Differentiation of
Transformed and Non-transformed
Human Mammary Luminal Epithelial Cells

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Artist may pursue Beauty
Priest may pursue Virtue
Scientist may pursue Truth
Only when he understands
and finds within
all of these qualities,
he shall complete his pursuit.

If he is to grow
his growth is said to be in the order of Truth, Virtue, Beauty and Mystery.
Mystery may be understood as a true form of freedom.

Dr and Master Yoshikazu Kawaguchi

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Abstract

Differentiation and its therapeutic potential were examined in the context of breast cancer. First, hormonal induction of differentiation was investigated *in vivo* using human breast tissues obtained at the 12\(^{\text{th}}\), 15\(^{\text{th}}\) and 18\(^{\text{th}}\) weeks of pregnancy. Multiple immunofluorescence labelling for markers of proliferation and markers of differentiation demonstrated that human mammary luminal epithelial cells (HMLEC) were capable of proliferation when they were phenotypically and functionally differentiated.

Second, pharmacological induction of differentiation was investigated *in vitro* using a longitudinal model of differentiation. This model was derived from a single parental line that represents immortalised but non-transformed HMLEC and consists of its transformed and control variants. Unlike horizontal models of cell lines obtained from different individuals, the longitudinal model allowed direct comparisons between transformed and non-transformed phenotypes as well as cause-and-effect correlation with a single oncogene such as *ErbB2* and *ras*. The present study conducted immunofluorescence labelling analysis to ensure that the cell lines utilised in the model exhibited stable and expected phenotypes. Pharmacological agents were then examined for their growth inhibitory effects. It was demonstrated that TNF\(\alpha\) selectively inhibited
the growