did not cross react with endogenous canine proteins (data not shown).

By contrast, the chimeric receptor containing the whole cytoplasmic domain of ErbB-2 (P75B2(680-1255)) was directed to the basolateral membrane demonstrating the presence of an autonomous basolateral sorting signal(s) in the cytoplasmic domain of ErbB-2 (Figure 3.14A). A P75NTR chimera containing amino acids 680-690 of ErbB-2 (P75B2(680-690)) was localised to the apical membrane, whereas a similar chimera containing ErbB-2 amino acids 680-706 localised to the basolateral membrane of MDCK cells, although a significant amount of intracellular staining was also detected (Figure 3.14A). That inclusion of amino acids 691-706 of ErbB-2 were sufficient to reverse the polarised distribution of a truncated P75NTR confirms the presence of a basolateral targeting signal in the juxtamembrane domain of ErbB-2 and also demonstrates that this signal can function in an autonomous or dominant fashion.

Recently, it was demonstrated that fusion of the last 15 carboxy-terminal amino acids of ErbB-2 (containing the ERBIN binding site) to the P75NTR were sufficient
to redirect it from the apical to the basolateral membrane (Jaulin-Bastard et al., 2001). Given the contradictory findings described in section 3.5, the polarised distribution of a similar chimeric receptor consisting of the extracellular and transmembrane domains of P75NTR fused to the last 15 carboxy-terminal amino acids of ErbB-2 (P75B2(1241-1255)) was assessed. As can be seen in Figure 3.14B, the last 15 carboxy-terminal amino acids of ErbB-2 were sufficient to redirect a heterologous apically localised protein to the basolateral membrane, in agreement with the published literature (Jaulin-Bastard et al., 2001).

3.7.3 The ERBIN Binding Site of ErbB-2 Contains a Cryptic Basolateral Targeting Signal

This last result concerning the distribution of the chimeric receptor P75B2(1241-1255) is in contradiction to the data presented earlier in the chapter that demonstrates the ERBIN binding site of ErbB-2 has no basolateral targeting activity. However, a closer examination of the last 15 carboxy-terminal amino acids of ErbB-2 incorporating the ERBIN binding site (PTAENPEYLGLDVPV) shows that it contains two motifs that conform to the consensus sequence of tyrosine based basolateral targeting signals (underlined) - NPXY and YXXΦ where Φ represents a bulky hydrophobic residue. To determine whether this sequence, transferred into a juxtamembrane context as seen in the chimeric receptor P75B2(1241-1255), might act as a cryptic basolateral targeting motif, the putative critical tyrosine residue at position 1248 was mutated to alanine P75B2(1241-1255(YA)). As this tyrosine is also involved in ERBIN binding (Jaulin-Bastard et al., 2001), a second mutation that would affect ERBIN binding (valine to alanine at position 1255) but not the putative cryptic basolateral targeting motif was also generated (P75B2(C15)VA). These receptors were expressed from the constructs pc4-P75B2(1241-1255(YA)) and pc4-P75B2(1241-1255(VA)) which were created in the same way as pc4-P75B2(1241-1255), described in section 3.7.1, except that primer pairs C15-YAf, C15YAr and C15-VAf, C15-VAr were used to generate the polylinker DNA. Both of these constructs were verified by DNA sequencing and their predicted chimeric receptor expression products are depicted in Figure 3.13. Using ME20.4 as a primary antibody in an immunoblot analysis, it was determined that both of these constructs produced proteins of the predicted size after their transient transfection into MDCK cells (data not shown).

pc4-P75B2(1241-1255(YA)) and pc4-P75B2(1241-1255(VA)) were transiently expressed in MDCK cells that were seeded onto PET membranes, and the
Figure 3.14 - Localisation of chimeric P75NTR:ErbB-2 receptors

A) Stable clones of MDCK cells were induced to express the indicated proteins by addition of tetracycline (1µg/ml) into the growth medium. Co-immunofluorescence revealed the location of the P75NTR:ErbB-2 chimeric receptors (green) and the tight junctions (ZO-1, red). XY and XZ confocal images are shown.

B) MDCK cells transiently expressing P75NTR chimeric receptors containing normal and mutated forms of the isolated ERBIN binding site of ErbB-2 shown in Figure 3.13 were analysed as in (A). Scale bar represents 10µm.
distribution of the resultant proteins, P75B2(1241-1255(YA)) and P75B2(1241-1255(VA)), assessed by indirect immunofluorescence and confocal microscopy. The results show that mutation of tyrosine causes a significant redirection of the P75NTR to the apical membrane whereas mutation of the carboxy-terminal valine gives a predominantly basolateral localisation similar to the unmutated chimeric receptor P75B2(1241-1255) (see Figure 3.14B). This indicates that the basolateral targeting activity of the isolated ERBIN binding site is most likely due to a cryptic tyrosine based basolateral targeting motif.

### 3.8 Localisation of ErbB-2 in LLC-PK1 Cells

The trafficking of some basolateral proteins requires the presence of the heterotetrameric adaptor complex AP1B. Expression and functional studies have demonstrated that the complex is expressed in cells of an epithelial lineage, and suggest that AP1B may be involved in the trafficking of membrane-bound proteins in polarised epithelia. Specifically, AP1B is associated with clathrin coated vesicles and has been implicated in the trafficking of proteins containing tyrosine based basolateral signals from the TGN to the cell membrane (see Introduction section 1.3.2c, and Bonifacino and Dell'Angelica, 1999; Folsch et al., 1999).

LLC-PK1 cells are derived from porcine kidney epithelium and were shown to be deficient for the μ1B subunit of the AP1B adaptor complex that is thought to recognise the tyrosine based basolateral sorting signals. Hence, these cells fail to make a functional AP1B complex and consequently mis-sort many basolateral proteins containing a tyrosine based sorting motif to the apical membrane (Folsch et al., 1999; Ohka et al., 2001). The ErbB-2 juxtamembrane basolateral sorting signal does not contain an essential tyrosine residue, nevertheless, the distribution of ErbB-2 in LLC-PK1 cells was examined to determine whether AP1B might also participate in the sorting of ErbB-2.

#### 3.8.1 LLC-PK1 Cells Form a Polarised Monolayer

LLC-PK1 cells were seeded onto PET membranes and allowed to grow to confluency. After a further two days in culture, cells were washed in PBSA, fixed in 4% (w/v) paraformaldehyde and the distribution of ZO-1 and β-catenin was assessed by indirect immunofluorescence and confocal microscopy as described for MDCK cells. β-catenin and ZO-1 were found to be localised to the lateral domain
distribution of the resultant proteins, P75B2(1241-1255(YA)) and P75B2(1241-1255(VA)), assessed by indirect immunofluorescence and confocal microscopy. The results show that mutation of tyrosine causes a significant redirection of the P75NTR to the apical membrane whereas mutation of the carboxy-terminal valine gives a predominantly basolateral localisation similar to the unmutated chimeric receptor P75B2(1241-1255) (see Figure 3.14B). This indicates that the basolateral targeting activity of the isolated ERBIN binding site is most likely due to a cryptic tyrosine based basolateral targeting motif.

### 3.8 Localisation of ErbB-2 in LLC-PK1 Cells

The trafficking of some basolateral proteins requires the presence of the heterotetrameric adaptor complex APIB. Expression and functional studies have demonstrated that the complex is expressed in cells of an epithelial lineage, and suggest that APIB may be involved in the trafficking of membrane-bound proteins in polarised epithelia. Specifically, APIB is associated with clathrin coated vesicles and has been implicated in the trafficking of proteins containing tyrosine based basolateral signals from the TGN to the cell membrane (see Introduction section 1.3.2c, and Bonifacino and Dell'Angelica, 1999; Folsch et al., 1999).

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3.8.2 LLC-PK1 Cells Mis-Sort a Large Proportion of ErbB-2 to the Apical Membrane Domain

LLC-PK1 cells were seeded onto PET membranes and allowed to recover overnight. The following day, expression constructs containing full length ErbB-2 and the ErbB-2 truncated protein Δ690 (pc4-B2 and pc4-Δ690) were transiently transfected into LLC-PK1 cells. The cells were then left for two days to form a polarised monolayer. The distribution of the expressed proteins was then assessed by indirect immunofluorescence and confocal microscopy. Surprisingly, significant amounts of full length ErbB-2 were mis-sorted to the apical membrane of this cell line, although the apical localisation was not as complete as the truncated ErbB-2 receptor Δ690 lacking the basolateral targeting signal (Figure 3.15B). These results suggest that the basolateral targeting signal is only partially effective in LLC-PK1 cells, and suggest that μ1B might recognise other types of basolateral sorting motif in addition to those containing a tyrosine such as that identified in ErbB-2. Alternatively, LLC-PK1 cells may possess other deficiencies in protein trafficking.

3.8.3 Reintroduction of μ1B Into LLC-PK1 Cells Partially Rescues ErbB-2 Basolateral Localisation

To test the involvement of μ1B in ErbB-2 sorting, this protein was reintroduced into LLC-PK1 cells and the sub-cellular distribution of ErbB-2 assessed. A human μ1B fragment was amplified from a cDNA preparation of reverse transcribed total RNA isolated from MCF10A cells (gift from Dr. L.M. Martins, Cancer Research UK, London), using Stratagene's *Pfu* Turbo® polymerase and the primers mu1bF-H3 and mu1bR-N1. The amplified fragment was digested with *Hind*III and *Not*I, gel extracted, and then cloned into *Hind*III/*Not*I digested pcDNA3.1 (Invitrogen Life Technologies) to form the plasmid pc3-μ1B, which was then verified by DNA sequencing.

The transferrin receptor (Tfr) was used as a functional control for μ1B expression in LLC-PK1 cells in the absence of specific μ1B antibodies, as basolateral localisation of Tfr (shown to be localised to the apical domain in LLC-PK1 cells) is rescued by reintroduction of μ1B into LLC-PK1 cells (Folsch et al., 1999). An expression
2.5 x 10^5 LLC-PK1 cells were seeded onto PET membranes and the localisation of endogenous and exogenously expressed proteins analysed by indirect immunofluorescence two days after the cells had reached confluence. Representative XY and XZ confocal images are shown.

A) Localisation of β-catenin (green) and ZO-1 (red, in all images) in polarised LLC-PK1 cells. Transient expression of full length ErbB-2 and the truncated receptor Δ690 (B, green). C) Localisation of exogenously expressed transferrin receptor (Tfr). D) Basolateral localisation of ErbB-2 and Tfr can be partially rescued by co-expression with μ1B. Scale bar represents 10μm.
plasmid containing the TFr (a kind gift from Dr. Colin Hopkins, Imperial College, London) was transiently transfected into LLC-PK1 cells following the same protocol described in section 3.8.2. Indirect immunofluorescence using antibodies against the TFr (B325, gift from C. Hopkins) and ZO-1 revealed a predominantly apical distribution for exogenously expressed TFr in agreement with the published literature (Figure 3.15C, Folsch et al., 1999). When the expression plasmid encoding full length ErbB-2 was transfected into LLC-PK1 cells together with pc3-μ1B (in a 1:10 ratio), basolateral localisation of ErbB-2 could be partially rescued (Figure 3.15D). Whilst the cells expressing ErbB-2 alone showed mainly apical and a mixed apical and basolateral localisation (Figure 3.15B), those receiving the μ1B cDNA showed many more cells localising ErbB-2 basolaterally (Figure 3.15D). A similar change in localisation was seen with the transferrin receptor control when co-expressed with μ1B as previously demonstrated (Folsch et al., 1999). It should be noted that although the majority of cells transfected with both constructs displayed a partial localisation rescue of ErbB-2 and TFr, a great deal of heterogeneity was apparent indicating that the rescue attributed to μ1B expression was not as complete as previously described.

3.9 Discussion

The ability of growth factors to effectively activate their cognate receptor tyrosine kinases must presumably depend on their close interaction with the receptor. In epithelial cells, the ErbB RTKs are predominantly localised to the basolateral membrane domain where they are in close proximity to stromally derived growth factors (Borg et al., 2000; Carraway and Sweeney, 2001; Darcy et al., 2000; De Potter et al., 1989). Thus, receptor localisation undoubtedly plays an important role in the reception of paracrine signals from adjacent cells. As ErbB-2 is a central component of the ErbB signalling network, acting as a preferred co-receptor for other family members (Graus-Porta et al., 1997; Tzahar et al., 1996), its localisation to the basolateral membrane domain should be paramount for the fidelity of ErbB signalling between these two cell lineages. In this chapter, MDCK cells were utilised to gain insights into the signals that control the basolateral targeting of ErbB-2 in epithelia.

Extensive mutation analyses of ErbB-2 demonstrated the presence of a basolateral sorting signal in the cytoplasmic juxtamembrane region, between amino acids 692-701. Alanine substitution mutations revealed a bipartite motif composed of the
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amino acids QETE and EPLT. As substitution of AA for EP in the second part of the motif had no effect on the polarised sorting of ErbB-2, it is possible that the LT represents the more important residues. However, as single alanine substitutions throughout the whole region had no significant effect on targeting, this suggests a degree of plasticity in the sorting sequence that might be reflected by the lack of effect on localisation in the EP to AA mutation. The requirement for both elements of the signal is supported by the results of internally deleted forms of ErbB-2 (Figure 3.7 and Figure 3.8). For example, a receptor protein missing QETE but retaining EPTL (Δ684-695) was essentially apical, as was a receptor protein retaining QETE but missing EPTL (Δ696-701).

The juxtamembrane basolateral sorting signal also appears to control localisation of ErbB-2 in CACO-2 cells since the truncated receptor Δ706 was found largely on the basolateral membrane when expressed in these cells, but a similar receptor lacking the targeting signal (Δ690) was apically localised. CACO-2 cells tend to utilise both direct and indirect routes to traffic proteins to the cell surface, whereas MDCK cells normally transport proteins to the cell surface directly (Matter and Mellman, 1994). The juxtamembrane basolateral targeting signal of ErbB-2 can therefore function effectively in two different epithelial cell types which use distinct but related mechanisms for protein trafficking. This is in contrast to a cryptic basolateral targeting signal in mutants of the normally apically localised P75NTR which are differentially interpreted by MDCK and CACO-2 cells (Monlauzeur et al., 2000).

The mutational analyses demonstrated that ablation of the basolateral targeting signal led to an apical, rather than a random or intracellular distribution of ErbB-2. As MDCK cells apparently do not traffic proteins by default to a particular membrane domain, this suggests that ErbB-2 contains recessive extracellular or transmembrane apical determinants that are functionally revealed in the absence of the basolateral targeting signal. This hierarchy is a common feature of many basolateral membrane proteins. For example, removal of the basolateral targeting signal of ErbB-1 results in an apical accumulation of the receptor that is dependent on N-linked glycosylation of the extracellular domain (Hobert et al., 1997). It would be of interest to see if treating some of the MDCK clones described in this chapter with tunicamycin to prevent N-linked glycosylation would inhibit the apical accumulation of ErbB-2 receptors lacking the basolateral targeting signal. The functional consequences of having opposing localisation signals in the same molecule are unknown, however, it may have important implications during development or disease, where ErbB-2 is known to have an apical location in some epithelial cells as discussed later.
A number of membrane proteins have been shown to contain a dihydrophobic amino acid pair that is necessary for basolateral membrane sorting. These include E-cadherin, CD44 and the FC receptor (Hunziker and Fumey, 1994; Miranda et al., 2001; Sheikh and Isacke, 1996), and substitution of alanine for one or more of the hydrophobic residues abrogates their basolateral targeting. This is in contrast to the ErbB-2 basolateral sorting signal where single alanine substitutions have no detectable effect on its trafficking. The ErbB-2 receptor contains two dihydrophobic residue pairs (LLQETELVEPLT) within the region containing the basolateral sorting motif (underlined), but mutation of either or both residue pairs did not affect basolateral targeting (Figure 3.12). Furthermore, basolateral proteins which rely on a dihydrophobic sorting signal, for example E-cadherin, are correctly sorted in both MDCK and LLC-PK1 cells (Miranda et al., 2001). In the experiments described here, ErbB-2 was sorted to the basolateral membrane in MDCK cells but mainly to the apical membrane in LLC-PK1 cells. This observation is consistent with ErbB-2 not using a dihydrophobic sorting motif.

Bipartite basolateral sorting motifs are not unusual. For example, the LDL receptor and the aquaporin 4 (AQP4) water channel, both have a tyrosine containing element as well as one encompassing a cluster of acidic residues (Madrid et al., 2001; Matter et al., 1992, see Table 1.3). However, while the ErbB-2 basolateral sorting signal contains two/three acidic residues they are dispersed and do not qualify as a true acidic cluster. In the LDL receptor and AQP4 water channel, three out of four key amino acid residues are acidic. Moreover, AQP4 also has a dihydrophobic pair of amino acids adjacent to the acidic cluster that is needed for efficient basolateral sorting. A similar dihydrophobic pair of amino acids (LV) follows the two acidic residues in ErbB-2 but they did not appear to contribute to basolateral sorting. Furin endopeptidase also appears to contain a bipartite basolateral sorting sequence (Simmen et al., 1999). In this case, a separate cluster of four acidic residues is followed by a separate phenylalanine-isoleucine pair. The FI pair may be equivalent to a dihydrophobic signal as found in many other receptors. Therefore it appears that the ErbB-2 sorting sequence is similar but distinct from those previously described.

LLC-PK1 cells mis-sort proteins such as the transferrin receptor and the LDL receptor that utilise a tyrosine-containing basolateral sorting signal (Folsch et al., 1999). While the ErbB-2 receptor is also aberrantly directed to the apical membrane in LLC-PK1 cells, interestingly it does not contain a tyrosine in the identified sorting signal. The defect in the basolateral targeting of receptor proteins with tyrosine-containing sorting signals in LLC-PK1 cells appears to reside in the loss of the $\mu$1B
subunit of the AP1B adaptor complex, since reintroduction of a cDNA encoding the μ1B subunit rescues basolateral sorting of these receptors (Folsch et al., 1999). Basolateral localisation of ErbB-2 and transferrin receptor can be partially rescued by co-transfection with a μ1B expression plasmid, although the rescue is less complete than previously demonstrated for the transferrin receptor (Folsch et al., 1999). Hence, the mis-sorting of ErbB-2 in LLC-PK1 cells suggests that AP1B, at least in part, may also be instrumental in sorting receptors with non-tyrosine containing motifs, although this would need to be firmly established through binding studies using the adaptin heteromeric complex proteins. Unfortunately, initial attempts to identify adaptor complex subunits that interact with the ErbB-2 basolateral signal using a yeast two-hybrid approach were unsuccessful. As basolateral targeting of the LDL receptor is controlled by both the AP1B and AP4 adaptor complexes (Borg et al., 2000; Simmen et al., 2002), it would be of interest to inactivate the AP4 complex in LLC-PK1 cells to see if ErbB-2 becomes exclusively localised to the apical domain. This could be achieved by using anti-sense oligonucleotides or small interfering RNAs (siRNA) directed against the specific subunits of the AP4 complex.

Interestingly, a very recent study implicates the AP1B complex in the sorting of basolateral proteins post-endocytically. In other words, AP1B may be involved in the recycling of internalised proteins to the basolateral membrane from sorting endosomes, and not in the initial targeting of basolateral proteins from the TGN to the cell surface. Experimental evidence for this was provided through the use of an LDL receptor mutant containing a partially inactivated endocytosis signal. When this mutated receptor was expressed in LLC-PK1 cells lacking the AP1B complex it localised predominantly to the basolateral membrane (Gan et al., 2002). This raises the possibility that the large apical population of ErbB-2 receptors observed in LLC-PK1 cells (Figure 3.15B) is due to internalised basolateral receptors being redistributed to the apical membrane. The reintroduction of μ1B into these cells would then restore the basolateral recycling machinery, resulting in the predominantly basolateral ErbB-2 distribution seen in Figure 3.15D. If this hypothesis is true then it would require ErbB-2 to be internalised from the cell surface. Compared to ErbB-1 however, endocytosis is relatively impaired in ErbB-2 and proceeds with slower kinetics (Baulida et al., 1996; Waterman and Yarden, 2001). Nevertheless, the constitutively expressing constructs used in this chapter to express ErbB-2 in LLC-PK1 cells would presumably allow detectable levels of internalised basolateral ErbB-2 to accumulate on the apical membrane. Specific antibodies to the extracellular domain of ErbB-2, that induce receptor endocytosis
but not degradation, could possibly be used to follow the intracellular path of receptor to clarify this issue. If these antibodies were presented to the basolateral chamber of PET membranes containing tight monolayers of LLC-PK1 cells, and were later detected on the apical membrane by direct or indirect immunofluorescence, then this would suggest that APIB is involved in the basolateral recycling of ErbB-2.

The extreme carboxy-terminal region of ErbB-2 contains a binding domain for the PDZ domain-containing protein ERBIN that co-localises with ErbB-2 at basolateral membranes (Borg et al., 2000; Jaulin-Bastard et al., 2001). The studies by Borg and colleagues found that ERBIN was necessary for the basolateral localisation of ErbB-2, since mutations that abrogate ERBIN binding also lead to the mis-localisation of ErbB-2 to apical membranes. ERBIN was therefore predicted to function in an analogous way to the PDZ domain-containing protein complex Lin-2/Lin-7/Lin-10 that controls the basolateral localisation of the epidermal growth factor receptor orthologue Let-23 in Caenorhabditis elegans vulval epithelial cells (Introduction section 1.3.2c, Kaech et al., 1998). However, the results presented here are not consistent with these findings, since the ERBIN binding site was found not to be necessary for basolateral targeting of the ErbB-2 receptor in MDCK cells (Figure 3.10A). Indeed, the same HER1/2 chimeric receptor lacking the ERBIN binding site used in the studies by Borg et al., and reported to mis-localise to the apical membrane of MDCK cells, in fact localised to the basolateral membrane in the experimental system used in this chapter (Figure 3.10B).

It is not clear why these results differ from those of Borg et al. A possible explanation could reside in subtle differences in experimental technique. In the studies presented in this chapter, cells were cultured on PET membranes which have been well characterised in previous studies to allow MDCK cells to form a tight polarised monolayer. By contrast, the MDCK cells used in the studies by Borg et al. were cultured upon glass cover slips, and so may not have achieved a fully polarised epithelial phenotype. Furthermore, their use of a chimeric receptor containing the extracellular domain of ErbB-1 fused to the cytoplasmic domain of ErbB-2 may have compromised the juxtamembrane targeting signal in some way causing a greater emphasis to be placed on the PDZ domain. However, the internal deletion analysis (Figure 3.8) and the localisation studies of the Borg chimeras (Figure 3.10B) presented here would not support this notion. Yet another explanation may lie in the levels of protein expression. It was apparent from these studies that cells expressing very high levels of exogenous protein localised more of the protein to the apical domain compared with cell expressing moderate levels of
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protein, even in the presence of a complete basolateral targeting signal. This phenomenon has also been observed in MDCK cells over expressing ErbB-1 (Hobert et al., 1999) and suggests that the pathways controlling protein trafficking can become saturated. As a consequence, the results presented within this chapter were determined by analysing many cell clones or individual cells expressing varying amounts of exogenous protein. If the MDCK cell clones used by Borg et al. were expressing very high levels of chimeric receptor, then the trafficking pathways of these cells may have become saturated, resulting in significant apical localisation of the receptors that would normally be found on the basolateral membrane.

The results presented in this chapter conclude that the targeting of ErbB-2 to the basolateral membrane does not rely on the interaction with ERBIN, but its persistence at the basolateral membrane might be dependent on the PDZ domain acting as a retention determinant, as seen in the GABA transporter (Perego et al., 1999). The cell culture model described in this chapter could be used to determine if this is indeed the case by comparing the longevity of pulse labelled ErbB-2 receptors with and without the ERBIN binding domain (FL ErbB-2 and Δ1249) at the cell surface. Although ERBIN was originally identified using a yeast two-hybrid screen as a protein that associates with the carboxy-terminus of ErbB-2, and was also characterised to preferentially interact with unphosphorylated ErbB-1/ErbB-2 receptors (Borg et al., 2000), its role as a binding partner of ErbB-2 has recently been called into question. In a follow-up study, surface plasmon resonance analysis showed that the interaction between ERBIN and ErbB-2 was of very low-affinity and was outside the range observed for other PDZ domain-peptide interactions (Jaulin-Bastard et al., 2002). By contrast, a catenin-related protein (p0071) interacted with high affinity to ERBIN. Although the authors speculate that a low affinity interaction between ERBIN and ErbB-2 may allow for a more dynamic regulation of this interaction to occur in vivo, there is no evidence for this. In an independent study, ERBIN was also found to bind with high affinity to the catenin related proteins δ-catenin and ARVCF, but ErbB-2 could not be precipitated from transfected cell extracts with a GST-ERBIN fusion protein (Laura et al., 2002). Whilst neither study could determine if the low affinity interaction between ERBIN and ErbB-2 was physiologically relevant, it seems most likely that ERBIN is not involved in the targeting or retention of ErbB-2. Rather, ERBIN probably functions as a linking protein participating in cell-cell and cell-basement membrane adhesion (Jaulin-Bastard et al., 2002).

The basolateral targeting signal of ErbB-2 was able to function autonomously. This finding was based on the observation that the signal, when fused onto the
intracellular domain of the apically localised P75NTR, was able to redirect this heterologous protein from the apical to the basolateral domain. This is a common feature of many juxtamembrane basolateral targeting signals. For instance, the basolateral targeting signal of the follicle-stimulating hormone receptor is also able to redirect the P75NTR to the basolateral membrane domain (Beau et al., 1998). Similarly, the basolateral signal of NPP1 is able confer a basolateral localisation when fused on to apical NPP3, although in this study it is not clear whether the signal is able to redirect an unrelated apical protein to the basolateral membrane (Bello et al., 2001). However, some basolateral sorting signals differ in their ability to redirect apical proteins to the basolateral cell surface, and may not always be dominant over apical signals (Renold et al., 2000).

The basolateral targeting signal identified in ErbB-1 shows homology to the signal identified in this chapter for ErbB-2 (discussed below), and is able to redirect a GPI anchored protein (decay accelerating factor) to the basolateral domain (Hobert et al., 1997). It is likely therefore that the basolateral targeting signal of ErbB-2 can act autonomously when fused to a number of proteins that depend on apical targeting signals present in O-glycosylation sites or GPI anchors. It would be interesting to determine whether this signal is also able to relocate apical proteins to the basolateral membrane that rely on apical targeting information present in N-glycosylation sites (e.g. gp-80) or transmembrane domains (e.g. Influenza virus haemagglutinin).

The carboxy-terminal 15 amino acid residues of ErbB-2 also have a capacity to act as a basolateral targeting motif, since they are able to direct a heterologous protein to the basolateral membrane (Figure 3.14). Interestingly, the ability of the isolated ERBIN binding site to direct the P75NTR to the basolateral membrane most likely relies on the presence of a cryptic sorting signal that functions when placed in a juxtamembrane position. This is strongly supported by the mutation analysis of the ERBIN binding site shown in Figure 3.14B. Moreover, similar sorting signals incorporating critical tyrosine residues have been previously demonstrated to act as basolateral targeting signals in the LDL receptor (NPVY, Matter et al., 1992) and the HIV-1 envelope glycoprotein (YSPL, Lodge et al., 1997).

This finding raises an interesting question concerning the interpretation of basolateral targeting signals. How does the cellular machinery controlling basolateral targeting distinguish between signals that confer basolateral delivery of a protein, and seemingly identical signals elsewhere in the protein that do not? For instance, most proteins would be likely to contain numerous di-hydrophobic motifs
ErbB-2 Basolateral Targeting

capable of functioning as such a signal, but only one of which might actually be involved in basolateral sorting. This seems to be the case in E-cadherin which contains two di-leucine motifs in its cytoplasmic domain, but only one of which appears to function as a basolateral targeting signal (Miranda et al., 2001). It is likely therefore that a combination of factors, such as the surrounding structural context and proximity to the transmembrane domain, contribute to the recognition and interpretation of these motifs as basolateral targeting signals. By creating progressive internal deletions within the cytoplasmic domain of the mutated, apically localised 12(A) ErbB-2 receptor (Figure 3.12), the cryptic carboxy-terminal basolateral targeting signal could be brought closer towards the transmembrane domain of the receptor. This strategy could provide a means of assessing how the position of a basolateral targeting motif in relation to the transmembrane domain impinges on its activity.

A region of 23 amino acids in the cytoplasmic juxtamembrane domain of ErbB-1 has been shown to encompass a basolateral sorting signal (Hobert and Carlin, 1995; Hobert et al., 1997). In this study it was suggested that these amino acids might form a pair of amphipathic helices that confer targeting information. However, the sequence defined in this chapter for ErbB-2 is no more than 10 amino acids long, and locates on the basis of amino acid similarity only to the distal helix and the region between the two proposed helical domains of ErbB-1. Nevertheless, this study together with the results described in this chapter provides strong evidence for a basolateral sorting sequence in the juxtamembrane region in this family of receptors. Figure 3.16 compares the basolateral targeting signal of ErbB-2 with similar sequences found in other members of the Type I RTKs.

As can be seen, ErbB-1 contains a very similar bipartite motif (QERE/EPLT) in its basolateral targeting signal, suggesting that these are the critical residues within the described 23 amino acid signal that direct receptor targeting. ErbB-4 also shows a high degree of conservation with the bipartite motif (ETE/EPLT), suggesting again that this signal may control its basolateral targeting. ErbB-4 has also been shown to interact with the PDZ protein PSD-95 which may influence its localisation. Conversely, ErbB-3 and DER show a poor degree of homology with the targeting signal. Interestingly, ErbB-3 has been shown to be apically localised (Darcy et al., 2000), although exogenous expression of ErbB-3 using the system described in this chapter led to an accumulation of ErbB-3 on the basolateral membrane (data not shown). Let-23 shows no homology with the basolateral targeting signal of ErbB-2, however, basolateral localisation of this receptor is controlled by the Lin-2/Lin-7/Lin-10 complex (Kaech et al., 1998).
A PSI-BLAST search for other proteins containing the basolateral targeting motif of ErbB-2 led to a number of significant hits, but these were all ErbB related proteins from a variety of species. This suggests that the basolateral targeting signal is relatively conserved within the family, but is not a common basolateral sorting signal used by membrane proteins from other families.

The localisation of endogenous ErbB-2 (and ErbB-1) to the apical membranes of epithelial cells has recently been reported in autosomal recessive polycystic kidney disease (ARPKD) (Nakanishi et al., 2001). Although the functional importance of this ‘mis-localisation’ is unclear, it has been postulated that apical ErbB receptors may contribute to cyst formation and enlargement in the diseased kidney (Nakanishi et al., 2001). Alternatively, the apical expression of ErbB receptors could be due to the general deregulation of vesicle trafficking and epithelial cell polarity that is characteristic of ARPKD. Apical ErbB-2 receptors have also been described in non-diseased states, and are thought to be differentially localised in various tissues. For instance, in epithelial cells of the female rat reproductive tract, ErbB-2 receptors have been identified by immunohistochemical analysis both on the apical and basolateral membrane (Idris et al., 2001). At the apical membrane in these tissues ErbB-2 was shown to co-localise and form a complex with the cell surface glycoprotein Muc-4. Moreover, this interaction occurs via an EGF-like domain within the transmembrane subunit of Muc-4 and has been shown to potentiate ErbB-2 activity in response to neuregulin signalling (Carraway et al., 1999). ErbB receptors are also differentially localised during mammary development in the rat (Darcy et al., 2000). Apical localisation of ErbB-2 was shown to be particularly prominent at puberty when proliferation of mammary epithelial cells is decreased. As ErbB-2 signalling is implicated in the later stages of pregnancy (lobulo-alveolar
development), could the apical localisation of the receptor observed after puberty be a mechanism for down-regulating its interaction with stromal ligands and hence its activity?

These studies, together with the results presented in this chapter highlight an intriguing problem – during normal developmental processes, how does a cell differentially localise a protein that contains dominant-acting targeting information? Regulation could potentially occur at the level of the targeting signal. For instance, the interaction with Muc-4 or other proteins might mask the juxtamembrane basolateral targeting signal and therefore expose extracellular apical determinants in ErbB-2. In another scenario, Muc-4 might form a stable complex with ErbB-2 which then hitches a ride with Muc-4 to the apical membrane. Regulation could also occur at the level of the trafficking machinery. A particularly relevant postulation pertaining to the present study concerns the APIB complex. A cell could accumulate apically localised basolateral proteins if expression of the μ1B subunit was down-regulated, or if the APIB complex was post-translationally inactivated. If this were true then the lack of μ1B in LLC-PK1 kidney cells may not be a trafficking defect as described in the literature, but could actually be a unique mechanism for overriding basolateral targeting signals and consequently redirecting proteins to the opposing membrane.

Another key issue that has yet to be addressed is whether differential localisation of ErbB-2 also leads to differential signalling activity. Considering that signalling proteins are thought to form higher-order signalling modules that are likely to be compartmentalised in a polarised epithelial cell, it is probable that the signal transduction pathways and cellular responses associated with ErbB-2 activation would differ from the apical and basolateral domains. An analysis of ErbB-1 activation in an over-expression system that causes both apical and basolateral localisation of the receptor has been used to examine the subtleties of differential signalling. It was found that only basolateral ErbB-1 could activate PLC-γ, and that receptor endocytosis and downregulation were less efficient at the apical membrane. Both populations of receptors however were competent to activate Shc, Erk1 and Erk2; and basolateral ErbB-1 was more effective at phosphorylating focal adhesion kinase (Amsler and Kuwada, 1999; Hobert et al., 1999; Kuwada et al., 1998). Although informative, these studies are inherently hampered by the simultaneous expression of different levels of the receptor on each membrane domain. Also, the potential for ErbB-1 signalling through heterodimeric complex formation at the apical membrane would be severely limited due to the basolateral localisation of the endogenous ErbB proteins.
For future work, the studies presented in this chapter allow a means to address the issue of differential signalling of ErbB-2 by analysing both the signal transduction pathways, and cellular responses elicited by receptor dimerisation. This could be achieved by comparing the full length ErbB-2 receptor (basolateral) with the 12(A) (apical), and EPLT(A) (shared apical/basolateral) ErbB-2 proteins. As ErbB-2 does not possess a direct and specific ligand, a system allowing controlled dimerisation of receptors with small chemical molecules could be developed. Such a system has already been used successfully with chimeric ErbB receptors where the cytoplasmic domain of the receptor has been fused to the synthetic ligand-binding domain from the FK506-binding protein (FKBP). Receptor activation in this model was achieved with the bivalent FKBP ligand AP1510 (Muthuswamy et al., 1999; Muthuswamy et al., 2001). By stably expressing chimeric ErbB-2/FKBP receptors containing the localisation mutations described above in mammary epithelial cells, one could analyse differential signalling from apically and basolaterally localised ErbB-2 after chemically-induced dimerisation. Moreover, by growing these cells in three-dimensional basement membrane gels to form mammary epithelial acini, the effects of ErbB-2 activation from the apical or basolateral membrane could be compared in an in vitro model of mammary development. When basolateral ErbB-2 is chemically dimerised in this model, cell proliferation in the quiescent acini is reinitiated and their structure changes leading to a phenotype that shares many properties with the early stages mammary carcinogenesis (Muthuswamy et al., 2001). If apical ErbB-2 was unable to induce these changes in mammary acini, then it would suggest that blocking the identified juxtamembrane basolateral targeting signal could be an effective mode of therapeutic intervention for breast cancer.

To conclude, the ERBIN binding domain of ErbB-2 is unlikely to direct basolateral sorting in MDCK and CACO-2 cells, rather basolateral sorting is dependent upon a novel bipartite juxtamembrane signal. This signal appears to be autonomous and may utilise the AP1B adaptor complex as one means of trafficking to the cell surface.
Chapter 4 | Role of Met Receptor in Postnatal Mammary Gland Development

4.1 Introduction

To study the physiological role of a particular gene within a developmental system, the best strategy is often to remove the function of this gene \textit{in vivo}, and then determine the effects of its loss. Gene targeting approaches, such as the one used in Chapter 5, are a very effective way of performing such functional deletions. However, germline deletion of many developmentally important genes can be lethal to the embryo at an early stage of development, thus complicating the use of this technology in the study of postnatal organogenesis. The transgenic expression of dominant negative proteins is an effective alternative to gene targeting strategies, and has been used successfully to delineate the roles of numerous genes in mouse mammary gland development, as well as in other developmental systems. The procedure relies on the overexpression of a non-functional version of a protein within the mammary gland, to abrogate the functions of specific endogenous gene(s). In this way, genes can be rendered ineffective within the mammary gland, whilst maintaining their normal function in other tissues.

Signalling by the Met receptor, through its activation by hepatocyte growth factor (HGF), has been postulated to have important functions in mouse mammary development (see section 1.2.1c). In the adult mammary gland, both Met and HGF are expressed in a developmentally regulated, temporal pattern in the epithelium and stroma respectively. Expression of both genes is highest in the developing virgin gland, and remains high during the initial phases of pregnancy. The levels of both transcripts then decrease progressively towards the end of pregnancy and lactation, before increasing during the late stages of involution (Niranjan et al., 1995; Pepper et al., 1995). Coupled with early studies demonstrating that HGF/Met signalling induces epithelial tubulogenesis in 3-dimensional cell culture matrices (Montesano et al., 1991a; Montesano et al., 1991b), and in \textit{in vitro} models of mammary development (Niemann et al., 1998), these expression studies suggest that HGF/Met signalling is involved in the branching morphogenesis of epithelial cells in the mammary gland. Despite these inferences, a direct role for the
HGF/Met signalling in mammary development has yet to be established in vivo. To investigate the in vivo role for HGF/Met signalling in the mammary gland, a dominant negative strategy was employed. The mouse mammary tumour virus long terminal repeat (MMTV-LTR), the promoter region of the MMTV, is strongly active in the mammary epithelial component of the gland. This promoter was used to direct expression of a truncated dominant negative Met receptor, lacking its tyrosine kinase domain, to the mammary gland. In this way, the transgene would be expressed in the same tissue compartment, the mammary epithelium, as the endogenous Met receptor, allowing the activity of this gene to be blocked in vivo.

The MMTV-LTR does not act as a truly mammary epithelial specific promoter as it has been shown to be highly active in the salivary glands and male testes/epididymis amongst other tissues. However, it has been widely used in vitro, and to direct expression of transgenes to the mammary glands of mice with great success (see Cardiff, 1996). MMTV-LTR driven gene expression can be regulated by glucocorticoid hormones, and it is thought that this is the mechanism for its activation in the mammary gland. In cultured cells, gene expression from the MMTV-LTR can be induced by the addition of exogenous glucocorticoids.

As activation of Met associated signalling pathways requires the homodimerisation and trans-phosphorylation of Met receptors, the truncated Met receptor was hypothesised to act as a dominant negative by preferentially forming inactive Met dimers that would be unable to initiate downstream signalling cascades in response to HGF. These dimers could potentially form between mutant truncated Met receptors, and therefore lead to the sequestration of the ligand. Alternatively, dimers could form between the truncated and endogenous Met receptors, in which case trans-phosphorylation of the wild type receptor would not occur. By placing the transgene under the control of the MMTV-LTR, it was envisaged that the levels of expression attained would be sufficiently high to effectively block Met signalling within the mammary gland. To distinguish between the expressed transgene and the endogenous murine Met receptors, the transgene was constructed to express a truncated human Met receptor within the mammary gland.

4.2 Generation and In Vitro Assessment of Transgene Function

The targeted expression of a truncated Met receptor in the mouse mammary gland required the generation of a number of DNA plasmids. These plasmids were used
to assess the function of truncated versions of the Met receptor in vitro, and also for the generation of transgenic mice. These analyses included an assessment of the sub-cellular localisation of the human Met receptor, and of the ability of the truncated protein to function as a dominant negative receptor.

4.2.1 Generation of pMet-DN

To generate the transgene for injection into mouse embryos, the vector pMet-DN was created. A 1937 bp fragment of cDNA incorporating the MMTV-LTR was digested using BglII and HindIII from the vector pMMTV (Muller et al., 1990). Sequencing of this cDNA showed that it was 99.7% related to the MMTV-LTR from the C3H mouse strain (accession J02274). The cDNA fragment was gel extracted, and then cloned into a BglII/HindIII digested pGL3-Basic vector (Promega) to form the vector pGL3M. This vector consisted of the following functional units: polylinker (containing SmaI site)_MMTV-LTR_luciferase gene_SV40 poly(A) signal_unique Sall site (see Appendix A6.2). A SmaI site was then introduced into the unique Sall site using a polylinker generated from the oligonucleotides SmaPL1 and SmaPL2, this site would later be used to release the transgene from the vector backbone.

A further polylinker (generated using the oligonucleotides pGL3pl1 and pGL3pl2) was cloned into the HindIII and XbaI sites of the vector to replace the luciferase gene with a 63 bp stretch of cDNA that introduced unique SphI and AatII restriction sites, forming the vector pM2P. This polylinker also contained stop codons in each reading frame 3' of the AatII site, and a unique SpeI restriction site 3' of the stop codons. A rabbit β-globin intron sequence was then inserted into the HindIII site, 3' of the MMTV-LTR and 5' of the polylinker, to form the intermediate vector pM2PI. The β-globin intron was amplified by PCR using a DNA template containing the intron, pHR2 (gift from Dr. S. Werner, ETH, Zurich), and the primers bglob1 and bglob2 containing HindIII sites at their 5' ends. The rabbit β-globin intron was included in the construct as it has been shown to increase the levels of transgene expression when placed between a promoter and an expressed gene (Yew et al., 1997).

A SphI/AatII digested cDNA fragment encoding a human Met receptor (Met-DN), truncated within the intracellular juxtamembrane region of the β-chain (at residue 982, see Appendix A6.4.3), was ligated into the polylinker by SphI/AatII digestion of pM2PI. The Met receptor fragment was amplified by PCR from a plasmid containing the full length human Met cDNA (pMOGl, kind gift from Dr. G Vande
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Woude, Van Andel Research Institute, Michigan) using the forward primer cMETpr1, and the reverse primer cMETpr3, containing_SphI and_AatII restriction sites respectively. Digestion of this construct using_AatII then allowed the ligation of a further polylinker encoding an in-frame double FLAG tag (generated using oligonucleotides FLAG1 and FLAG2) between the Met receptor cDNA and the stop codons to form the final construct, pMet-DN (Figure 4.1, Appendix A6.2.4). The FLAG tag was included at the carboxy-terminus to aid the identification of the receptor within transgenic animals, this provided an alternative to using anti-Met receptor antibodies that could cross-react with the endogenous Met receptors. All constructs, and subsequent constructs described in this chapter were verified by DNA sequencing.

Figure 4.1 – pMet-DN construct (transgene)

Schematic depiction of transgene created to express a truncated Met receptor (Met-DN) with a C-terminal FLAG tag (F) under the regulatory control of the MMTV-LTR. The figure shows a Smal (S) digested fragment of pMet-DN. Rabbit β-globin intron (intron), and SV-40 late poly adénylation signal (Poly(A)) are shown. Enzyme sites: BglII (B), HindIII (H), SphI (Sp), SpeI (E), and AatII (A).

4.2.2 Testing of pMet-DN construct

Before pMet-DN was used to generate transgenic mice, the construct was tested to verify that it was able to express the truncated Met receptor. This was essential to confirm that: i) the Met cDNA fragment was translated effectively, and ii) the MMTV-LTR promoter was active.

4.2.2a Transcription and translation (in vitro)

In order to determine that a protein could be generated from the cDNA fragment encoding Met-DN, an in vitro transcription and translation experiment was performed. This experiment required a new plasmid to be created, as an appropriate promoter was not present in the pMet-DN construct. The Met-DN cDNA fragment was therefore excised from pMet-DN by digestion with SphI (blunted) and SpeI. The resulting Met-DN cDNA was then purified from the remainder of the vector by gel extraction, after separation of the digested products through a 1% (w/v) TAE agarose gel. This fragment was cloned directly into the EcoRV and SpeI sites of pBluescript SK- (Stratagene) to bring the Met-DN cDNA.
under the transcription control of the T7 promoter (pBSMet-DN, see Appendix A6.2.5).

In vitro transcription and translation using pBSMet-DN was carried out using a rabbit reticulocyte lysate kit, that couples transcription and translation into a single reaction (described in section 2.12.6). A pET21a vector (Novagen) containing a cDNA fragment encoding Fgf-8a (an alternatively spliced form of Fgf-8) under the regulatory control of a T7 promoter was used as a positive control. Translated products incorporating L-[\(^{35}\)S] methionine were separated on a 12% SDS-containing polyacrylamide gel, which was then fixed and exposed to autoradiographic film. The Met-DN cDNA fragment was predicted to express a protein of approximately 116kD as estimated using the Staden Software Suite molecular biology package. As can be seen in Figure 4.2A, a product of approximately this size is translated efficiently from the pBSMet-DN vector. The transcription/translation reaction using the Fgf-8a vector also produced a band of the predicted size (26kD). These data show that the Met-DN cDNA gives rise to a product of the correct size when transcribed and translated in vitro.

4.2.2b Expression in mammalian cells

The ability of pMet-DN to express the truncated Met receptor product (Met-DN) in response to activation of the MMTV-LTR was assessed in mammalian cells. 1.8x10^5 MDCK cells, an epithelial cell line that expresses the Met receptor (Joseph et al., 1992), were grown in the wells of a six well cell culture plate and transfected the following day with 2.5 μg pMet-DN (section 2.9.4a). One day following transfection, the cells were stimulated overnight with varying concentrations of dexamethasone - a synthetic glucocorticoid which is known to increase the transcriptional activity of the MMTV-LTR. The cells were then lysed in a RIPA buffer, and the presence of Met-DN determined by immunoblotting, after separation of the proteins through an 8% SDS-containing polyacrylamide gel. The antibody DL-21, directed against the extracellular portion of the β-chain of the human Met receptor, was used to detect exogenous Met-DN receptor expression. Protein lysates prepared from A431 human colon carcinoma cells, which strongly express the Met receptor, were used as a positive control for detecting Met expression. Untransfected MDCK cell lysates were included as a negative control.

After detection of bound DL-21 complexes, two strong bands of approximately 170kD and 145kD were present in the lane containing the A431 cell lysates (Figure 4.2B). These two protein species correspond to the β-chain of Met (145kD), and the
Figure 4.2 - In vitro analysis of Met-DN

A) In vitro transcription and translation of pBSMet-DN (left lane) using the T7 RNA polymerase. The open arrowhead shows a major product migrating at approximately 116kD. The right lane shows a control reaction producing an Fgf-8a product of approximately 26kD (closed arrowhead). The positions of known molecular weight marker proteins run in parallel are shown.

B) Immunoblot of lysates prepared from MDCK cells transiently transfected with pMet-DN and induced to express Met-DN (open arrowheads) after overnight induction with dexamethasone as indicated. A431 cells which strongly express Met provided a positive control. Lysates from untransfected MDCK cells were used as a negative control.

C) Indirect immunofluorescence showing localisation of Met proteins (green) after transient transfection of the pcDNA4/TO based vectors indicated. The tight junction protein ZO-1 is stained in red. XY and XZ confocal images are shown. Scale bar represents 10μm.
single inactive precursor chain of Met (170kD) that is eventually cleaved between residues 306 and 307 to form the mature α and β-chains (Appendix A6.4.3, Naldini et al., 1991, see Figure 1.4 and section 1.2.1a). No signal was detected in untransfected MDCK cells, indicating that the DL-21 antibody did not cross-react with canine Met receptors. By contrast, MDCK cells transiently transfected with pMet-DN, and stimulated with dexamethasone, produced two major bands at around 90kD and 125kD. Analogous bands were also detected when cell lysates were incubated with an anti-FLAG antibody (data not shown). These bands were of the predicted size for the β-chain (90kD), and the single chain uncleaved precursor (125kD), of the truncated Met receptor. The results show that incubation with 10^4M dexamethasone overnight induces high levels of Met-DN expression, and that the majority of the detected proteins are of the mature cleaved form of the receptor. The level of Met expression decreased progressively as the concentration of dexamethasone was reduced, such that induction with 10^10M dexamethasone led to very little Met expression. In the absence of dexamethasone, the levels of Met-DN expression were virtually undetectable using this technique. Similar results were obtained using the HC11 murine mammary epithelial cell line, although in these cells the basal level of Met-DN expression was higher than in MDCK cells (data not shown). Taken together, these results show that pMet-DN is capable of expressing a truncated Met receptor under the regulatory control of the MMTV-LTR in mammalian cells. This product is expressed as a single chain peptide, and appears to be efficiently cleaved to produce a Met β-chain of the predicted size. Unfortunately, an antibody was not available to check that cleavage of the Met precursor product also gave rise to an α-chain of the predicted size (50kD).

### 4.2.3 Basolateral Localisation of Met-DN

The Met receptor is normally localised to the basolateral membrane domain in polarised epithelial cells, where presumably it is activated by stromally derived HGF (Crepaldi et al., 1994). As the truncation of many membrane bound proteins normally localised to the basolateral membrane, such as ErbB-2 (see Chapter 3), may lead to their accumulation on the apical surface, the sub-cellular distribution of Met-DN was assessed in polarised epithelial cells. This was an important issue to consider, as the ability of the truncated Met receptor to act as a dominant negative would rely on its co-localisation on the basolateral cell surface with endogenous Met receptors, and its close proximity to stromally derived HGF.
The effect of Met receptor truncation on its polarised distribution was assessed using cDNA fragments of Met, cloned into the mammalian expression vector pcDNA4/TO, and MDCK cells. This system was used extensively to analyse the signals involved in the polarised trafficking of ErbB-2 as discussed in Chapter 3 (section 3.2).

To confirm that the full length human Met receptor localised to the basolateral membrane domain in polarised MDCK cells, a cDNA fragment encoding the full length Met receptor was amplified by PCR from the vector pMOG1. A forward primer containing a 5′ HindIII site (MetHIII-F), and a reverse primer (NI4173-R) that introduced an extra stop codon and a NotI site in the amplified product immediately 3′ of the natural stop codon were used. This fragment was then digested with HindIII/NotI, purified as described in section 2.8.5c, and cloned into the HindIII/NotI sites of pcDNA4/TO to form the vector pc4-Met-FL. A similar vector was constructed using the primers MetHIIII-F and NI2946-R to amplify a cDNA fragment from pMOG1 that would encode the first 982 amino acids of Met followed by a double stop codon. This vector, pc4-Met-A982, was engineered to express the same truncated Met receptor protein as the vector pMet-DN, this protein should therefore localise to the same membrane domain as the product of the M-MetDN transgene.

Expression of full length and truncated Met proteins was determined by immunoblot analysis using the DL-21 antibody. Both pc4-Met-FL and pc4-Met-A982 produced products of the predicted size when lysates prepared from transiently transfected MDCK cells were immunoblotted (data not shown).

To assess the location of these proteins in polarised epithelial cells, 2.5x10⁵ MDCK cells were seeded on to PET membranes, and transfected the following day with pc4-Met-FL or pc4-Met-Δ982. Two days post-transfection, the cells were fixed in cold methanol and the sub-cellular distribution of ZO-1, a marker for the tight junctions, and the expressed Met receptors were analysed by indirect-immunofluorescent staining and confocal microscopy. Cells were incubated with primary antibodies DO-24 (a mouse monoclonal antibody directed against the extracellular domain of human Met) and MAB1520 (a rat monoclonal antibody detecting ZO-1). Immunofluorescent staining was achieved using FITC-conjugated goat anti-mouse and TRITC-conjugated goat anti-rat antibodies as described in section 2.9.5.
The full length Met receptor was found to locate exclusively to the basolateral membrane of polarised MDCK cells, in agreement with the published observations (Figure 4.2C, Crepaldi et al., 1994). As MDCK cells do not sort proteins to the basolateral membrane by default, this suggests that the Met receptor possesses a basolateral targeting signal(s). The truncated Met receptor (Met-Δ982) also localised predominantly to the basolateral membrane, although significant apical staining was observed, signifying that the truncation may have minimally compromised a basolateral targeting signal within the intracellular tail of Met. Nevertheless, this result suggests that the Met-DN receptor should act as a dominant negative receptor in epithelial cells expressing endogenous Met receptors, as the basolateral membrane contains a large proportion of the mutated receptors.

Given that basolateral targeting signals in membrane-bound proteins are usually present as distinct amino acid sequences located within the intracellular domain, these observations would suggest that a basolateral targeting signal is present in the Met receptor between the end of the transmembrane region (residue 956), and residue 982. As can be seen in Figure 4.3A, this juxtamembrane region contains a pair of di-hydrophobic residues (LV) that could potentially act as a basolateral sorting signal (residues 968 and 969). In order to determine whether the LV pair, or any other juxtamembrane residues in Met act as a basolateral targeting signal, a new vector was constructed containing a carboxy-terminal truncation adjacent to the transmembrane domain, that would delete all but five intracellular amino acids of Met (Figure 4.3A). This vector, pc4-Met-Δ960, was created in the same way as pc4-Met-Δ982 except that the primers MetHIII-F and MetTr2881 were used for the PCR amplification.

Transient expression of pc4-Met-Δ960 in MDCK cells produced bands of the predicted size, correlating with the truncated single chain precursor, and β-chain Met products after immunoblotting with the DL-21 antibody (data not shown). The localisation of the Met-Δ960 receptor in polarised MDCK cells was assessed by indirect immunofluorescence and confocal analysis as described above. Unexpectedly, Met-Δ960 was shown to distribute to the basolateral and apical membrane domains of polarised MDCK cells, with a localisation pattern that was indistinguishable from Met-Δ982 (Figure 4.2C). These results suggest, in contrast to other basolateral proteins such as ErbB-2, that the Met receptor contains basolateral targeting information within the transmembrane or extracellular domain of the receptor.
Figure 4.3 – Mutated Met receptor proteins

A) Diagram detailing the domain structure of the human Met receptor (TM = transmembrane) and the amino acid sequence of the intracellular juxtamembrane domain (red). The positions of 2 truncation mutations (at amino acid positions 982 and 960) within this domain are shown. B) schematic depiction of a chimeric receptor composed of the extracellular and transmembrane domains of the P75NTR, and the intracellular domain of the human Met receptor. (-) indicates intervening Met amino acids not shown. The (*) represents the position of a stop codon.

The shared basolateral/apical localisation of the truncated Met receptors indicates that some basolateral targeting information is also present within the intracellular domain of the receptor. In order to determine whether the intracellular domain of the Met receptor contained dominant basolateral targeting information, a chimeric receptor was created as a fusion between the apically localised P75NTR, and the intracellular domain (residues 961-1390) of Met (see section 3.7 for further details). The construct expressing this receptor was created using pcDNA4/TO in the same way as the P75NTR:ErbB-2 chimeric receptor constructs described in section 3.7.1, except that an EcoRI linker was used to join the receptor cDNAs together. The
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primers used for the amplifications were P75-FoH3 and P75-ReR1 (for the P75NTR extracellular and transmembrane domains), and Met-FoR1 and NI4173-R (for the Met intracellular domain). This vector was named pc4-P75Met(961-1390) and the product expressed from this construct is illustrated in Figure 4.3B.

Expression of pc4-P75Met(961-1390) in MDCK cells revealed a product of the correct size when cell lysates were immunoblotted with the anti-P75NTR ME20.4 antibody (data not shown). When expressed in polarised MDCK cells, the P75Met(961-1390) protein localised to the basolateral membrane (Figure 4.2C). Thus, the intracellular domain of the human Met receptor is able to redistribute the apically localised P75NTR to the basolateral membrane domain, suggesting that it contains dominant or autonomous basolateral sorting information.

Altogether, these results show that Met-Δ982, a truncated Met receptor similar to Met-DN but lacking the FLAG tag, localises to the basolateral membrane domain when expressed in polarised epithelial cells. This localisation is key to Met-DN functioning as a dominant negative receptor in polarised mammary epithelial cells. However, these results also show that intracellular truncation of the Met receptor reduces the efficiency of basolateral targeting, and indicates that the intracellular domain of Met contains dominant basolateral targeting information. Furthermore, the data indirectly suggests that the Met receptor also contains basolateral targeting information within the transmembrane or extracellular portion of the receptor.

4.2.4 Activation of the Met Receptor

In order to demonstrate that exogenously expressed human Met receptor could become activated by HGF, an immunoprecipitation experiment was carried out. This was an important experiment to perform as the ability to bind ligand is probably essential for the truncated Met receptor to act in a dominant negative fashion in vivo. As the extracellular ligand binding portion of Met-DN is identical to that found in the full length Met receptor, this experiment was performed using a new construct containing a cDNA fragment encoding the full length Met receptor. This approach enabled the ligand binding capability of the Met extracellular domain to be assessed using a simple kinase assay, rather than through more complicated chemical cross-linking experiments.

A full length Met receptor cDNA was created by PCR using the DNA template pMOG1 and the primers MetHIII-F and NI4173T-R. These primers placed a HindIII, and a NotI restriction site at the 5' and 3' end of the encoding cDNA
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respectively. The product was then digested with HindIII/NotI, gel extracted, and cloned into the vector pcDNA4/TO/Myc-HisC (Invitrogen Life Technologies) using the same restriction sites to form the vector pc4-Met-FLt. pcDNA4/TO/Myc-HisC is an equivalent vector to pcDNA4/TO, but places a carboxy-terminal myc and His tag onto the cloned gene. Thus, pc4-Met-FLt was essentially the same vector as pc4-Met-FL, except that the full length Met receptor was tagged at the carboxy-terminus with a myc, and a His tag. Transient expression of this vector into MDCK cells produced products of the predicted size, that were indistinguishable from the those expressed from the pc4-Met-FL construct (data not shown).

20μg pc4-Met-FLt was used to transfect a 10cm petri dish of MDCK cells that were plated at 60% confluency using the protocol described in 2.9.4a. The following day, the transfected cells were split into the wells of a 6-well plate. Two days post-transfection the cells had reached confluency and were starved for six hours in E4 medium containing 0.1% (v/v) newborn calf serum. Cells were then stimulated with 20ng/ml human recombinant HGF for 30 seconds, 5, 15, 30, or 60 minutes, or left unstimulated. Samples were then lysed in NET buffer, and the expressed Myc tagged Met receptors immunoprecipitated using the 9E10 anti-myc antibody as described in section 2.12.3. Purified immuno-complexes were then separated on an 8% SDS-containing polyacrylamide gel and immunoblotted using the PY99 antibody to determine the phosphorylated state of the captured Met receptors. These experiments were performed using TBS-T instead of PBS-T for the washing steps, and TBS-T containing 1% (w/v) BSA as a blocking reagent.

The presence of two bands indicating a basal level of Met phosphorylation could be detected in transfected cells that were not stimulated with HGF (Figure 4.4A). These bands were of the predicted sizes of the β-chain and single chain precursor of the full length Met receptor, suggesting, in agreement with the published findings, that both the precursor and mature Met receptor are partially phosphorylated when overexpressed in mammalian cells (Bardelli et al., 1998). The level of phosphorylation noticeably increases after 5 minutes of stimulation with HGF, consistent with the exogenously expressed receptors being activated by ligand. Phosphorylation of Met peaks at 15 minutes stimulation with HGF, and remains high up to 60 minutes stimulation. An important observation from these experiments is that only the smaller band, detecting the presence of the mature Met β-chain, shows an increase in its phosphorylative state in response to HGF. This strongly suggests that the transfected human Met receptor is processed correctly in mammalian cells, with the single chain translated inactive precursor giving rise,
through its intracellular cleavage, to a mature α-β receptor complex at the cell surface. No bands were detected with the PY99 antibody when the same experiment was performed with untransfected MDCK cells.

These results demonstrate the ability of the full length human Met receptor to bind, and become activated by HGF. As Met-DN contains the same extracellular ligand binding domain, and appears to be cleaved into an α-β complex in the same way as the full length receptor, it is highly probable that Met-DN is also able to bind HGF.

4.2.5 Dominant Negative Activity of Met-DN

In order to demonstrate Met-DN was able to act in a dominant negative fashion, and block the activation of endogenous murine Met receptors in response to HGF, HC11 mouse mammary epithelial cells were utilised. HC11 cells were separately transfected with pcDNA4/TO, pc4-Met-FL, and the pc4-Met-Δ982 construct that encodes a protein with same open reading frame as Met-DN. Pooled stable HC11 cell clones were obtained after selection with 150 μg/ml Zeocin™, and Met expression was confirmed by immunoblot analysis using the DL-21 antibody. Products of the predicted size were detected in cells transfected with pc4-Met-FL, and pc4-Met-Δ982, and no exogenous Met expression could be detected in cells transfected with the empty pcDNA4/TO vector (Figure 4.4B).

2x10⁶ cells from each stable HC11 cell line were plated into 4 wells of a 6 well cell culture dish and allowed to grow to confluency. The cells were then washed in RPMI 1640 3.7% medium containing 0.1% (v/v) newborn calf serum, and then starved using the same medium for 48 hours. Cells were then stimulated with either 20ng/ml HGF for 5 or 15 minutes, or with 20ng/ml EGF for 5 minutes, or alternatively left unstimulated. Protein lysates were prepared by lysing the cells directly in Laemmli buffer, and the products were then separated on an 8% SDS-containing polyacrylamide gel. The cell lysates were then immunoblotted with antibodies to detect i) phosphorylated Met receptors (P-Met), ii) phosphorylated Erk1 and Erk2 (downstream targets of Met receptor activation), and iii) β-catenin as a loading control. The P-Met antibody is specifically reactive against the Met receptor when it is phosphorylated on residues Tyr-1230, Tyr-1234, and Tyr-1235, and therefore only detects activated Met receptors.

As can be seen in Figure 4.4B, HGF stimulation caused an increase in the levels of endogenous murine Met receptor phosphorylation in the cells transfected with pcDNA4/TO and pc4-Met-FL, when compared with cells that were left
A) Immunoblot (IB) of lysates from MDCK cells, unstimulated (U) or stimulated with HGF as indicated, using the PY99 antibody to detect phosphorylated tyrosine residues. The figure shows two immunoblots using lysates from MDCK cells that were either transfected with pc4-Met-FLt, or left untransfected. Immunoprecipitation (IP) was performed with an anti-myc tag antibody.

B) Immunoblots of lysates from HC11 cells stably transfected with the constructs shown (top labels), and stimulated with 20ng/ml HGF for 5 minutes (5H), or 15 minutes (15H), or with 20ng/ml EGF for 5 minutes (5E) - U = unstimulated. Cell lysates were immunoblotted with antibodies to detect phosphorylated Met receptors (P-Met), phosphorylated Erk1/2. β-catenin was detected as a loading control. The bottom panel shows the exogenous expression of Met receptor products using lysates obtained from unstimulated stable HC11 cells.
unstimulated. This increase was not readily detectable when the cells were stimulated with EGF as expected, however, EGF stimulation led to an increase in Erk1/2 phosphorylation as observed with HGF. By contrast, phosphorylated Met receptors could not be detected in lysates prepared from cells transfected with pc4-Met-A982, even when these cells were stimulated with HGF. This indicates that the truncated Met-A982 receptor, and therefore Met-DN, specifically abrogates signalling through endogenous murine Met receptors by acting in a dominant negative manner as predicted. Interestingly, even though Met receptor signalling was blocked in these cells, HGF was still able to activate Erk1/2 as observed in the control samples. The reasons for this are unclear, although it is possible that Erk1/2 activation occurs via a Met receptor independent pathway, perhaps through activation of the Ron receptor which is closely related to Met. Alternatively, the HGF used in these experiments could contain impurities that are able to cause the activation of the Erk1/2 pathway.

In summary, these experiments demonstrate that expression of a truncated human Met receptor is able to effectively block the activation of endogenous murine Met receptors.

### 4.3 Generation and Analysis of Transgenic Mice

Having demonstrated that pMet-DN was correctly expressing the desired product, which could act as a dominant negative receptor, the construct was used to create transgenic mice. pMet-DN was digested with *Sma*I to produce the transgene *M-MetDN* (see Figure 4.1). The transgene was separated from the pMet-DN vector backbone, and eluted to 5ng/µl in sterile transgenic injection buffer as described in section 2.8.16. *M-MetDN* was sent for injection into the male pronucleus of fertilised F1 x F1 hybrid (CBA x C57BL/6 strain) mouse embryos and then implanted into pseudopregnant foster mothers to generate transgenic mice (Cancer Research UK, Transgenic Services).

Three independent rounds of injections with the *M-MetDN* transgene were performed by the Cancer Research UK Transgenic Services (Table 4.1). Genomic DNA isolated from potential transgenic founder mice was screened for correct integration of the transgene. Those mice that contained the transgene, and were transmitting it to subsequent generations, were then screened to detect for expression of *Met-DN* in the mammary gland.
4.3.1 Screening for Transgene Integration

The presence of the integrated M-MetDN transgene was detected by Southern blotting. Genomic DNA was isolated from tail biopsies and resuspended in 100μl TE buffer (section 2.10.1). 10μl genomic DNA was then digested overnight with PstI in the presence of 2.5μg/ml RNase A and 1mM spermidine (section 2.10.1). The digested DNA was separated on a 0.8% (w/v) agarose gel, and transferred to Nytran® N SuPerCharge membrane for Southern blotting (described in section 2.10.4). A 1041 bp fragment was used as a probe to detect M-MetDN. This probe was isolated by digestion of pMet-DN with Apal, followed by gel extraction of a fragment of cDNA encompassing the 3' end of the rabbit β-globin intron, and the first 977 bases of the human Met gene (see Figure 4.5A).

141 potential founder mice were screened using this strategy. A total of four heterozygous founders were identified that contained an intact transgene, as determined by the presence of two bands of 2784 and 1257 bps in the exposed blot. Positive mice were then bred to F1 wild type mice, and the resulting offspring screened using the same method to determine whether the transgene was being transmitted through the germline. Analysis of these offspring showed that the transgene was being transmitted at roughly the expected Mendelian ratios (50%) in all four transgenic lines. This indicates transgene integration occurred before division of the injected embryos. Figure 4.5B shows a representative result from these experiments. Breeding colonies of each transgenic line were established, and the resulting litters screened for the presence of M-MetDN by PCR using the primers IntF1 and MetIntR1: an annealing temperature of 46°C was used for the reactions (see Figure 4.5A, and section 2.10.3).

<table>
<thead>
<tr>
<th>Round</th>
<th># Injected Embryos</th>
<th># Embryo Transfers</th>
<th># Potential Founder Mice</th>
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<tr>
<td>1</td>
<td>201</td>
<td>9</td>
<td>26</td>
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<tr>
<td>2</td>
<td>244</td>
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<tr>
<td>Total</td>
<td>596</td>
<td>27</td>
<td>141</td>
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*The table shows the number of embryos injected with the transgene, the number of successful embryo transfers into pseudopregnant females, and the number of potential founder mice born that needed to be screened for transgene integration.*
4.3.2 Screening for Transgene Expression

Screening for transgene expression was carried out by Northern analysis of mammary gland total RNA. Sexually mature female transgenic mice from each transgenic line were mated with available male mice. The number 4 right hand side inguinal mammary gland was then dissected at day 14.5 of pregnancy, and total RNA extracted as described in section 2.11.1b. Mammary glands from this stage were chosen for the detection of transgene expression, as previous studies had demonstrated the MMTV-LTR to be highly active in the mid-late pregnant gland (Jackson et al., 1997). 20μg total RNA was separated through a 1.2% (w/v) denaturing agarose gel, transferred to Nytran® N membrane, and then probed to detect expression of Met-DN. As a negative control, total RNA extracted from equivalent mammary glands of 14.5 day pregnant female non-transgenic littermates were used. An extra set of controls was provided by total RNA isolated from MDCK cells stably expressing Met-Δ982 under the regulatory control of a tetracycline-on inducible CMV promoter (cells were transfected with pcDNA6/TR and pc4-Met-Δ982, see Figure 3.2). Total RNA from these cells was extracted after overnight incubation without (further negative control), or with (positive control) 1μg/ml tetracycline.

Membranes containing cross-linked total RNA were hybridised with a radio-labelled probe generated from the same cDNA fragment used for the Southern analysis (Figure 4.5A, section 2.10.4a). Samples were also hybridised with a control probe to detect a housekeeping gene, glyceraldehydephosphate dehydrogenase (GAPDH), that is expressed in all cells. The GAPDH probe was generated using a 260 bp BamHI/EcoRI digested cDNA fragment of the rat GAPDH gene (gift from Vera Fantl, Cancer Research UK).

Representative results of a Northern blot analysis are shown in Figure 4.5C. GAPDH expression was clearly detected as a strongly positive band in all tissue samples. The weaker GAPDH signal detected in MDCK cells may reflect the variation in sequence between the rat and canine GAPDH message, or a reduced expression level. The sample containing stable MDCK cells induced to express Met-Δ982 (lane 6) were positive for Met expression as expected. No Met-Δ982 expression was detected when the same cells were grown in the absence of tetracycline (lane 5), or when total RNA from wild type mammary glands was probed (lane 1). These results confirmed that the probe was detecting expression of the truncated Met receptor. Overall, it was found that just one transgenic line (lane 3, line 2668B) was expressing the transgene at detectable levels.
A) Schematic of Met-DN transgene showing position of the probe generated by Apal (A) digestion. Southern blotting was carried out after digestion of genomic DNA with PstI (P), leading to mice positive for the transgene showing two bands of 1257 and 2784 bps. The positions of two primers are shown that were used for genotyping transgenic litters by PCR.

B) Southern blot of genomic DNA from a litter of transgenic mice with probe shown in (A) demonstrating germline transmission of the Met-DN transgene. The blot shows one negative (1) and three positive mice (2-4). (L) 1kb DNA ladder.

C) Northern blot of total RNA extracted from mammary glands of a wild type mouse (1) and mice from three independent transgenic lines (2-4), showing that sample 3 is expressing Met-DN. Control lanes: 5 & 6, total RNA isolated from MDCK cells stably integrated with pcDNA6/TR and pc4-Met-Δ982 after overnight induction without (5), and with (6) 1μg/ml tetracycline. Samples were probed with the Met-DN probe shown in (A), and a GAPDH probe.
Given the low number of expressing transgenic lines, further attempts to generate transgenic mice expressing a truncated Met receptor, under the regulatory control of the MMTV-LTR, were undertaken. For these experiments an alternative transgene consisting of a similar truncated Met receptor cDNA was used. However, a similar pattern emerged: 13 additional transgenic founders were established, of these 4 did not transmit the transgene to the next generation, and none of the remaining 9 lines were found to express the truncated Met receptor in the mammary gland by Northern analysis. Similar results were obtained when total RNA extracted from mature transgenic male testes and the epididymis, both strong potential sites of MMTV-LTR activity, were probed for transgene expression.

4.3.3 Morphological Analysis of Transgenic Mammary Glands

In order to examine whether the transgenic expression of Met-DN in the mammary glands affected its growth and differentiation, glands from transgenic line 2668B were analysed. Sexually mature female mice positive for the transgene were mated and then sacrificed on days 11.5, 14.5, and 18.5 of pregnancy. The morning that the vaginal plug was found was considered to be day 0.5. Sexually mature virgin mice were also sacrificed. The number 4 inguinal mammary glands were then dissected and prepared for morphological examination. This was achieved by fixing the right hand side gland in carnoys fixative and then staining it with carmine stain for a wholemount preparation (section 2.13.2). The left hand side gland was fixed overnight in NBF, dehydrated through a series of graded alcohols, and embedded in paraffin wax and then sectioned at 5μm intervals before being histologically stained with haematoxylin and eosin. As a control, littermate or age matched wild type female mice were used.

At the stages analysed, no significant discernible morphological differences could be detected between wild type and transgenic mice (Figure 4.6). Both sets of mice showed a similar degree of mammary branching morphogenesis after puberty and during pregnancy, and exhibited apparently normal lobulo-alveolar development. The histological analysis shown in Figure 4.7 further demonstrates this. Moreover, transgenic mice suckled their young normally, indicating that mammary morphogenesis was not seriously affected by the presence of the M-MetDN transgene.
Figure 4.6 - Wholemount analysis of *M-MetDN*⁺ mammary glands

The number 4 right hand side inguinal mammary gland was dissected from *Met-DN*⁺ and control mice from various stages of development: virgin, days 11.5, 14.5, and 18.5 of gestation. Glands were fixed and stained with carmine stain. Scale bar represents 1mm.
The number 4 left hand side inguinal mammary gland was dissected from Met-DN⁺ and control mice from various stages of development: virgin, days 11.5, 14.5, and 18.5 of gestation. Glands were fixed, paraffin embedded, sectioned and stained with haematoxylin and eosin. Scale bar represents 100μm.
4.4 Discussion

This study attempted to elucidate a biological role for Met receptor signalling in the development of the postnatal mammary gland. A transgene was constructed to express a truncated version of the human Met receptor, that would act in a dominant negative manner when expressed in the mammary glands of mice. Similar truncated RTKs, that lack their intracellular tyrosine kinase domain, have been shown previously to act as dominant negative receptors when expressed in the mammary gland. For instance, expression of truncated forms of the FgfR-2IIIb and ErbB-2 RTKs, under the regulatory control of the MMTV-LTR, has revealed roles for these receptors in mammary lobulo-alveolar development (Jackson et al., 1997; Jones and Stern, 1999).

The in vitro experiments demonstrated that the truncated Met receptor localised correctly to the basolateral membrane domain of polarised epithelial cells, and that binding of HGF induced receptor phosphorylation. It was also shown that the activity of the MMTV-LTR was upregulated upon addition of synthetic glucocorticoids into the growth medium of cells transfected with the transgene. These findings supported the notion that a truncated Met receptor should act in a dominant negative fashion within the mammary gland. This was further substantiated through experiments using mouse mammary epithelial cells. Dominant negative activity was demonstrated in these cells using an antibody specific for the activated form of the Met receptor (Figure 4.4B). In cells that were not expressing the truncated receptor, Met phosphorylation could be easily detected in response to HGF, but not EGF stimulation. However, in cells that were expressing the truncated Met receptor, HGF stimulation did not lead to an increase in the levels of endogenous Met phosphorylation, indicating that the introduced receptor was acting as a dominant negative receptor. These findings are in agreement with previous studies showing that exogenously expressed Met receptors can function in a dominant negative manner when truncated within the intracellular juxtamembrane region (Aoki et al., 1997), or when the tyrosine kinase domain is rendered inactive (Firon et al., 2000). Furthermore, they show that a dominant negative human Met receptor can effectively block signalling through the mouse Met receptor in agreement with (Furge et al., 2001).

The molecular mechanism underlying the dominant negative effect is not clear. The truncated receptors may act by forming inactive heterodimers with the endogenous wild type receptors, or by sequestration of available ligand through the formation.
of homodimeric complexes, or both. In either case the dimeric complex would not be able to function, as the tyrosine kinase domain is required for the intermolecular trans-phosphorylation event that is necessary for receptor activation (see van der Voort et al., 2000).

Attempts to generate transgenic mice using the dominant negative transgene were largely unsuccessful. The number of founder mice born to foster mothers tended to be very low for each round of transgenic injections, and of the 17 founder lines produced, only one of these expressed the transgene at detectable levels. In this transgenic line the levels of transgene expression appeared quite low in the mammary gland, and it is not clear whether these levels would be sufficiently high for the expressed receptors to function as a dominant negative. As the locus for transgene integration into the genome is random, it is generally accepted that two independent transgenic lines carrying the same transgene should be analysed to assess any potential phenotype. Therefore no firm conclusions can be drawn from the experiments performed on the single transgenic line 2668B. Nevertheless, a brief analysis of the mammary glands from these mice revealed that they appear to develop normally.

In addition to the development of another transgenic line that expresses the transgene, a full characterisation of line 2668B needs to be undertaken to establish whether the lack of a mammary phenotype represents a non-essential role for Met in mammary development, or a problem with the transgene, such as an inactivating mutation, in this line. This characterisation would include a quantification of mammary branching and side branching in the glands of control and transgenic mice at different stages of development, and also a comprehensive examination of transgene expression throughout the different stages of mammary morphogenesis by Northern analysis and in situ hybridisation. The in situ hybridisation analysis would be a particularly important experiment to perform, as variegated transgene expression in the mammary gland is a well documented phenomenon (Dobie et al., 1997), and has been described for transgenes driven by the MMTV-LTR (Muller et al., 1988). As the dominant negative Met transgene would be expected to have growth inhibitory effects within the mammary gland, a phenotype associated with its expression may be missed if the surrounding cells were not expressing the transgene, and thereby acting in a compensatory manner.

Although it is not clear why this strategy failed to generate sufficient transgenic mice, it seems possible that expression of the transgene could be toxic to the embryos. This would explain why the number of founder lines were below the
expected average from each round of injections, and also provides an explanation as to why only one founder line was expressing the transgene. Transgene toxicity is a distinct possibility given that the Met receptor is essential for embryonic development (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995), and that the MMTV-LTR can be active early in embryogenesis and in a broad range of tissues (Wagner et al., 2001). Thus the generation of transgenic mice in this study may only have been successful in cases where the transgene had integrated into a region of the genome that suppresses its expression during embryogenesis.

The observation that a Met receptor lacking all but five of its intracellular amino acids still localises predominantly to the basolateral membrane of polarised MDCK cells is an interesting one. Usually single membrane-spanning basolateral proteins contain intracellular signals that specify basolateral delivery from the TGN. This is certainly the case for the Met receptor, as its intracellular domain is able to redirect the P75NTR from the apical to the basolateral membrane. However, the fact that the truncated receptor Met-Δ960 localises to the basolateral membrane, as well the apical membrane, suggests that some basolateral targeting information could be present in the transmembrane or extracellular domain. This basolateral targeting could be direct by recognition of a signal, or indirect through an interaction with a chaperone molecule. It would be of interest to determine whether these domains are present and able to specify basolateral deliver of Met in polarised epithelial cells as this would be the first demonstration of a basolateral sorting signal residing outside the intracellular domain of a membrane bound receptor.

In conclusion, a transgene was created to express a truncated Met receptor under the control of the MMTV-LTR. Analysis of transgene function in cultured cells demonstrated that it was functioning correctly, however, attempts to generate mice expressing the transgene within the mammary gland gave inconclusive results.
Chapter 5 | Role of FgfR-2IIIb in Embryonic and Postnatal Mammary Gland Development

5.1 Introduction

The mouse mammary gland has been extensively studied as a model system for understanding developmental processes, and tumour progression. Its development is usually considered to occur in two distinct phases, embryonic and postnatal. Whereas the development of the postnatal mammary gland is regulated by the endocrine action of steroid and peptide hormones, growth of the embryonic gland is largely independent of systemic cues. Despite these apparent differences, tissue remodelling during both stages is essentially controlled by locally-acting factors that are expressed in the mammary epithelium and mesenchyme (Dunbar and Wysolmerski, 2001; Robinson et al., 1999; Silberstein, 2001b). Several members of the Fgf family, and their receptors are expressed in the mammary gland, suggesting a functional role in mammary development through instructive epithelial-mesenchymal interactions (Coleman-Krnacik and Rosen, 1994; Pedchenko and Imagawa, 2000).

The IIIb isoform of FgfR-2 has been shown to have important roles in mesenchymal-epithelial signalling during mouse organogenesis (De Moerlooze et al., 2000). In addition to an essential role in limb and lung development, FgfR-2IIIb is also required for the development of several ectodermal rudiments such as teeth and hair follicles (De Moerlooze et al., 2000, Dr. A. Petiot, Cancer Research UK, London, personal communication). As might be expected for an organ that is derived from the budding and subsequent branching of ectodermal cells, gene expression studies have detected $FgfR-2IIIb$ transcripts in the mammary gland. In the embryonic mammary gland, $FgfR-2IIIb$ transcripts were detected by in situ hybridisation in the epithelial bud at E14 (Cunha and Hom, 1996). $FgfR-2IIIb$ gene expression in the adult mammary gland has been demonstrated by RNase protection assay and RT-PCR, and follows a developmentally regulated pattern, with peak expression occurring in the mature nulliparous adult (Pedchenko and Imagawa, 2000). Furthermore, two activating ligands for FgfR-2IIIb have been identified in the mammary gland: Fgf-7 in the embryonic mammary mesenchyme (Cunha and Hom,
Mice containing a targeted disruption of the IIIb isoform of FgfR-2 were used to study the physiological role of FgfR-2IIIb in the embryonic and postnatal mammary gland. As mice lacking FgfR-2IIIb can only survive in utero due to gross developmental defects, two strategies were employed (De Moerlooze et al., 2000). The role of FgfR-2IIIb signalling in embryonic mammary development was investigated using a mouse containing a non-conditional deletion of the IIIb exon of FgfR-2. Conversely, a conditional gene deletion approach was utilised to assess the role of FgfR-2IIIb in postnatal mammary development. This latter strategy relied on the specific transgenic expression of the cre-recombinase enzyme (a 38kD protein of the integrase family of site-specific recombinases isolated from the P1 bacteriophage) in the adult to functionally disrupt FgfR-2IIIb in the mammary gland, whilst leaving an intact FgfR-2IIIb in other tissues.

5.1.1 Targeted Disruption of FgfR-2IIIb in Mice

Mice containing a targeted disruption of the IIIb exon of FgfR-2 were previously generated in the laboratory (De Moerlooze et al., 2000). These mice were created by removing exon IIIb of FgfR-2 from the mouse genome using cre-recombinase to excise exon IIIb from the genome of targeted embryonic stem (ES) cells (outlined in Figure 5.1). A brief overview of the strategy used to generate these mice is set out below.

A targeting vector was created containing a selection cassette flanked by two loxP sites (floxed) in the same orientation. The loxP site is a 34 bp sequence identified in the P1 bacteriophage consisting of a core spacer sequence of 8 bp and two 13 bp palindromic flanking sequences. The selection cassette was cloned into the intronic sequences between the IIIa and IIIb exons of FgfR-2, and contained two genes driven by a herpes simplex virus (HSV) promoter: i) a neomycin resistance gene (HSV-neo), and ii) a thymidine kinase gene (HSV-tk) that confers sensitivity to gancyclovir. A further loxP site in the same orientation was cloned into the intron between exons IIIb and IIIc of FgfR-2 (Figure 5.1).

The targeting vector was electroporated into ES cells (derived from 129 strain inbred mice) and clones containing stably integrated vector were selected using G418 (neomycin). Neomycin resistant clones containing a homologously recombined allele were transiently transfected with a plasmid constitutively
expressing cre-recombinase. This enzyme catalyses the recombination of DNA between loxP sites through the binding of a single molecule of enzyme to each palindromic sequence. This allows a tetramer of recombinase molecules to form, bringing the two loxP sites together and resulting in the recombination of DNA between the spacer sequences of the sites. With the resulting excision a post-recombination single loxP site is also formed as a hybrid of the two original sites. Given that a specific 34 bp sequence would occur by chance every $10^{18}$ base pairs, and that the mammalian genome is only approximately $3 \times 10^9$ base pairs in length, it is highly unlikely that fortuitous loxP sites would be present in the genome of mice. Cre-recombinase mediated DNA excision is therefore only likely to occur between introduced recognition sites (reviewed in Nagy, 2000).

Transient expression of cre-recombinase in the targeted ES cells resulted in excision of the HSV-neo/HSV-tk selection cassette, and the generation of a conditional IIlb allele floxed by two unidirectional loxP sites (selected for on the basis of gancyclovir insensitivity). The recombined ES cells were injected into 4 day old mouse blastocysts and transferred to pseudopregnant foster mice (strain C57BL/6) to produce C57Bl/6 / 129 chimeric mice. Chimeric mice were then mated with wild type C57BL/6 mice and germline transmission of the conditional allele obtained (FgfR-2IIlb<sup>floxed</sup>). The non-conditional allele was generated by microinjecting the pronucleus of fertilised mouse eggs from C57BL/6 X FgfR-2IIlb<sup>floxed</sup> crosses with a cre-recombinase expressing plasmid, and then transferring these eggs to pseudopregnant mothers (Figure 5.1B).

The absence of the IIlb exon in homozygous non-conditional mice (referred to as FgfR-2IIlb<sup>+</sup>) did not affect splicing of the IIIc exon into FgfR-2, and these mice consequently showed normal expression patterns of FgfR-2IIIc during development. In addition, a novel splice isoform of FgfR-2 (from exon IIIa to exon TM) was generated as a result of exon IIIb removal. However, the resulting product of this splice variant was predicted to be severely truncated and unable to function as a signalling molecule due to the presence of a termination codon in the TM exon (De Moerlooze et al., 2000).
Strategy used to generate mice defective for FgfR-2IIIb, as described (De Moerlooze et al., 2000). A) Exon structure around the third Ig loop of FgfR-2 (see Figure 1.6) showing isoform specific splicing events (TM = exon encoding transmembrane domain). B) Simplified vector map containing mouse genomic DNA incorporating IIIa/b/c exons and surrounding introns shown in (A) used to target ES cells. Figure shows position of introduced selectable cassette and loxP sites (black triangles). HSV-neo allowed for the selection of targeted candidate homologous recombinant ES cell clones using G418. Transient expression of cre-recombinase in correctly recombined ES cells generated a conditional allele for the IIIb exon of FgfR-2 after selection for the loss of the cassette (HSV-tk) in the presence of gancyclovir. Injection of a cre-recombinase expression plasmid into fertilised eggs containing the conditional allele of FgfR-2IIIb allowed the generation of the non-conditional allele. The positions of genotyping primers (P1-P4) are shown.

5.2 Embryonic Mammary Gland Development

Mice containing the non-conditional deletion of the IIIb exon of FgfR-2 were used to investigate the role of FgfR-2IIIb in embryonic mammary development. This was achieved through comparative histological and in situ hybridisation analyses of mutant homozygous (FgfR-2IIIb−/−), and wild type (FgfR-2IIIb+/*) mice. As mice heterozygous for the disrupted allele (FgfR-2IIIb+/*) were shown to be
indistinguishable from wild type littermates, they were substituted for wild type embryos in some experiments (De Moerlooze et al., 2000).

5.2.1 Identification of FgfR-2IIIb<sup>−/−</sup> Mice

In order to generate homozygous mutant embryos (FgfR-2IIIb<sup>−/−</sup>) for analysis, breeding pairs of heterozygous (FgfR-2IIIb<sup>+/−</sup>) mutant mice were established. Interbreeding of these mice gave rise to litters containing the expected Mendelian ratio of all three possible genotypes: 25% wild type (FgfR-2IIIb<sup>+/+</sup>), 50% heterozygous mutants (FgfR-2IIIb<sup>+/−</sup>), and 25% homozygous mutants (FgfR-2IIIb<sup>−/−</sup>).

Embryos were dissected from pregnant female mice and placed into fixative. Genotyping was performed on genomic DNA isolated from digested yolk sacs using a standard PCR protocol (see sections 2.10.1b and 2.10.3). Two independent reactions, with annealing temperatures of 55°C, were performed using two separate pairs of primers (see Figure 5.1B). Primer P1 (annealing to sequences in the IIIb exon) and P2 (annealing to intronic sequences between exons IIIb and IIIc) were used to determine the presence of a wild type allele, and primers P3 (annealing to sequences within exon IIIa) and P4 (annealing to sequences in the loxP site between exons IIIb and IIIc) were used to determine the presence of a IIIb null allele. As homozygous mutant FgfR-2IIIb<sup>−/−</sup> embryos lacked limbs, had a curly tail, and were smaller than wild type and heterozygous littermates, genotyping could also be performed visually (De Moerlooze et al., 2000).

In all pregnancies, the morning the vaginal plug was identified was considered to be embryonic day 0.5. The age of all embryos was also confirmed by measuring the crown-rump distance (Kaufman, 1992). The sex of embryos obtained at E14.5 or E15.5 were determined, and only female embryos were used for experiments due to the sexual dimorphism of the mammary gland at these stages (see section 1.1.1a). Sex determination was carried out by PCR of yolk sac DNA, using primers ZfyF and ZfyR, to amplify a Y-chromosome specific fragment of DNA. An annealing temperature of 60°C was used with an elongation time of 2 minutes for these reactions (section 2.10.3).

5.2.2 Histological Identification of Embryonic Mammary Glands

To provide a histological picture of embryonic mammary development that would assist in the identification of glands for future experiments, E11.5, E13.5, and E15.5
wild type embryos were obtained and fixed overnight in NBF. The following day embryos were transferred to 70% ethanol, embedded laterally in paraffin wax, and sectioned in a sagittal plane at 5μm intervals through to the midline of the embryo. Sections were histologically stained with haematoxylin and eosin and mounted in DPX (see section 2.13.1).

Embryonic mammary glands were clearly identified as epithelial invaginations in the thoracic and inguinal regions, surrounded by condensed mesenchyme (Figure 5.2, see Figure 1.1 for schematic depiction). No major morphological differences were noted between glands within the same embryo.

5.2.3 Expression of FgfR-2IIIb in Embryonic Mammary Glands

In order to confirm the presence of FgfR-2IIIb in the mammary epithelium of E14 embryos (Cunha and Hom, 1996), in situ hybridisation was performed. Female wild type embryos at E14.5 were obtained as described above and fixed in 4% (w/v) paraformaldehyde in PBSA containing 0.1% (v/v) DEPC. Embryos were embedded in paraffin wax, sectioned as described above, and stored at 4°C. Every fifth slide was stained with haematoxylin and eosin, and these were used to identify embryonic mammary buds histologically under a Zeiss Axiophot microscope. Once identified, adjacent unstained sections were used for the in situ hybridisation experiments.

In addition, similar experiments were performed on mammary glands from wild type E11.5 and E15.5 female embryos. Glass microscope slides containing 5μm paraffin wax sections of these mammary glands were obtained in the same way as above, except that every third rather than fifth slide containing E11.5 embryo sections were histologically stained due to the smaller size of the glands.

A 1039 bp fragment of the intracellular region of FgfR-2 (nucleotides 1726-2764) encompassing the tyrosine kinase domain was used to generate a probe to detect expression of FgfR-2. This fragment had previously been cloned into pBluescript IISK+ using HindIII and BamHI to produce the plasmid FGFR2/pbluescript (De Moerlooze et al., 2000). 10μg FGFR2/pbluescript was digested using BamHI to linearise the plasmid at the 5' end of the FgfR-2 fragment. The digested plasmid was then separated through a 1% (w/v) agarose gel to check the digestion was complete, the linearised DNA was then gel extracted. An antisense [35S]-labelled riboprobe (referred to as TK) was then generated using T7 RNA polymerase, and used to detect FgfR-2 transcripts in the embryo sections.
Figure 5.2 - Histological analysis of early embryonic mammary development

Haematoxylin and eosin staining of embryonic mammary glands from E11.5, E13.5, and E15.5 embryo sections. The E15.5 mammary gland is shown labelled: mammary epithelium (ME), mammary mesenchyme (MM), and epidermis (E). Scale bar represents 100μm.
containing E11.5, E14.5, and E15.5 mammary glands (see section 2.13.5b, and see Appendix A6.3 for further details of all probes used for in situ hybridisation in this chapter). Hybridised TK probe was left to expose photographic emulsion on the slides for two weeks before developing. Exposure time was estimated by developing a test slide containing adjacent tissue sections one week after dipping the slides into photographic emulsion, and then using this to judge the strength of the exposed signal. Hybridised slides were mounted in DPX and digital images obtained in bright- and darkfield.

As can be seen in Figure 5.3A, a strongly positive radioactive signal representing FgfR-2 expression, identifiable as black dots in the brightfield image and white dots in the darkfield image, is present in the mammary epithelium, but not in the mesenchymal cell layers. A strong signal was also observed in the basal keratinocytes surrounding the mammary bud in agreement with previous findings (De Moerlooze et al., 2000).

FgfR-2 expression was also detected in the mammary epithelium of E11.5 and E15.5 placodes/buds (Figure 5.3B). This suggests that FgfR-2 may play a functional role in the earlier stages of embryonic mammary development during placode induction and maturation, as well as during the latter stages of the ‘resting phase’. On closer examination it is apparent that the highest levels of FgfR-2 expression in the E14.5 and E15.5 mammary glands locates to the epithelial cells that are adjacent to the underlying mesenchyme at the edge of the bud.

The TK probe is directed against the invariant intracellular tyrosine kinase domain of FgfR-2 and therefore does not distinguish between the IIIb and IIIc spliced isoforms of the gene. However, the IIIb isoform has previously been shown to be expressed in epithelial cell lineages, whereas the IIIc isoform is expressed in some cells of mesenchymal origin (Orr-Urtreger et al., 1993). It is therefore likely that the TK probe is hybridising to FgfR-2IIIb transcripts in the mammary epithelium in agreement with the published data (Cunha and Hom, 1996). No discernable differences in FgfR-2 expression were detected between thoracic and inguinal glands (data not shown).

To confirm that the TK probe was hybridising to RNA transcripts of the IIIb, and not the IIIc isoform of FgfR-2 in the mammary epithelium, in situ hybridisation was performed on sections containing E14.5 mammary glands using [³⁵S]-labelled IIIb or IIIc specific FgfR-2 riboprobes generated from the IIIb and IIIc exons of FgfR-2 respectively (referred to as 'IIIb' or 'IIIc' probe). Both isoform specific probes have
In situ hybridisation of embryo sections containing embryonic mammary glands using a radio-labelled TK probe directed against the cytoplasmic domain of FgfR-2

A) Bright- and darkfield images identifying FgfR-2 transcripts in the mammary epithelium and surrounding epidermis in E14.5 embryos. No detectable expression of FgfR-2 is observed in the mammary mesenchyme.

B) FgfR-2 expression in a mammary epithelial placode at E11.5, and a mammary epithelial bud at E15.5. Expression in the bud is greatest in the cells directly adjacent to the underlying mammary mesenchyme. Bright- and darkfield images are shown. Scale bar represents 100μm.
Figure 5.4 - Identification of the Illb isoform of FgfR-2 in the embryonic mammary gland

In situ hybridisation of E14.5 mammary glands showing a strong signal in the mammary epithelium and epidermal layers with a probe directed against FgfR-2Illb (A). A much weaker signal is observed in adjacent tissue sections using a probe directed against the Illc isoform of FgfR-2 (B). Bright- and darkfield images are shown. Scale bar represents 100µm.
been previously characterised to identify sites of known \( FgfR-2\)IIlb or \( FgfR-2\)IIlc expression in other tissues, and furthermore, the \( IIlb \) probe does not produce a positive signal in \( FgfR-2\)IIlb\(^+\) mice (De Moerlooze et al., 2000; Kettunen et al., 1998; Revest et al., 2001a; Revest et al., 2001b).

Figure 5.4A shows a strongly positive signal in the mammary epithelium of sections hybridised with the probe against \( FgfR-2\)IIlb. The intensity of this signal is a little weaker than the signal obtained in adjacent E14.5 embryo sections using the TK probe (compare with Figure 5.3A). Nevertheless, the pattern of expression overlaps precisely, and the differences in signal intensity are probably reflective of the difference in the sizes of the two probes. Strangely, a very weak signal in the mammary epithelium was also obtained using the \( IIlc \) probe (Figure 5.4B). However, as \( FgfR-2\)IIlc is not normally expressed in epithelial cell lineages this signal probably represents a small degree of non-specific hybridisation with a gene expressed in epithelial cells, such as \( FgfR-2\)IIlb. Alternatively it may merely reflect a higher level of background signal in areas of higher cell density. These results indicate that the TK probe is detecting the \( IIlb \) isoform of \( FgfR-2 \) in the mammary epithelium, and also suggest that the \( IIlc \) isoform of \( FgfR-2 \) is not expressed in the mammary gland at appreciable levels.

### 5.2.4 Agenesis of Mammary Glands in \( FgfR-2\)IIlb\(^+\) Mice

#### 5.2.4a Histological analysis of \( FgfR-2\)IIlb\(^+\) mammary development

The \textit{in situ} hybridisation analysis described above reveals that \( FgfR-2\)IIlb is expressed in the mammary epithelium from the beginning of placode formation (E11.5) to at least E15.5, just prior to epithelial sprouting. In order to determine whether loss of both copies of \( FgfR-2\)IIlb affects the development of the mammary gland, \( FgfR-2\)IIlb\(^+\) and littermate wild type mice were obtained on day 14.5 of gestation. Embryo sections were prepared as described in section 5.2.2, and then examined under a light microscope to determine if there were any obvious differences in the morphology of the mammary glands.

As expected, all 5 mammary glands could be identified along each flank of wild type embryos. Surprisingly, analysis of tissue sections from E14.5 \( FgfR-2\)IIlb null mice failed to identify any structures resembling E14.5 mammary glands, suggesting that they fail to develop in the absence of \( FgfR-2\)IIlb (Figure 5.5A). \( FgfR-2\)IIlb\(^+\) mice are smaller than wild type mice of an equivalent stage, and show a slight delay in their development. However, as structures characteristic of the
Figure 5.5 - Analysis of E14.5 FgfR-2lllb^-/- mammary development

A) Haematoxylin and eosin stained embryo sections showing position of a thoracic mammary bud (M) in wild type embryos (left panel). No mammary buds were detected in FgfR-2lllb null embryos at the same stage (right panel). The position of a whisker follicle (W), and the rib cage (R) are marked for orientation. Scale bar represents 0.5mm

B) In situ hybridisation of embryo sections from E14.5 FgfR-2lllb^-/- and FgfR-2lllb^-/- embryos using an antisense Lef-1 riboprobe. Representative bright- and darkfield images are shown. Scale bars represent 200µm (A) and 100µm (B).
earlier stages of mammary development were not detected in E14.5 sections of 
FgfR-2IIIb<sup>−/−</sup> mice, it appears that loss of this gene results in the absence (agensis) of 
mammary glands, and not in a delay of their development. Analysis of serially 
sectioned E11.5, E12.5 and E13.5 FgfR-2IIIb null embryos stained with haematoxylin 
and eosin also failed to detect mammary placodes (data not shown).

5.2.4b Analysis of FgfR-2IIIb<sup>−/−</sup> mammary development by Lef-1 in situ 
hybridisation

The HMG family transcription factor Lef-1 has been shown to be important for 
embryonic mammary development and can be used as a molecular marker to detect 
mammary epithelium during the early stages of mammary morphogenesis (van 
Genderen et al., 1994), and mammary mesenchyme after E15 (Foley et al., 2001). To 
assay for the presence of mammary tissue in FgfR-2IIIlb null mice, FgfR-2IIIlb<sup>−/−</sup> 
embryos at E14.5 were prepared for in situ hybridisation. An antisense [<sup>35</sup>S]-labelled 
Lef-1 riboprobe was generated using a cDNA fragment of Lef-1 that had been cloned 
into a pBluescript vector (gift from Dr. P. Kettunen). Embryo sections containing 
mammary glands from wild type littermates acted as experimental controls. A 
number of sagittal sections between the midline and lateral flank of the FgfR-2IIIlb 
null embryos were used to ensure that areas of prospective mammary tissue were 
included. Sections hybridised with the antisense Lef-1 riboprobe were exposed to 
photographic emulsion for 2 weeks.

Figure 5.5B shows the results of the in situ hybridisation experiments. A strong 
positive signal, indicating the presence of high levels of Lef-1 transcription, were 
present in the mammary epithelium of wild type (FgfR-2IIIlb<sup>+/+</sup>) mice in agreement 
with previous findings (van Genderen et al., 1994). Lef-1 was also detected in the 
basal keratinocyte layer. Although Lef-1 expression was detected in the 
kera
tinocytes of FgfR-2IIIlb<sup>−/−</sup> mice, no strong patches of positive hybridisation signal 
that could indicate the presence of a mammary epithelium were detected at E14.5. 
These results agree with the histological analysis of FgfR-2IIIlb<sup>−/−</sup> null mice, and 
indicate that mammary glands fail to develop in the absence of a functional 
FgfR-2IIIlb receptor. A similar result was obtained using an [<sup>35</sup>S]-labelled TK 
riboprobe as a marker for mammary development (data not shown).
5.2.5  *FgfR-2IIIb* is Required for the Maintenance But Not Induction of an Inguinal Mammary Placode

5.2.5a  Wholemount study of *Lef-1* expression

The *Lef-1* expression experiments described in the previous section gave a snapshot of mammary development in individual sections of E14.5 wild type and *FgfR-2IIIb* null embryos. However, there was a possibility that mammary tissue (positive for *Lef-1*) had developed in the null embryos, but had been missed in both this experiment, and in the histological analysis of haematoxylin and eosin stained embryo sections (section 5.2.4). In order to give an overall view of mammary development in wild type and *FgfR-2IIIb* null embryos, a wholemount *in situ* hybridisation experiment was performed to mark positions of *Lef-1* expression. Wholemount *in situ* hybridisation analysis is an ideal technique to visualise the developing mammary glands of an embryo, as their proximity to the surface of the skin allows them to be easily identified without the need for dissection. Moreover, this proximity circumvents any potential problems associated with the penetration of the riboprobes and other reagents during the hybridisation.

*FgfR-2IIIb*/*FgfR-2IIIb* embryos at E11.5, E12.5, E13.5, and E14.5 were obtained by the interbreeding of *FgfR-2IIIb*/*FgfR-2IIIb* parents. Two embryos of each genotype and developmental stage were fixed in 4% (w/v) paraformaldehyde in PBSA overnight and then dehydrated through a series of graded methanol/PBS-T steps to 100% methanol. A digoxigenin-labelled *Lef-1* antisense riboprobe was then generated as described in Materials and Methods section 2.13.5a. Wholemount *in situ* hybridisation was performed using 100ng/ml of *Lef-1* riboprobe, and littermate wild type and *FgfR-2IIIb* null embryos were grouped together throughout the experiment to ensure similar experimental conditions. Embryos were digested in proteinase K (10μg/ml) in PBS-T for the following times prior to hybridisation: E11.5 = 30 minutes, E12.5 = 35 minutes, E13.5 = 45 minutes, and E14.5 = 50 minutes. Embryos were developed in the dark for two hours at room temperature to visualise regions of *Lef-1* expression (see section 2.13.5a).

Figure 5.6 shows the results of the wholemount *Lef-1 in situ* hybridisation experiments. At E11.5 *Lef-1* was detected with a similar expression pattern in both the wild type and *FgfR-2IIIb* null embryos. Prominent *Lef-1* expression was apparent in the snout of the embryo, consistent with its previously defined expression pattern in the mesenchyme of the snout at E12.5 (van Genderen et al., 1994). Expression of *Lef-1* could also be detected at the tip of the tail in both
genotypes (hidden in the wild type embryo), and at the tips of the limb buds in wild type mice. Limited expression was also detected in the mesencephalon and hindbrain. No strong patches of Lef-1 positive cells, indicating the presence of mammary placodes, could be detected at E11.5 in the thoracic and inguinal regions along the lateral flank of the embryos. However, a closer examination of the wild type embryo (top left panel) reveals some Lef-1 positive cells in the middle of the flank between the fore- and hindlimb (black arrowhead, Figure 5.6). This area of Lef-1 expression correlates well with the position of mammary placode number 3 (see Figure 1.1, and Mailleux et al., 2002), and could be marking the immature placode.

In the wild type embryo at E12.5, a pattern of Lef-1 expression similar to that obtained at E11.5 was detected, however, all 10 mammary placodes were also clearly visible as round patches of Lef-1 positive cells (see Figure 5.6, mammary placodes 1 and 5 are hidden by the limbs). Thus, Lef-1 can be used as a molecular marker for identifying mammary glands in both embryo sections (using radiolabelled antisense riboprobes), and also in whole embryos (using digoxigenin-labelled antisense riboprobes). In the FgfR-2IIIb<sup>−/−</sup> embryos Lef-1 expression was detectable at the tip of a rudimentary hindlimb bud, however no clusters of positive cells were detected along the flank of the embryo. This finding is in agreement with the histological analysis of FgfR-2IIIb<sup>−/−</sup> embryo sections described in section 5.2.4a.

At E13.5, expression of Lef-1 remains strong in the mammary buds of wild type mice (black arrowheads, Figure 5.6). In addition, expression is maintained in the snout and the tips of the tail and limbs. Further expression of Lef-1 was detectable in the interdigital tissue of the limbs, the ear, and in the follicles of the whiskers (vibrissae) in agreement with the published findings (van Genderen et al., 1994). In the FgfR-2IIIb<sup>−/−</sup> embryo, Lef-1 is expressed in a similar set of tissues with the exception of the limb buds, which have degenerated. Notably, Lef-1 can be detected in the whisker follicles at this stage which are derived through a similar epithelial budding process to the mammary gland. Close examination of the flank of the embryo revealed no thoracic mammary glands as determined by Lef-1 expression. Surprisingly, a halo of Lef-1 expression (white arrowhead, Figure 5.6) was observed in the region where the number 4 inguinal mammary gland would be expected to develop. This halo was not detectable a day earlier (E12.5), and had disappeared by E14.5, suggesting that an inguinal mammary placode may form transiently in the absence of FgfR-2IIIb. If this locus of Lef-1 expression represents a rudimentary mammary placode, then it is not clear why it is halo- rather than the normal disc-shape. It is possible that the halo-shape represents a mammary gland that is
Figure 5.6 - Lef-1 wholemount in situ hybridisation analysis of embryonic mammary development

Wholemount in situ hybridisation of E11.5, E12.5, E13.5, and E14.5 embryos using an antisense riboprobe against Lef-1. FgfR-2IIIb+/+ and FgfR-2IIIb−/− littermates are shown. Visible mammary placodes/buds are shown (black arrowheads). The location of a possible inguinal mammary bud is indicated, and is magnified in the dotted box (white arrowhead). Also magnified is an E14.5 wild type mammary placode. Scale bar represents 1mm.
degenerating; this would agree with the histological analysis described in section 5.2.4a which could not identify any structures resembling embryonic mammary glands. Alternatively, the halo shape may suggest a mesenchymal rather than epithelial pattern of expression for Lef-1 in this region.

Expression of Lef-1 at E14.5 extended to the hair follicles in the wild type embryo which complicated the identification of the mammary placodes. Nevertheless, at this stage of development the Lef-1 positive cells in the mammary gland remained clustered in a round shape, suggesting that Lef-1 was still expressed in the mammary epithelium and had not yet shifted to the mammary mesenchyme as reported (Foley et al., 2001). No hair follicles were detected by Lef-1 expression in the FgfR-2IIIb" embryo at this stage, although the whisker follicles were strongly positive. No further circular patches of Lef-1 expression indicative of mammary epithelium were seen along the flank of the FgfR-2IIIb" embryo.

Taken together these findings suggest that inguinal gland number 4, but no thoracic mammary glands develop in the absence of FgfR-2IIIb signalling. This mutant inguinal mammary placode is induced after E12.5 (as determined by expression of Lef-1), but prior to E13.5. No mammary placodes were identified in FgfR-2IIIb null embryos by histological analysis of sagittal embryo sections from E11.5, E12.5, or E13.5 embryos (section 5.2.4a). This suggests that the putative mammary placode develops extremely transiently and degenerates shortly after its induction.

5.2.5b Apoptosis causes the degeneration of the inguinal mammary placode in FgfR-2IIIb" embryos.

In order to determine whether an inguinal mammary placode developed in the absence of FgfR-2IIIb, embryos null for this gene were obtained at various stages between E12.5 and E13.5. Embryos sections were prepared and histologically stained with haematoxylin and eosin. Analysis of these slides under a light microscope identified an area containing a mass of epithelial cells surrounded by condensed mesenchyme in the inguinal region of the FgfR-2IIIb null embryo, that resembled a mammary bud. This putative mammary bud was only identified in two embryos at around E13 and could not be detected in the thoracic region, suggesting that its formation was both extremely transient, and limited to the inguinal region. Inguinal and thoracic mammary buds were easily identifiable in embryo sections of littermate wild type control embryos. In contrast to wild type mammary placodes at this stage of development, the mutant inguinal placode in
the ~E13 FgfR-2IIIb null embryo appeared to be detached from the epidermal surface and was surrounded by mesenchymal tissue.

The transient appearance of the inguinal mammary placode in FgfR-2IIIb null mice suggests that it degenerates shortly after its formation. A TUNEL assay was performed on paraffin embedded embryo sections, containing the mutant inguinal mammary placode, to detect the presence of apoptotic cells (see section 2.13.4). As an experimental control, sections containing mammary placodes from wild type littermate mice were used. As can be seen in Figure 5.7, the wild type mammary placode did not appear to contain any apoptotic cells whereas the mutant inguinal placode was undergoing extensive apoptosis. The apoptotic cells (as identified by their brown nuclear staining) were present predominantly within the epithelial compartment of the placode, and presumably cause the complete degeneration of the gland within a few hours.

Taken together, the data presented in this section demonstrates that an inguinal mammary placode develops in the FgfR-2IIIb⁺ mouse. This placode develops approximately a day later than wild type placodes containing a functional FgfR-2IIIb gene, and is destroyed by apoptosis soon after its induction at around E13. Thus, FgfR-2IIIb is required for the induction of the thoracic mammary placodes, but is not required for the induction of an inguinal mammary placode. However, the maintenance and/or further development of this placode is dependent on signalling through FgfR-2IIIb.

5.2.6 Expression of Fgf-3, Fgf-7 and Fgf-10 in the Embryonic Mammary Gland

The requirement for FgfR-2IIIb expression in the mammary gland has been demonstrated through the analysis of mammary development in FgfR-2IIIb null embryos. This reflects the requirement for activation of FgfR-2IIIb, and the initiation of downstream signalling cascades by specific ligands of the receptor in mammary gland development. Fgfs-1, -3, -7, and -10 have all been shown to activate FgfR-2IIIb in vitro and so are likely candidates to act as ligands for FgfR-2IIIb in the mammary gland during embryogenesis (Lu et al., 1999; Ornitz et al., 1996). Analysis of gene expression has shown that Fgf-1 is not expressed in the mammary gland (Mailleux et al., 2002), and Fgf-7 is expressed in the mammary mesenchyme at E14 (Cunha and Hom, 1996). The expression patterns of Fgf-3 and Fgf-10 in the embryonic mammary gland have not been characterised to date. An in situ hybridisation analysis of Fgf-3, Fgf-7 and Fgf-10 expression in the mammary
Figure 5.7 - Apoptosis in the mutant FgfR-2IIib/- inguinal mammary placode

TUNEL assay to detect apoptotic cells in wild type (FgfR-2IIib+/+), and FgfR-2IIib/- mammary placodes. The brown nuclei indicate cells that are undergoing apoptosis and are only detected in the mutant FgfR-2IIib/- mammary placode epithelium. A higher magnification image of each gland is shown (right panels). Scale bars represent 50μm (left panels) and 25μm (right panels).
gland was therefore undertaken using [³⁵S]-labelled riboprobes and embryo sections of wild type E14.5 embryos containing mammary buds.

[³⁵S]-labelled antisense riboprobes were generated as described in section 2.13.5b using plasmids containing cDNA fragments of Fgf-3, Fgf-7, and Fgf-10 (see Appendix A6.3 for further details).

The analysis shows that at E14.5 in the wild type embryo, neither Fgf-3 nor Fgf-10 are expressed at detectable levels in the mammary gland (Figure 5.8). However, Fgf-3 and Fgf-10 transcripts were detected in the brain and hair follicles respectively providing a positive control for the hybridisation (data not shown). In agreement with the published findings, the Fgf-7 antisense probe gave a weakly positive signal in the mesenchyme surrounding the mammary epithelium (Cunha and Hom, 1996). Importantly, Fgf-7 appears to be expressed in a complementary pattern to FgfR-2IIIb suggesting that the ligand and receptor could participate in signalling through a paracrine mechanism in the mammary gland (compare Figure 5.8 and Figure 5.4).

As a requirement for FgfR-2IIIb signalling in mammary development appears to be at the early stages of placode formation, the expression of Fgf-7 and Fgf-10 was investigated in E12.5 and E11.5 mammary glands respectively using in situ hybridisation with specific antisense riboprobes on embryo sections. To further expand the expression data of these genes in the mammary gland, embryo sections containing E15.5 mammary glands were included in the experiment.

Hybridisation of E12.5 wild type embryo sections with the antisense Fgf-7 riboprobe revealed a high level of expression in the mesenchyme surrounding the mammary epithelial bud (Figure 5.9A). Its proximity to the site of FgfR-2IIIb expression in the epithelial bud (see Figure 5.3 and Figure 5.4) suggests that Fgf-7 may also be a paracrine mediator of FgfR-2IIIb signalling in the mammary gland at this stage of development. Hybridisation of the Fgf-7 probe to sections from E15.5 wild type embryos failed to detect expression in the mammary mesenchyme. Thus Fgf-7 is highly expressed during placode formation in the mammary mesenchyme, but then expression decreases by E14.5 and is undetectable by E15.5.

The expression analysis of Fgf-10 was performed on sections from E11.5 and E15.5 wild type embryos. At neither stage could Fgf-10 be detected at high levels in the mammary mesenchyme (see Figure 5.9B), however a strong positive signal was detected in the hair follicles at E15.5 adjacent to the mammary mesenchyme. As the hair follicles are known to express Fgf-10, this acted as a control for detecting Fgf-10 expression (Suzuki et al., 2000). A weakly positive Fgf-10 signal was detected at
Figure 5.8 - Expression of Fgf-3, Fgf-7, and Fgf-10 in E14.5 mammary glands

In situ hybridisation analysis of gene expression in wild type E14.5 mammary glands using antisense radio-labelled riboprobes against Fgf-3, Fgf-7, and Fgf-10. Bright- and darkfield images are shown. Scale bar represents 100µm.
Figure 5.9 - Expression of *Fgf-7* and *Fgf-10* in the embryonic mammary gland

**A**

*Fgf-7*

E12.5  
Brightfield  Darkfield

E15.5  
Brightfield  Darkfield

**B**

*Fgf-10*

E11.5  
Brightfield  Darkfield

E15.5  
Brightfield  Darkfield

*In situ* hybridisation analysis of gene expression in embryo sections containing wild type mammary glands at the stages indicated, using antisense radio-labelled riboprobes against *Fgf-7* and *Fgf-10*. Bright- and darkfield images are shown. HF = hair follicle. Scale bar represents 100µm.
E15.5 in the mammary fat pad precursor tissue underlying the condensed mammary mesenchyme.

These results show that mesenchymally expressed Fgf-7 may act as a ligand initiating FgfR-2IIIb signalling in the embryonic mammary gland.

5.2.7  *Fgf-8* Is Not Expressed in the Embryonic Mammary Placodes Between E11.5 and E13.5

During the development of some organs that require FgfR-2IIIb signalling, Fgf-8 has been shown to be expressed and required for organogenesis. For instance, in the developing limb bud both FgfR-2IIIb and Fgf-8 are expressed in a group of specialised epithelial cells, the apical ectodermal ridge (AER), that act as a signalling centre at the tips of the limb bud to control anterior-posterior patterning of the limb (Martin, 1998; Revest et al., 2001a). In tooth morphogenesis, which shares a similar process of ectodermal budding to the mammary gland during its development, both FgfR-2IIIb and Fgf-8 are expressed in the dental ectoderm (Bei and Maas, 1998; Kettunen et al., 1998; Neubuser et al., 1997). To determine whether Fgf-8 may play a role in the development of the embryonic mammary gland, an analysis of *Fgf-8* expression was undertaken by wholemount in situ hybridisation. Given that *Fgf-8* is expressed during the early phases of limb and tooth development, the expression of *Fgf-8* was assessed during the initial stages of mammary development in wild type embryos between E11.5 and E13.5. A digoxigenin-labelled antisense *Fgf-8* probe was kindly provided by Dr. J.M. Revest (Cancer Research UK, London, Revest et al., 2001a). After hybridisation with the antisense *Fgf-8* probe, embryos were developed for approximately two hours in the dark as described in section 2.13.5a.

The results of the wholemount analysis of *Fgf-8* expression are shown in Figure 5.10. As can be seen, the AER at the tips of the limb buds stained strongly positive for *Fgf-8* expression at all the stages of development analysed, consistent with its role in limb bud development. *Fgf-8* expression was also detected in the brain. However, no positive signals were detected in the mammary placodes along the flank of the embryos between the hind- and forelimb suggesting that, at least between E11.5 and E13.5, *Fgf-8* is not required for the normal development of the mammary gland.
Figure 5.10 - Expression of Fgf-8 in the embryonic mammary gland

Wholemount in situ hybridisation of E11.5, E12.5, and E13.5 wild type embryos using an antisense probe against Fgf-8. Scale bar represents 1 mm.
5.3 Postnatal Mammary Gland Development

Mice containing the conditional mutation of FgfR-2IIIb were used to study the role of FgfR-2IIIb signalling in the development of the postnatal mammary gland (see Figure 5.1). As the IIIb exon in the third Ig-loop of FgfR-2 in these mice was flanked by unidirectional loxP sites rather than deleted, the mice were able to produce a functional FgfR-2IIIb receptor, and were consequently viable and phenotypically indistinguishable from wild type mice (De Moerlooze et al., 2000).

The aim of this work was to assess the role of FgfR-2IIIb signalling in postnatal mammary development by deleting the IIIb exon of FgfR-2IIIb to abrogating FgfR-2IIIb signalling within the mammary gland of adult mice, whilst maintaining a fully-functional FgfR-2IIIb in non-mammary tissues. To achieve this, mice containing the conditional floxed allele of FgfR-2IIIb were crossed with transgenic mice expressing cre-recombinase under the control of a mammary gland specific promoter.

5.3.1 Expression of FgfR-2 in the Adult Mammary Gland

To assess the temporal and spatial expression pattern of FgfR-2 in the adult mammary gland, an in situ hybridisation analysis of mammary gland sections was undertaken. Mammary glands were dissected from female wild type mature virgin, 7.5 day, and 13.5 day pregnant mice. In addition, mammary glands were obtained from wild type mice that were 1 day post-partum, and also from mice with involuting mammary glands (sacrificed 7 days after the pups had weaned). All mammary glands were dissected from the left hand side of the mouse (number 4 inguinal gland), and fixed overnight at 4°C in 4% (w/v) paraformaldehyde in PBSA that had been treated with 0.1% (v/v) DEPC. 5μm mammary gland sections were then prepared through the middle of the gland, and some sections were stained with haematoxylin and eosin to check that mammary epithelium was contained in the sections. Adjacent sections were then dewaxed and hybridised with an antisense [35S]-labelled TK riboprobe to detect FgfR-2 expression (see sections 2.13.5b and 5.2.3).

The expression analysis shows a strong positive signal indicating the presence of FgfR-2 transcripts in the ductal epithelial cells of the mature virgin mammary gland (Figure 5.11). This signal was present in the epithelia throughout all portions of the gland examined (data not shown). No expression of FgfR-2 could be detected in the
mammary fat pad or mammary mesenchyme surrounding the ductal epithelia, strongly suggesting that the TK probe detects the expression of the epithelially expressed IIIb, and not the mesenchymally expressed IIIc isofom of FgfR-2. The intensity of the FgfR-2 signal in the mammary epithelium decreased progressively during pregnancy indicating that FgfR-2IIIb expression is downregulated during alveolar and lobulo-alveolar development. FgfR-2 expression could not be detected following birth (1 day post-partum) when the mammary gland is fully differentiated and the epithelial component of the gland fills the majority of the mammary fat pad. However, during involution when the mammary gland undergoes extensive epithelial apoptosis and ductal remodelling, FgfR-2 transcripts were clearly detected with the antisense TK riboprobe. The expression of FgfR-2IIIc was also assessed in adjacent mammary sections using an antisense IIIc probe (see section 5.2.3), as expected no positive signal was detected (data not shown).

These results agree with previous findings using RNase protection assay and RT-PCR to show that FgfR-2IIIb is expressed in the epithelial compartment of the adult mammary gland (Pedchenko and Imagawa, 2000).

5.3.2 Conditional Deletion of FgfR-2IIIb by Transgenic Cre-Recombinase Expression

A large number of transgenic mice have been generated that express the cre-recombinase gene from a diverse set of promoters (see http://www.mshri.on.ca/nagy/Cre-pub.html for a database of published cre-recombinase expressing transgenic mice). By crossing these transgenic mice with mice containing a gene flanked by loxP sites, the gene of interest can be deleted in a pattern that reflects the activity of the transgenic promoter (for review see Nagy, 2000).

This strategy was used to assess the role of FgfR-2IIIb in the adult mammary gland. Mice containing the conditional mutation of FgfR-2IIIb were crossed with a transgenic mouse line expressing cre-recombinase under the regulatory control of the whey acidic protein promoter (WAP-Cre mice, kind gift from Dr. Lothar Hennighausen, NIH, Bethesda). The WAP-Cre mice were shown by RT-PCR to express cre-recombinase specifically in the adult mammary gland, in a pattern that resembled the endogenous WAP gene (Wagner et al., 1997). Thus deletion of FgfR-2IIIb in the mammary ductal and alveolar epithelium was predicted to commence at around day 14 of pregnancy, concurrent with the expression of the endogenous WAP gene, and to persist through late pregnancy and lactation. This time frame of
Figure 5.11 - Expression of FgfR-2 in the adult mammary gland

In situ hybridisation analysis of FgfR-2 expression in adult mammary gland sections using an antisense TK probe that recognises the tyrosine kinase domain of FgfR-2. FgfR-2 expression during different stages of mammary gland development are shown in bright- and darkfield: virgin, 7.5 days pregnant, 13.5 days pregnant, 1 day post-partum (1 day PP), and involuting glands. Scale bar represents 100 μm.
cre-recombinase expression coincides with the developmental stage at which a defect in mammary gland development is noticeable when FgfR-2IIIb signalling is perturbed with a dominant negative receptor (Jackson et al., 1997).

5.3.2a Identification of WAP-Cre transgenic mice

Mice were genotyped for the presence of the WAP-Cre transgene using genomic DNA isolated from tail snips as described in Materials and Methods. PCR was used to amplify a fragment of DNA specific to the transgene using primers that annealed to sequences in the WAP promoter and cre-recombinase gene (primers: WAPScreen and CreScreen respectively). PCR was performed using a standard protocol described in section 2.10.3 using an annealing temperature of 50°C. WAP-Cre mice appeared phenotypically normal and developed mammary glands that were morphologically indistinguishable from wild type glands (data not shown).

5.3.2b Identification of FgfR-2IIIb^loxP/loxP^ mice

Genotyping of mice to ascertain the presence of the IIIb exon of FgfR-2 floxed with loxP sites was achieved by PCR using genomic DNA isolated from mouse tail snips (see sections 2.10.1a and 2.10.3). Primers P1 and P2 were used to amplify a DNA fragment between the IIIb exon and the intronic sequences upstream of the IIIc exon (see Figure 5.1). The resulting products of the PCR potentially gave two bands when separated by electrophoresis on a 1% (w/v) agarose gel: a band of 320 bp corresponded to a wild type FgfR-2IIIb allele, and a 420 bp band corresponded to the conditional floxed allele of FgfR-2IIIb. If only one band of the correct size was visible on the agarose gel, then the mouse was homozygous for the wild type or floxed allele of FgfR-2IIIb (FgfR2-IIIb^loxP/loxP^ or FgfR2-IIIb^loxP/loxP^ respectively). If however two bands were present at the correct size on the agarose gel, then the mouse was heterozygous, containing one copy of the wild type, and one copy of the conditional floxed allele (FgfR2-IIIb^loxP/loxP^). 5.3.2c Mouse breeding strategy

Breeding pairs of FgfR-2IIIb^loxP/loxP^ mice were established to obtain progeny of the genotype FgfR-2IIIb^loxP/loxP^. Male or female mice containing this genotype were then crossed with WAP-Cre^+ transgenic mice and the litters screened to obtain mice of the genotype WAP-Cre^+ .FgfR-2IIIb^loxP/loxP^ . These mice were then interbred or back-crossed to FgfR-2IIIb^loxP/loxP^ mice to generate mice with the genotype WAP-Cre^+ .FgfR-2IIIb^loxP/loxP^ . Finally, WAP-Cre^+ .FgfR-2IIIb^loxP/loxP^ mice were bred with FgfR-2IIIb^+ mice (heterozygous mice containing the non-conditionally deleted
allele of FgfR-2IIlb, see section 5.2.1). The female progeny from these litters with the genotype WAP-Cre*.FgfR-2IIlb\textsuperscript{floxed/-} were used for the analysis of mammary development described in the next section as they were positive for the WAP-Cre transgene, and only contained one functional floxed copy of the IIlb exon of FgfR-2. Thus, the transgenic expression of cre-recombinase under the control of the WAP promoter in these mice would potentially lead to the genotype WAP-Cre*.FgfR-2IIlb\textsuperscript{-/-} within the mammary epithelial cells, whilst retaining the genotype WAP-Cre*.FgfR-2IIlb\textsuperscript{floxed/-} in all other cells of the body. As an experimental control for the effects of cre-recombinase mediated gene deletion, littermate or age matched female mice with the genotypes WAP-Cre*.FgfR-2IIlb\textsuperscript{-/-} or WAP-Cre*.FgfR-2IIlb\textsuperscript{floxed/-} were used. These control mice would always retain at least one functional allele of FgfR-2IIlb, even after DNA recombination, and no discernible differences could be detected in mammary gland development in these mice when compared with wild type mice (data not shown).

5.3.2d Analysis of mammary development in WAP-Cre*.FgfR-2IIlb\textsuperscript{floxed/-} mice

In order to examine the effect of transgenic cre-recombinase expression from the WAP promoter in mice carrying a single floxed copy of the IIlb exon of FgfR-2, the mammary glands of WAP-Cre*.FgfR-2IIlb\textsuperscript{floxed/-} and control mice were analysed. Mature female WAP-Cre*.FgfR-2IIlb\textsuperscript{floxed/-} and control mice were mated with available male mice and sacrificed at 11.5 days and 15.5 days gestation, and 2 days postpartum. In addition, mature virgin WAP-Cre*.FgfR-2IIlb\textsuperscript{floxed/-} and control mice were sacrificed. The number 4 right hand side inguinal gland was dissected from sacrificed mice and stained with a carmine dye as described in section 2.13.2. The number 4 left hand side inguinal gland was also dissected and fixed in NBF for a histological analysis of gland morphology as described in section 2.13.1.

As can be seen from the wholemount and histological preparations of virgin and 11.5 day pregnant mammary glands, no gross differences can be detected between WAP-Cre*.FgfR-2IIlb\textsuperscript{floxed/-} and control mice (Figure 5.12 and Figure 5.13). This result was expected despite the high levels of FgfR-2 expression in the virgin mammary gland (see Figure 5.11), as the cre-recombinase transgene is not expressed until late pregnancy (Wagner et al., 1997). Excision of the single floxed IIlb exon in WAP-Cre*.FgfR-2IIlb\textsuperscript{floxed/-} mice would be expected to start at around day 14 and increase towards the end of pregnancy and lactation. However, wholemount and histological analysis of WAP-Cre*.FgfR-2IIlb\textsuperscript{floxed/-} mammary glands during the late stage of pregnancy or lactation (15 days gestation and 2 days postpartum) revealed no visually noticeable morphological differences when compared with control
Figure 5.12 - Wholemount analysis of WAP-Cre⁺,FgfR-2ІІіb<sup>floxed⁻</sup> mammary glands

The number 4 right hand side inguinal mammary gland was dissected from WAP-Cre⁺,FgfR-2ІІіb<sup>floxed⁻</sup> and control mice from various stages of development: virgin, days 11.5 and 15.5 of gestation, and 2 days postpartum (2 days PP). Glands were fixed and stained with carmine stain. Scale bar represents 1mm.
The number 4 left hand side inguinal mammary gland was dissected from WAP-Cre⁺.FgfR-2IIibfloxed/- and control mice from various stages of development: virgin, days 11.5 and 15.5 of gestation, and 2 days postpartum (2 days PP). Glands were fixed, paraffin embedded, sectioned and stained with haematoxylin and eosin. Scale bar represents 100µm.
glands (Figure 5.12 and Figure 5.13). A similar result was obtained when mice containing the transgene were crossed with mice homozygous for the floxed IIIb exon (WAP-Cre^*.FgfR-2IIlb^floxed/floxed, data not shown). This could signify that FgfR-2IIlb is not required for mammary development during the stages of pregnancy when the WAP promoter becomes active, or that there is some redundancy of function with other Fgf receptors. The absence of an effect on mammary development may also be due to non-uniform expression of cre-recombinase in the mammary epithelia, resulting in a mosaical pattern of FgfR-2IIlb inactivation. It also remains possible that FgfR-2IIlb signalling may be required for mammary development during the early phases of pregnancy, or even prior to pregnancy (for example during pubertal development). The expression data presented in Figure 5.11 support this notion; however, mammary glands expressing a dominant negative FgfR-2IIlb in the epithelium show a retardation of lobulo-alveolar development, suggesting a later requirement for FgfR-2IIlb at a stage after the WAP promoter becomes active (Jackson et al., 1997; Wagner et al., 1997).

The initial characterisation of the WAP-Cre mouse demonstrated that mammary epithelial cells that had undergone cre-recombinase mediated DNA excision could be detected 30 days after weaning, and at higher levels during the second pregnancy (Wagner et al., 1997). This is consistent with floxed DNA excision occurring in putative stem cell compartments within the mammary gland. Thus in the second pregnancy, a degree of DNA recombination remains from the first pregnancy, allowing the effect of DNA excision to be examined prior to the onset of cre-recombinase expression during late pregnancy. In addition, the higher levels of recombination in the second pregnancy could reveal a role for FgfR-2IIlb signalling during the later stages of mammary development that was missed in the first pregnancy. In order to determine if deletion of FgfR-2IIlb could affect mammary gland development in a second pregnancy, female WAP-Cre^*.FgfR-2IIlb^floxed/ and control mice were mated with available males and allowed to litter. After weaning, the males were reintroduced for a second mating. The mice were sacrificed at 11.5 and 15.5 days of gestation, before and after the reported onset of cre-recombinase expression from the WAP promoter, the mammary glands were then dissected and prepared for wholemount and histological analysis as described above. The results of this experiment are shown in Figure 5.14.

Mammary glands from day 11.5 of the second pregnancy were essentially similar in WAP-Cre^*.FgfR-2IIlb^floxed/ and control mice. Both glands demonstrated comparable levels of ductal side branching and alveolar development, and had reached a similar differentiative state as judged by their histology. Interestingly, both glands
appeared slightly more differentiated when compared with the analogous stage of the first pregnancy (compare Figure 5.14 and Figure 5.12). This probably reflects the fact that involution remodels the mammary gland to a slightly more differentiated state than the virgin gland. By day 15.5 of the second pregnancy, mammary glands of WAP-Cre*;FgfR-2IIIb\textsuperscript{loxP/loxP} and control mice also appear similar in morphology as judged by the wholemount preparation. There does appear to be a slight retardation in the differentiation of mammary epithelia in the WAP-Cre*;FgfR-2IIIb\textsuperscript{loxP/loxP} mammary gland as evidenced by the absence of milk fat droplets (histological mammary section, Figure 5.14), although this could be accounted for by slight variations in the timing of the matings, or by the region and plane of sectioning of the gland.

Taken together, these results provide no firm evidence defining a role for FgfR-2IIIb signalling in the postnatal mammary gland. However, the interpretation of this data is complicated by the relatively late transgenic expression of cre-recombinase during pregnancy, which may have resulted in an early role for FgfR-2IIIb signalling in mammary development being overlooked. Although a PCR-based strategy confirmed that excision of the floxed IIIb allele was occurring in late pregnancy (data not shown), these experiments have not given any indication of, i) the extent of cre-recombinase mediated DNA excision, and ii) the uniformity of cre-recombinase expression driven from the WAP promoter in the mammary epithelium. If either, or both of these factors were not at a sufficiently high level in the mammary gland, then a requirement for FgfR-2IIIb at any stage of mammary development could be missed using this system. It therefore remains possible that FgfR-2IIIb signalling may be an important regulator of postnatal mammary development, but its role can not be sufficiently elucidated using the current transgenic model.

5.3.3 Detection of Transgenic Cre-Recombinase Expression and Activity

The characterisation of the WAP-Cre mouse was originally performed using RT-PCR to monitor cre-recombinase expression in the mammary gland as a whole, and an in vivo functional assay of cre-recombinase activity using an adenovirus β-galactosidase reporter construct (Wagner et al., 1997). Although this report provided indisputable evidence that cre-recombinase is expressed in a developmentally regulated pattern during mammary development, and that the cre-recombinase was able to excise a fragment of DNA floxed by loxP sites in
Wholemount and histological analysis of number 4 inguinal mammary glands from WAP-Cre\(^+\).FgfR-2\(\text{IIlb}\)\(^\text{floxed}\)/- and control mice from day 11.5 and 15.5 of a second pregnancy. Wholemount glands were fixed and stained with carmine stain, histological sections were stained with haematoxylin and eosin. Scale bars represent 1mm (wholemount) and 100\(\mu\)m (histology).
infected mammary cells, it did not address the extent of cre-recombinase expression and activity at a cellular level in the whole gland. In order to further characterise the WAP-Cre mouse, a number of experiments were performed to investigate the degree of cre-recombinase expression, and subsequent DNA recombination, at the cellular level in the mammary gland. It was hoped that these experiments would provide additional information that would help explain the lack of a significant mammary phenotype in the WAP-Cre* FgfR-2IIIb/+ mouse.

As experiments that attempted to detect the cre-recombinase protein in the mammary glands of WAP-Cre mice had failed due to the absence of an effective antibody, WAP-Cre mice were bred with reporter mouse strains to analyse the activity, and hence expression, of cre-recombinase in the mammary gland.

5.3.3a Chicken β-actin β-galactosidase reporter mouse (LacZ Reporter)

Mice carrying a transgene that allows the activity of cre-recombinase to be monitored were obtained, and breeding colonies started (LacZ Reporter mice, gift from Dr. A. Berns, Netherlands Cancer Institute, Akagi et al., 1997). These mice contain a transgene consisting of the promoter for chicken β-actin (CBA) followed upstream of two loxP sites and the β-galactosidase (LacZ) transcriptional unit. An intervening reporter/silencing gene was placed between the two loxP sites (chloramphenicol acetyl-transferase, CAT) to form the transgene CBA-loxP-CAT-loxP-LacZ, such that the CAT gene prevents the expression of LacZ from the CBA promoter. When cells containing this transgene are exposed to active cre-recombinase, the enzyme catalyses the recombination of DNA between the two loxP sites. This has the effect of excising the CAT gene and bringing LacZ under the transcriptional control of the strong CBA promoter. Thus, by utilising mice that contain this transgene in every cell, the activity of cre-recombinase can be monitored using the X-gal reagent which stains cells blue in the presence of β-galactosidase activity. In cells that have not been subjected to cre-recombinase, β-galactosidase expression is suppressed leaving these cells clear after incubation with X-gal.

WAP-Cre mice were therefore crossed with LacZ Reporter mice. Identification of mice containing both transgenes (WAP-Cre* LacZ*) was achieved by screening genomic DNA isolated from tail snips for the presence of each transgene by PCR. The WAP-Cre transgene was detected as described in section 5.3.2a. The CBA-loxP-CAT-loxP-LacZ transgene was detected using the primers CAT-5′ and CAT-3′ to amplify a fragment of DNA from the CAT gene.
WAP-Cre\(^*\).LacZ\(^*\) female mice were mated with available male mice and later sacrificed towards the end of pregnancy at a time when the transgenic WAP promoter is known to be active. The number 4 inguinal mammary glands were dissected, processed, and then incubated overnight with X-gal to report cre-recombinase mediated DNA excision of the CAT silencer gene (see section 2.13.3). Surprisingly, no blue staining could be detected in the mammary glands from WAP-Cre\(^*\).LacZ\(^*\) mice (data not shown). This suggests either that the WAP-Cre mice are not expressing the cre-recombinase as previously reported (Wagner et al., 1997), or that excision of the CAT gene was not occurring in these mice. Although a positive β-galactosidase tissue was not available as an experimental control, the lack of blue staining was unlikely to reflect a problem with the experimental technique as a standard protocol was followed. An alternative explanation may reside in the promoter activity of the LacZ Reporter mouse. Although DNA recombination in the mammary gland was not reported in the characterisation of the LacZ Reporter mouse strain, the authors demonstrated significant variation of CAT expression levels between tissues, suggesting differential activity of the chicken β-actin promoter. For instance, CAT expression was high in the heart and skeletal muscles, but lower in the liver and thymus. If transgene silencing occurred in the mammary epithelium then this would provide a satisfactory explanation as to why cre-recombinase activity could not be detected in mice carrying both transgenes. In this scenario, excision of the CAT gene in the mammary gland after activation of the WAP-Cre transgene would still occur, but remain undetectable due to silenced β-galactosidase expression.

5.3.3b ROSA26 β-galactosidase reporter mouse (ROSA26 Cre Reporter)

In light of the negative results obtained using the LacZ Reporter mouse, a similar strategy was employed to characterise the activity of the WAP-Cre transgene using an alternative cre-recombinase reporter strain. The ROSA26 Cre Reporter line was obtained and breeding colonies were established (originating from Dr. P. Soriano, Fred Hutchinson Cancer Research Center, Washington, Soriano, 1999). In contrast to the transgenic LacZ Reporter mouse the ROSA26 Cre Reporter was developed as a knock-in model, in which a DNA cassette containing a neomycin gene flanked by unidirectional \(\text{loxP}\) sites, and a downstream β-galactosidase gene was inserted into the ROSA26 locus. This locus acts as a constitutively active promoter in all cell types of the mouse during embryogenesis suggesting that it may also function in the adult mouse in a similar way (see Mao et al., 1999; Soriano, 1999). In the absence of cre-recombinase, the ROSA26 locus does not transcribe β-galactosidase expression.
due to a triple polyadenylation site at the end of the neomycin gene that prevents transcriptional read-through. However in the presence of cre-recombinase, DNA excision between the two loxP sites brings the β-galactosidase gene under the transcriptional regulation of the ROSA26 locus, leading to high levels of β-galactosidase expression and activity that is detectable by X-gal staining. As the ROSA26 locus is constitutively active in all tissues examined, this mouse model has the potential to assess cre-recombinase activity in any tissue, and circumvents problems associated with transgene silencing.

To establish the pattern of DNA recombination arising from the WAP-Cre transgene, WAP-Cre and ROSA 26 Cre Reporter mice were interbred to establish mice that were positive for the transgene and heterozygous for the knock-in reporter allele (genotype: WAP-Cre*.ROSA26+/−). Identification of the ROSA26 knock-in allele was performed by PCR screening of genomic DNA isolated from tail snips as previously described (sections 2.10.1a and 2.10.3). Three oligonucleotides were used as primers for the amplification of DNA fragments from the ROSA26 locus (ROSA1, ROSA2, and ROSA3) using an annealing temperature of 55°C. When the products of the PCR amplification were separated through a 1% (w/v) agarose gel by electrophoresis, two potential products could be identified – a ~500 bp band corresponding to the wild type allele, and a ~250 bp band corresponding to the mutant knock-in allele. Screening for the presence of the WAP-Cre transgene was performed as described above. The genotype WAP-Cre*.ROSA26+/− was achieved in mice by first interbreeding ROSA26 Cre Reporter mice to homozygosity, and then mating these to WAP-Cre heterozygous mice. The resulting litters were either the experimental genotype {WAP-Cre*.ROSA26+/−}, or alternatively a control genotype lacking the WAP-Cre transgene {WAP-Cre*.ROSA26−/−}.

Sexually mature female WAP-Cre*.ROSA26+/− and control mice were sacrificed, or mated and sacrificed to obtain mammary glands from mice that were either virgin, 11.5 and 15.5 days pregnant, 2 days postpartum, and 11.5 and 15.5 days pregnant from the second pregnancy (see section 5.3.2d). Two WAP-Cre*.ROSA26+/− mice were used for each developmental stage. Both number 4 inguinal mammary glands were dissected from sacrificed mice and spread on to separate glass microscope slides. Glands were fixed on the slides to maintain their shape and then stained overnight in 1mg/ml X-gal solution as described in the Materials and Methods. Both glands were dehydrated in ethanol, cleared in xylene and stored at room temperature in 100% ethanol. The right hand side gland was maintained for wholemount analysis, and the left hand side gland was sectioned after being
embedded in paraffin wax. These sections were then counterstained with nuclear
fast red.

As expected, mammary glands from control mice which did not contain the
WAP-Cre transgene but were genotypically ROSA26" exhibited no cre-recombinase
activity as indicated by the presence of blue cells (data not shown). However, mice
containing both the WAP-Cre transgene, and the ROSA26 Cre Reporter allele
(WAP-Cre*.ROSA26") showed varying amounts of blue staining in the mammary
epithelium, consistent with the regulated expression of cre-recombinase from the
WAP promoter (Figure 5.15 and Figure 5.16). A comparable degree of cre-
recombinase activity was detected between different mammary glands at the same
stage of development, suggesting a similar pattern of WAP promoter activation.
Analysis of the wholemount preparations of the sexually mature virgin mammary
glands revealed no blue staining after incubation with X-gal. This demonstrates
that no significant levels of DNA recombination have occurred, and agrees with the
published findings that the transgenic WAP promoter is silent in the virgin mouse
(Wagner et al., 1997). By day 11.5 of the first pregnancy, a small amount of blue
staining can be detected in a localised patch of mammary epithelia indicating that
limited DNA recombination takes place before the WAP promoter becomes fully
active during late pregnancy. However, at this stage of pregnancy the vast majority
of the gland remains unstained by X-gal suggesting that cre-recombinase expression
from the WAP promoter is still largely suppressed. This is confirmed by the
histological sections through the contralateral mammary gland which shows an
absence of blue cells.

The level of X-gal staining increases progressively during pregnancy. By day 15.5
of pregnancy and 2 days postpartum, blue mammary epithelia can be readily
detected throughout the mammary gland (see wholemount images, Figure 5.15).
Importantly however, at these developmental stages a significant proportion of the
ductal and alveolar network remains unstained after incubation with X-gal
suggesting that cre-recombinase is not expressed in these cells. The areas of the gland
that appear not to have undergone DNA recombination are the peripheral ducts
and alveoli, this is most noticeable at 2 days postpartum. Notably, the areas that
have not stained blue for β-galactosidase activity look to be morphologically
indistinguishable from the regions in the middle of the gland that did stain blue in
the presence of X-gal. This suggests the inactivity of the transgenic WAP promoter
in the peripheral mammary epithelium is not due to a delay in epithelial
differentiation, rather that the promoter is not uniformly active throughout the
mammary epithelia resulting in a mosaic cre-recombinase expression pattern.
Furthermore, the histological sections of these glands indicate that even in regions where cre-recombinase activity is high, the majority of ductal epithelial cells have not stained with X-gal. This is most clearly illustrated with the 15.5 day pregnant mammary gland, where a main epithelial duct remains unstained after treatment with X-gal, whereas some epithelial cells in close proximity are positively stained by X-gal (Figure 5.15).

During the second pregnancy, a similar pattern of cre-recombinase expression is found (Figure 5.16). Despite overall higher levels of β-galactosidase activity, significant portions of the gland did not stain blue around the peripheral ductal network indicating that cre-recombinase is only expressed in a subset of cells in the epithelial compartment of the gland. The histological sections of mammary glands from the second pregnancy provides further evidence for the variegated expression pattern of cre-recombinase from the WAP promoter.

Taken together, these findings show beyond doubt that the WAP-Cre transgene does not lead to the uniform excision of DNA that is flanked by loxP sites in the mammary epithelium. Instead, recombination appears in epithelial clusters suggesting that a common smaller set of parental cells have undergone DNA excision and passed the recombined allele onto their progenitors.

### 5.4 Discussion

The generation of transgenic and gene targeting technologies in mice has revolutionised the field of mouse genetics, and enabled considerable progress to be made in the understanding of the genetic control of development. By utilising mice containing targeted germline mutations in the FgfR-2IIIb gene, the results presented in this chapter have shed new light on the role of FgfR-2IIIb signalling in mammary gland development.

During embryonic mammary development FgfR-2IIIb signalling was shown to be required for the induction of thoracic mammary placodes. These placodes could not be detected in mice lacking FgfR-2IIIb at any stages of their development that were studied, either by examination of histologically stained embryo sections or by in situ hybridisation using a Lef-1 specific probe as a molecular marker for mammary tissue (Figure 5.5 and Figure 5.6). By contrast, an inguinal mammary placode (probably gland number 4) was detectable using these techniques at approximately E13 in mice deficient for FgfR-2IIIb, suggesting that it can be induced
Figure 5.15 - Analysis of cre-recombinase activity in mammary glands containing WAP-Cre transgene (first pregnancy)

Wholemount and histological analysis of number 4 inguinal mammary glands from WAP-Cre^+/-.ROSA26^+/+ mice at the developmental stages indicated during the first pregnancy (PP = postpartum). Blue stain indicates the cells in which cre-recombinase mediated DNA recombination has occurred. The histological analysis of the virgin gland is absent due to an error whilst processing the gland. Scale bars represent 2mm (left panels) and 100μm (right panels).
Figure 5.16 - Analysis of cre-recombinase activity in mammary glands containing WAP-Cre transgene (second pregnancy)

Wholemount and histological analysis of number 4 inguinal mammary glands from WAP-Cre^+/ROSA26^{fl/lox} mice from the developmental stages indicated during the second pregnancy. Blue cells indicate the cells in which cre-recombinase mediated DNA recombination has occurred. Scale bars represent 2mm (left panels) and 100 μm (right panels).
by a different signalling pathway to the remaining inguinal and thoracic glands. This placode was shown to have a different morphology to comparable wild type mammary placodes, appearing detached from the epidermis, and surrounded by mesenchymal tissue. The reasons for this difference are unclear, but may be explained by the extensive apoptosis that was detected in the epithelial cells of the mutant placode shortly after its formation (Figure 5.7).

The absence of limbs in these mice made it difficult to distinguish which inguinal mammary placode, number 4 or 5, had transiently developed. Its relative position to the tail suggests that it could be placode number 4, however, placodes 4 and 5 reside quite close to each other in the wild type embryo at the point where the hind limb bud extends from the body. If embryonic limb development, which occurs at around the same developmental stage as mammary placode formation, assists in pushing placodes 4 and 5 apart into distinct locations, then it is conceivable that the mutant placode in FgfR-2IIIb mice could be an amalgamation of placodes 4 and 5. If this is the case, then these results suggest that the signalling pathways that control inguinal mammary induction can differ from those controlling development of the thoracic glands, rather than placode 4 being unique. This would provide a more satisfactory explanation from an evolutionary point of view, given that different families of mammals possess diverse numbers and sets of glands. For instance, in the mouse both thoracic and inguinal glands develop, whereas in primates and ruminants only one set of glands develop (thoracic or inguinal respectively).

The degeneration of the mutant inguinal placode by apoptosis is typical of a number of organs in the FgfR-2IIIb-mouse (De Moerlooze et al., 2000; Revest et al., 2001a). The limbs are a good example of this; as can be seen clearly in Figure 5.6, development of the limb buds is initiated in the absence of FgfR-2IIIb signalling. However, the receptor is required for their maintenance and further development, and its absence results in limb bud degeneration by apoptosis. By contrast, thymus development is initiated and maintained in the absence of FgfR-2IIIb, but FgfR-2IIIb signalling is required for thymic epithelial cell proliferation and full differentiation (Revest et al., 2001b). Thus the requirement for FgfR-2IIIb in embryogenesis is organ specific, with FgfR-2IIIb signalling participating in the induction, as well as the maintenance and differentiation of various organs.

The requirement for FgfR-2IIIb signalling in mammary gland induction and maintenance is further supported by the in situ hybridisation experiments. These studies showed a high level of FgfR-2IIIb expression in the mammary epithelium throughout the induction and resting phases of embryonic mammogenesis (Figure
The nature of the molecules that activate this receptor during embryonic mammary development is a contentious issue. The expression analysis presented in this chapter strongly implicates the mesenchymal ligand Fgf-7 as the key regulator of FgfR-2IIIlb signalling. Fgf-7 is strongly expressed in close proximity to the receptor during mammary placode formation (Figure 5.9). It is also expressed in the mammary mesenchyme up to E14.5 suggesting that it could be required for the maintenance, or further development of the gland. The other identified activating ligands for FgfR-2IIIlb (Fgfs-1, -3, and -10) are not expressed in the mammary mesenchyme at the stages examined in this chapter. However, no mammary phenotype has been described for the Fgf-7 knock-out mouse, in fact these mice show very few defects in organs requiring mesenchymal-epithelial signalling for their development (Guo et al., 1996). This is somewhat surprising given the severity of the FgfR-2IIIlb<sup>+</sup> mouse phenotype, and the fact that Fgf-7 does not appear to signal through other receptors (Ornitz et al., 1996). Instead, Fgf-10 seems to be the major ligand for FgfR-2IIIlb, and mice lacking Fgf-10 show very similar defects to mice lacking FgfR-2IIIlb (Ohuchi et al., 2000 and Introduction section 1.2.2c).

The absence of Fgf-10 signalling in the mouse leads to a perturbation in mammary development that overlaps with the phenotype described in this chapter (Maillieux et al., 2002). The requirement for Fgf-10 signalling is somewhat surprising given the absence of detectable Fgf-10 expression in the embryonic mammary gland. It would be expected, as with the requirement for Fgf-10/FgfR-2IIIlb signalling in lung development, for there to be a complementary mesenchymal/epithelial Fgf-10/FgfR-2IIIlb expression pattern in the mammary gland (Bellusci et al., 1997, Dr. B. Spencer-Dene, Cancer Research UK, London, personal communication). Nevertheless, in Fgf-10 deficient mice, the thoracic mammary placodes fail to induce and are absent throughout development, but an inguinal mammary placode is induced in a similar position to that seen in FgfR-2IIIlb<sup>+</sup> mice. In contrast to the inguinal mammary placode identified in this chapter, the mutant Fgf-10<sup>+</sup> placode does not degenerate by apoptosis, but is maintained until birth and shows a limited amount of ductal branching (Maillieux et al., 2002). It therefore seems that the mutant inguinal placode does not require Fgf-10 or FgfR-2IIIlb signalling for its induction, but it does require FgfR-2IIIlb for its further development. The similarity between the Fgf-10<sup>+</sup> and FgfR-2IIIlb<sup>+</sup> inguinal mammary phenotypes strongly suggests that another ligand can compensate for the loss of Fgf-10 by signalling through FgfR-2IIIlb in the mutant placode. The expression analyses presented in this chapter would implicate Fgf-7 in this role, however, a double Fgf-7<sup>-/-</sup>.Fgf-10<sup>-/-</sup>
knockout mouse would need to be developed to test this hypothesis. Alternatively, another Fgf(s) may be able to compensate for the loss of Fgf-10 in the mutant inguinal placode by activating FgfR-2IIIb. Based on its sequence similarity to Fgf-7 and -10, the recently discovered Fgf-22 could possibly act as an activating ligand for FgfR-2IIIb in embryonic mammary development (Nakatake et al., 2001). Its expression in the mammary gland has not yet been assessed.

Based on their regulated expression patterns, a number of different genes have been implicated in the development of the mouse embryonic mammary gland (see Table 1.1). In spite of this, gene deletion studies have shown essential roles for only a few of these: Lef-1, Msx-1 and Msx-2, PTHrP and PPR1 (section 1.1.1b). The results described in this chapter add FgfR-2IIIb to this list, and together with the requirement for Fgf-10 demonstrate that the pathways controlling embryonic mammary development differ between glands. The scant information regarding the upstream modulators and downstream targets of FgfR-2IIIb signalling make it hard to place this receptor within the broader context of a morphogenetic pathway involved in mammary development. The dissimilarity between the FgfR-2IIIb^+ mammary phenotype, and other embryonic mammary phenotypes obtained from gene deletion studies further compounds this difficulty. The early role for FgfR-2IIIb signalling in mammary development would suggest that some of the genes expressed early on in the gland may form part of the same genetic pathways that control its development. However it should be noted that these early defects in mammary development may also mask later requirements for FgfR-2IIIb signalling. Therefore genes expressed later on in mammary development may also reside up- or downstream of FgfR-2IIIb signalling. It would be interesting to determine if any of the epithelial growth regulators identified in the early embryonic mammary gland, such as Wnt-10b, Bmp-2, and PTHrP, could partially compensate for the loss of FgfR-2IIIb, and allow mammary development to proceed past the induction stage. If this were the case then it would suggest they reside downstream of FgfR-2IIIb receptor activation.

In addition to establishing a differential role for FgfR-2IIIb signalling in thoracic and inguinal mammary placode development, the results presented in this chapter also emphasise important differences between mammary placodes and other organs derived from ectodermal budding. It has previously been suggested that these organs require a similar set of genes for their initial development. For instance, the development of the mammary placodes, whisker follicles, and tooth buds are all impaired when Lef-1 is functionally inactivated (Kratochwil et al., 1996; van Genderen et al., 1994). However in mice lacking FgfR-2IIIb, mammary placode
development is severely impaired, whereas whisker and hair follicles develop, albeit with a slight developmental delay (see Figure 5.6), and teeth only progress to the bud stage of their development (De Moerlooze et al., 2000). It is an interesting observation that Fgf-10, the major ligand for FgfR-2IIIb, can be strongly detected in the developing hair follicles of wild type mice, but cannot be detected in the mammary mesenchyme, yet deletion of FgfR-2IIIb severely affects mammary development and not hair follicle development. A further illustration of these differences can be seen from the expression pattern of Fgf-8. In tooth development, Fgf-8 is expressed in the dental ectoderm and may induce signalling pathways via activation of Msx-1 (Bei and Maas, 1998), however in the mammary placode, Fgf-8 expression could not be detected, suggesting that Msx-1 activation occurs through alternative pathways (Figure 5.10).

The role of FgfR-2IIIb signalling in the adult mammary gland remains less defined than in the embryonic gland. The in situ hybridisation analysis of FgfR-2 expression in the adult mammary gland showed a high level of expression in the virgin and involuting glands, but decreasing levels in the glands during pregnancy. This expression pattern perhaps suggests FgfR-2IIIb is involved in ductal morphogenesis and gland remodelling after pregnancy, rather than in the rapid proliferative and differentiative processes that are associated with mammary development during pregnancy. This hypothesis is not supported by previous findings using a dominant negative FgfR-2IIIb receptor truncated within the intracellular domain, and over expressed in the mammary gland using the MMTV-LTR promoter. In these experiments, expression of the dominant negative receptor was found to perturb lobulo-alveolar development during late pregnancy, at a stage when expression of the endogenous receptor is at its lowest (Jackson et al., 1997; Pedchenko and Imagawa, 2000 and Figure 5.11). This suggests that FgfR-2IIIb signalling may be involved in regulating the differentiation of lobulo-alveolar units. However, it is not clear from these studies whether the lobulo-alveolar defect is specific to the block in FgfR-2IIIb signalling, as the dominant negative receptor would have the effect of blocking signalling through other Fgf receptors. For example, as Fgfs-1, -3, -7, and -10 can bind FgfR-2IIIb, signalling in the mammary gland by these ligands would potentially be blocked by the dominant negative receptor. As Fgf-3 is not expressed in the mammary gland (Coleman-Krnacik and Rosen, 1994), Fgf-7 cannot activate other receptors, and Fgf-1 is unlikely to be an important ligand in the mammary gland as a dominant negative FgfR-1IIIc does not affect mammary development (Jackson et al., 1997), this leaves Fgf-10 as a potential candidate. Thus it is possible that the FgfR-2IIIb dominant negative receptor blocks
Fgf-10 signalling through other receptors in the mammary gland, and that the lobulo-alveoli defect is due to an indirect block on this signalling pathway. Although few studies have assessed the capacity of Fgf-10 to activate receptors other than FgfR-2IIIb, it has been shown to bind with high affinity to FgfR-1IIIb. Furthermore, FgfR-1 is expressed in the mammary gland and influences mammary development (Chodosh et al., 2000; Welm et al., 2002).

The experiments described in this chapter attempted to identify a specific role for FgfR-2IIIb signalling in the adult mammary gland. A conditional gene deletion approach, that would delete FgfR-2IIIb specifically in the mammary gland, was used due to the early lethality of mice containing the non-conditionally deleted alleles of FgfR-2IIIb. Unfortunately, using this model system, a specific role for FgfR-2IIIb signalling could not be identified in adult mammary gland development. It is not absolutely clear why this technique failed to identify a role for FgfR-2IIIb, however, a number of possible reasons are considered. Firstly, it is possible that FgfR-2IIIb signalling is not important for mammary development in the adult; although this seems unlikely given its regulated expression pattern, and the phenotype associated with expression of the dominant negative receptor. Secondly, the WAP-Cre transgene used in this study was only active during late pregnancy. Therefore a role for FgfR-2IIIb signalling in the mammary gland would not be detected during early pregnancy, or in the virgin, or involuting mouse. A third explanation could reside in the efficacy of cre-recombinase mediated DNA excision in the FgfR-2IIIb^flx^ mouse. However, as the non-conditional FgfR-2IIIb^/+ mouse was generated from this mouse this possibility is highly unlikely. The most likely explanation resides in the use of the WAP-Cre transgenic mouse to delete the floxed IIIb exon of FgfR-2 in the mammary gland.

The characterisation of the WAP-Cre mouse, using the ROSA26 Cre Reporter knock-in mouse, identified serious problems associated with the transgenic expression of cre-recombinase in the mammary gland (Figure 5.15 and Figure 5.16). These studies demonstrated a significant mosaicism in the pattern of DNA recombination within the mammary epithelium, revealing that the majority of epithelia are not exposed to cre-recombinase activity. The implications of this for identifying a role for FgfR-2IIIb in the mammary gland are clear. If deletion of FgfR-2IIIb in the mammary epithelium leads to a growth disadvantage, then it would likely be very difficult to detect as the mammary epithelial cells containing a functional copy of the gene could compensate for these mutant cells. Even in the second pregnancy, the pattern of DNA recombination may not be sufficiently widespread to allow the detection of a mammary phenotype associated with loss of
exon IIIb. The possibility remains that in subsequent pregnancies, enough mammary epithelial cells would contain the recombined allele of FgfR-2 to allow detection of a phenotype.

Additional attempts were made to understand the physiological role of FgfR-2IIIb in the adult mammary gland using alternative cre-recombinase expressing transgenic mice. Mice expressing cre-recombinase from the MMTV-LTR promoter (MMTV-Cre mice) presented an attractive transgenic model to use as an alternative to the WAP-Cre mouse. Although the MMTV-LTR promoter tends to be less specific than the WAP promoter (e.g. the MMTV-LTR is usually also active in the salivary glands and some other organs), it can be active in a broad range of developmental stages in the mammary gland, including the virgin. Two independent MMTV-Cre mouse lines were obtained (i. MMTV-Cre, a gift from Dr. W. Muller, McMaster University, Ontario, and ii. MMTV-Cre(line A), Jackson Laboratory, Maine). Frustratingly, MMTV-Cre mice did not recombine floxed DNA in the mammary gland when crossed with FgfR-2IIIb<sub>fl</sub>/<sub>fl</sub> mice (data not shown). MMTV-Cre(line A) mice were shown to express cre-recombinase after just 6 days postpartum in mammary epithelial cells, but unfortunately, attempts to breed these mice with mice containing the conditional allele of FgfR-2IIIb failed to produce significant numbers of progeny for a study (Wagner et al., 2001).

The problems reported in this chapter draw attention to a major limiting factor concerning the use of transgenic mice expressing cre-recombinase in the mammary gland. Namely, most mammary gland promoters identified to date are regulated by lactogenic hormones, and so only have a narrow spatial and temporal activity that may not overlap with the expression of the floxed gene of interest. A newly devised technique, that takes advantage of the regenerative capacity of the mammary gland, may circumvent these problems in the future, and could be used to study the role of FgfR-2IIIb in mammary gland development. This technique utilises an adenovirus-cre-recombinase construct to infect primary mammary epithelial cells and cause the recombination of floxed DNA in culture. Cells were isolated from ROSA26 Cre Reporter mice in the original report, but could equally be derived from the mammary glands of FgfR-2IIIb<sub>fl</sub>/<sub>fl</sub> mice. The infected mammary cells are then transplanted into the cleared mammary fat pad of recipient immunocompromised mice, and the ensuing mammary development analysed (Rijnkels and Rosen, 2001). This system would allow the consequence of FgfR-2IIIb deletion to be assessed at all stages of postnatal mammary development, and not just during pregnancy.
To conclude, the expression patterns of FgfR-2IIIb in the embryonic and postnatal mammary gland suggest that signalling through the receptor product of this gene could play a role in mammary development. Through the use of mice containing a non-conditional deletion of the IIIb exon of FgfR-2, it was shown that signalling through FgfR-2IIIb is essential for mammary placode development. Moreover, it was demonstrated that an inguinal placode utilises an FgfR-2IIIb independent pathway for its induction, but requires an FgfR-2IIIb pathway for its maintenance. The induction of the remaining placodes was shown to require an FgfR-2IIIb dependent signalling pathway. In the adult mammary gland, a specific role for FgfR-2IIIb signalling could not be deciphered using mice containing a conditional allele of FgfR-2IIIb. This probably reflects the inability of the WAP-Cre transgene to cause sufficient recombination of the floxed IIIb exon within the mammary gland, as demonstrated using the ROSA26 Cre Reporter mouse.
Different aspects of the roles of three RTKs in mammary gland development have been studied. The signals that control the basolateral targeting of ErbB-2 in epithelial cells were investigated utilising an *in vitro* model of epithelial cell polarity. In addition the developmental roles of the Met receptor and FgfR-2IIIb in the mammary gland were analysed using transgenic and knock-out mouse technologies.

At the cellular level, ErbB-2 localises to both the basolateral and apical membrane domains of mammary epithelial cells during mammary gland development, in a stage-specific manner (Chodosh et al., 2000; Darcy et al., 2000; Niranjan et al., 1995; Pedchenko and Imagawa, 2000; Pepper et al., 1995). Using MDCK cells that form tight epithelial monolayers with clear domain characteristics, basolateral targeting of ErbB-2 was shown to be dependent on a novel bipartite signal (QETE and EPLT). This signal resides in the intracellular juxtamembrane domain of the receptor, and is able to confer basolateral targeting information when fused to a heterologous apical membrane protein. Furthermore, the signal was shown to be distinct from other known basolateral targeting signals, and is both necessary and sufficient to target human ErbB-2 to the basolateral membrane domain of polarised epithelial cells.

These investigations also contradict a previous report describing the involvement of the PDZ protein, ERBIN, in the basolateral targeting of ErbB-2 (Borg et al., 2000). In essence, mutation analysis of the ERBIN binding site showed that it was not required for the basolateral targeting of ErbB-2, but that it did contain a cryptic basolateral targeting signal.

It is not clear which mechanisms control the differential localisation of ErbB-2 to the basolateral and apical membrane domains, nor is it apparent whether the receptor has different roles at each domain during mammary development. It has been reported that ErbB-2 is involved in lobulo-alveolar development, and that its function is necessary for proper lactation that ensues after parturition (Jones and Stern, 1999). However, this study used a dominant negative ErbB-2 transgene lacking the juxtamembrane basolateral targeting signal. Hence, given that ErbB-2 receptors lacking this signal were shown to locate predominantly to the apical membrane domain (Figure 3.5), it is conceivable that the role in mammary development of basolateral ErbB-2 receptors in the mammary epithelium were not truly revealed in the study by Jones and Stern. Instead, it is possible that the
observed phenotype is a reflection of blocking signalling through the apically localised ErbB-2 receptors. It would be interesting to determine whether additional mammary phenotypes would be uncovered using a dominant negative ErbB-2 receptor that localised both to the apical, and basolateral membrane domains.

The Met receptor was also shown to locate to the basolateral membrane domain in MDCK cells, and presumably displays a similar location within the mammary epithelium. The intracellular domain of the Met receptor was shown to possess basolateral targeting activity, however, unlike ErbB-2, deletion of its intracellular domain does not lead to the accumulation of Met on the apical membrane domain of polarised epithelial cells. This indicates either that the Met receptor does not possess strong apical determinants within the extracellular or transmembrane domains, or alternatively that these domains may contain extra basolateral targeting information. It would be interesting to assess the polarised distribution of Met within the mammary epithelium throughout the development of the mammary gland in order to determine whether it is differentially localised in a similar way to ErbB-2. If this were the case, then it would raise interesting questions concerning the mechanisms of apical Met receptor localisation, and the regulation of its function in mammary gland development.

To gain some insights into the role of Met receptor signalling in mammary gland development, a dominant negative approach was used to block Met signalling in the mammary gland. This was achieved by generating transgenic mice that express a truncated version of Met, which acted as a dominant negative receptor in vitro, within the mammary epithelium. Similar transgenic strategies have successfully identified roles for other RTKs in mammary gland development (Jackson et al., 1997; Jones and Stern, 1999). Analysis of transgenic mice revealed no abnormalities in mammary gland development, consequently a specific role for Met receptor signalling in the mammary gland was not defined using this method. It is unclear why this approach failed to yield a phenotype given the in vitro evidence for a function in mammary gland development. However, the low number of positive expressing transgenic founder lines suggest that the transgene may have toxic effects during embryogenesis. The development of new technologies utilising small interfering RNA (siRNA) molecules to inhibit endogenous gene function, in an organ specific manner in postnatal mice, may allow potential problems with transgene toxicity to be overcome in the future (Lewis et al., 2002). An alternative strategy might be to create a conditional knock-out mouse in which the Met gene, floxed by loxP sites, could be deleted by crossing with transgenic mice expressing cre-recombinase specifically in the mammary gland.
Concluding Remarks

A tissue specific conditional knock-out approach was used to determine if FgfR-2IIIb is involved in the postnatal development of the mouse mammary gland. Mice containing the IIIb exon of FgfR-2 flanked by loxP sites were crossed with WAP-Cre mice, that express cre-recombinase specifically in the mammary gland. In this study the low degree of recombination of floxed DNA in the mammary epithelium confounded the analysis. The WAP-Cre transgene was unable generate the homogeneous population of recombined mammary epithelial cells that would be necessary to identify a developmental role FgfR-2IIIb in the mammary gland. However, the receptor is likely to play a role in mammary gland development as it was shown to be differentially expressed during pregnancy, and because mammary gland development is impaired in the presence of a dominant negative FgfR-2IIIb receptor (Jackson et al., 1997; Pedchenko and Imagawa, 2000).

In the embryonic mammary gland, a clear essential role for FgfR-2IIIb was identified. FgfR-2IIIb signalling was shown to be required for the induction of all mammary placodes, with the exception of an inguinal placode. This mutant placode was induced in the absence of the receptor, but its maintenance required FgfR-2IIIb signalling, demonstrating that FgfR-2IIIb is indispensable for the development of all the mammary glands.

In conclusion, a number of issues have been addressed in this thesis that bring together aspects of cell and developmental mammary biology. An important issue for future consideration is to address how the polarised distribution of RTKs, and other membrane bound proteins, impinges on their activity. Furthermore, it would be of immense interest to try and understand the molecular mechanisms that control RTK localisation, and to determine how epithelial cells are able to differentially localise these proteins throughout development. The mammary gland provides a unique and well characterised developmental model that is open to genetic manipulation, and could therefore be used to answer some of these intriguing questions.
## A6.1 Oligonucleotide Primers

Oligonucleotide primers used for DNA sequencing are not listed in the following table.

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<td>GGCGGCTCATTACAGCGGCTCCGCTAGGTGTCAGCGGCTCCACCAGCTCCGTTTCCTGCAGCAGTCTCCGCTATGGAT</td>
</tr>
<tr>
<td>CAT-3'</td>
<td>ACTGGTGAAACTCACCCA</td>
</tr>
<tr>
<td>CAT-5'</td>
<td>CAGTCAGTGGTGTCAATGTACC</td>
</tr>
<tr>
<td>cMETpr1</td>
<td>CTGTCTGATGTCAATGAAAGGCGGCCGCCGCTGCTGGCTGCG</td>
</tr>
<tr>
<td>cMETpr3</td>
<td>CGTCTAGACGTCCCCTACCCCTAATGAGGAGGTGTTGCTGCTGCTG</td>
</tr>
<tr>
<td>CreScreen</td>
<td>CATCAGTGGTGTCAATGTACC</td>
</tr>
<tr>
<td>D6mut</td>
<td>AATCTCTAGAATATACAGGTACTCCGGTTTCTGCTGCGCCG</td>
</tr>
<tr>
<td>EP-AA</td>
<td>GAAACCGAGCCTGGTGCCGCCGCTGACACCTAGCGGA</td>
</tr>
<tr>
<td>EP-AA(R)</td>
<td>TCCGCTAGGTGTACGCGCCGCCACCCAGCCTGCTGGTTC</td>
</tr>
<tr>
<td>EPLT(forall12)</td>
<td>GGGCGGCGGAGGGCGGCGGACGAGACGACGACTTAGGGAGGCGGATG</td>
</tr>
<tr>
<td>EPLT(forall12)r</td>
<td>CATCGCTCCGCTAGGTGTCACGAGACGAGCGCACTACGAGC</td>
</tr>
<tr>
<td>EPLT-AAAA</td>
<td>GAGTCCTTGGTCGGCGCGGCGGCGGCGGCTACGAGCGAGCGG</td>
</tr>
<tr>
<td>EPLT-AAAA(R)</td>
<td>CATCGCTCCGCTAGGTGTCACGAGACGAGCGACGCTGACGAGCG</td>
</tr>
<tr>
<td>FLAG1</td>
<td>CGACTACAAAGGATGACGACGACAAAGGACGACTACAAGGATGACGACGCAAGAGCG</td>
</tr>
<tr>
<td></td>
<td>CAAGGACG</td>
</tr>
</tbody>
</table>
Appendices
Primer Name

Primer Sequences (6*

3’)

FLAG2

CCTTGTCGTCGTCATCCTTGTAGTCCTTGTCGTCGTCATCCTTGTA
GTCGACGT

IntFI

ACCATGTTCATGCCTTCTTC

LLQETELV

GTACACGATGCGGAGAGCAGCAGCAGCGGCTGCAGCGG

LLQETELVr

CCGCTGCAGCCGCTGCTGCTGCTCTCCGCATCGTGTAC

Met-FoR1

CCGGAATTCATTAAAGATCTGGGCAGTGAATT

MetHIII-F

CCCAAGCTTACTATGAAGGCCCCCGCTGTGCTTGC

MetIntRI

CACAGCTAATGAGTTGATCATC

MetTr2881

ATAAGAATGCGGCCGCTTATCATTGCTTTCTCTTTTTCAGCCACA

mu1bF-H3

CCCAAGCTTACTATGTCCGCCTCGGCTGTCTTCATTC

mu1bR-N1

ATAAGAATGCGGCCGCTTACTAGCTGGTACGAAGTTGGTAATCG

NI2946-R

ATAAGAATGCGGCCGCTTATCACCTATCCAAATGAGGAGTGTGTA

NI4173-R

ATAAGAATGCGGCCGCTTACTATGATGTCTCCCAGAAGGAGGCTG

NI4173T-R

ATAAGAATGCGGCCGCTGATGTCTCCCAGAAGGAGGCTG

P1

CTGCCTGGCTCACTGTCC

P2

CTCAACAGGCATGCAAATGCAAGGTC

P3

GGCAGTAAATACGGGCCTG

P4

CGTAAACTCCTCTTCAGACC

p75-FoH3

CCCAAGCTTATGGGGGCAGGTGCCACCGGCCGC

P75-R6R1

CCGGAATTCCCTCTTGAAGGCTATGTAGGCCAC

p75-ReR5

CCGGATATCCCTCTTGAAGGCTATGTAGGCCAC

p75St-ReR5

CCGGATATCTCATTACCTCTTGAAGGCTATGTAGGCCAC

pGL3pl1

AGCTTTCGCGAATGCTAGCATGCAAGCCTATGCGGACGTCGATAG
AGTAATCTGAACTAGTTACTCT

pGL3pl2

CTAGAGAGTAACTAGTTCAGATTACTCTATCGACGTCCGCATAGGC
TTGCATGCTAGCATTCGCGAA

QETEEPLT

GATGCGGAGACTGCTGGCAGCAGCAGCACTGGTGGCGGCGGCG

QETEEPLTr

CGCCGCCGCCACCAGTGCTGCTGCTGCCAGCAGTCTCCGCATC

QETELV

GCTGGCAGCGGCTGCAGCGGCGGAGCCGCTGACACC

QETELVr

GGTGTCAGCGGCTCCGCCGCTGCAGCCGCTGCCAGC

SmaPLI

TCGACCTTACTTATCCCGGGTTATTAGTGCATCCATTGCG

SmaPL2

TCGACGCAATGGATGCACTAATAACCCGGGATAAGTAAGG

VPAmut

AATCTCTAGAAATTACGCTGGCACGTCCAGACCCAGGTAC

WAPScreen

TAGAGCTGTGCCAGCCTCTTC

ZfyF

AAGATAAGCTTACATAATCACATGGA

ZfyR

CCTATGAAATCCTTTGCTGCACATGT

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A6.2 DNA Plasmid Maps

The details of all plasmids generated for these studies are depicted below. Plasmids that were used, but not manipulated, are not shown.

A6.2.1 pGL3M

A6.2.2 pM2P
A6.2.3 pM2PI

A6.2.4 pMet-DN
A6.2.5 pBSMet-DN

This plasmid was used to create the majority of expression vectors in Chapters 3 and 4, the gene of interest was cloned into the multiple cloning site shown.

A6.2.6 pcDNA4/TO

This plasmid was used to create the majority of expression vectors in Chapters 3 and 4, the gene of interest was cloned into the multiple cloning site shown.
A6.3 Probes For In Situ Hybridisation

The following table details the probes used for the in situ hybridisation experiments described in Chapter 5. Available details of the plasmid vector, the restriction endonuclease and RNA polymerase (RNA pol) used to generate antisense probes, and the source of each plasmid are given.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Details</th>
<th>Antisense</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fgf-10</td>
<td>627 bp fragment of full length Fgf-10 cloned into pBluescript KS (Stratagene)</td>
<td>HindIII / T3 RNA pol</td>
<td>Dr. S. Werner, Revest et al., 2001a</td>
</tr>
<tr>
<td>Fgf-3</td>
<td>500 bp 3' untranslated sequence cloned into the vector pGEM4 (Promega)</td>
<td>EcoRI / T7 RNA pol</td>
<td>Dr. D. Wilkinson, National Institute for Medical Research, London</td>
</tr>
<tr>
<td>Fgf-7</td>
<td>Entire Fgf-7 coding region cloned into pGEM3 vector (Promega)</td>
<td>Pvull / SP6 RNA pol</td>
<td>Revest et al., 2001b</td>
</tr>
<tr>
<td>Fgf-8</td>
<td>Fgf-8 fragment cloned into pGEM9 vector (Promega)</td>
<td>HindIII / T7 RNA pol</td>
<td>Revest et al., 2001a</td>
</tr>
<tr>
<td>FgfR-2 (TK)</td>
<td>Cytoplasmic domain of Fgfr-2 cloned into pBluescript IISK+ (Stratagene). Nucleotides 1726-2764 (accession M63503)</td>
<td>BamHI / T7 RNA pol</td>
<td>De Moerlooze et al., 2000</td>
</tr>
</tbody>
</table>
### Appendices

**Probe** | **Details** | **Antisense** | **Source/Reference**
--- | --- | --- | ---
FgfR-2IIlb | The whole IIlb exon of FgfR-2 cloned into pBluescript IISK+. Nucleotides 1267-1417 (accession M63503) | BamHI / T7 RNA pol | De Moerloose et al., 2000
FgfR-2IIlc | The whole IIlc exon of FgfR-2 cloned into pAMPI (GIBCO BRL). Nucleotides 1599-1737 (accession X55441) | EcoRI / SP6 RNA pol | Dr. P. Kettunen, Kettunen et al., 1998
Lef-1 | 660 bp 3' truncation of original Gli-1 clone in a pBluescript vector. | EcoRI / T3 RNA pol | Dr. P. Kettunen, University of Helsinki, Keranen et al., 1998

### A6.4 cDNA Sequences

#### A6.4.1 Human ErbB-2 sequence

| ATG | GAG | CGG | GCC | GCG | TGG | TTC | CGG | GCC | TGG | CCC | GCA | GGC | GCG | AGC | ACC | GTG | 75 |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| IME | LAL | CLR | CWR | WWL | LALL | LALL | LPP | PGA | GAA | STQ | V | 25 |
| 153 | TOC | ACC | GCC | ACA | GAC | AGT | AAG | CGG | CTG | AGA | CGG | ACC | CTG | AGT | CTC | CCC | CAC | 150 |
| 351 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |
| 352 | CTC | GTT | GCT | AGC | AGG | AAC | CGT | GAG | CTC | ACC | TGG | ACC | AGG | TCT | TGG | ACC | GAC | ACC | 225 |

| 1051 | GCA | GGG | TCG | CGG | TGG | GAG | AGA | TCT | GCT | ACC | GAG | ACC | CGG | TGG | ACC | GAC | ACC | TGG | 1050 |
| 352 | COT | QRC | EXC | CSX | KTP | CAR | VCY | CTG | GLO | MEX | L | 350 |
| 1126 | TTT | CCG | CGG | GAG | AGC | TTT | GAT | GGG | GCA | GCC | TCC | ACC | GAG | ACC | TGG | ACC | GAC | ACC | TGG | 1200 |
| 351 | R | E | K | E | G | L | Y | I | S | H | A | W | P | D | S | L | P | D | L | S | V | 425 |
| 1276 | CAC | ACC | CTG | CGG | GAG | AGC | TTT | GAT | GGG | GCA | GCC | TCC | ACC | GAG | ACC | TGG | ACC | GAC | ACC | TGG | 1350 |
| 426 | Q | N | L | Q | V | K | G | R | I | G | H | N | A | S | L | T | L | Q | L | 450 |

1351 | GCC | TCG | CGG | GAG | AGC | TTT | GAT | GGG | GCA | GCC | TCC | ACC | GAG | ACC | TGG | ACC | GAC | ACC | CTC | TGG | 1425 |
A6.4.2 Human P75NTR sequence

```
1 2 0 1 CTC CTG GCC GCC CTG CGC CGC ATC CAG CGA GCC GAC CTC GTG GAG AGT CTG TGC AGT GAG TCC ACT GCC ACA TCC 1 2 7 5
```

A6.4.3 Human Met Receptor Sequence

```
1 2 0 1 CTC CTG GCC GCC CTG CGC CGC ATC CAG CGA GCC GAC CTC GTG GAG AGT CTG TGC AGT GAG TCC ACT GCC ACA TCC 1 2 7 5
```

Appendices
Bibliography


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Bibliography


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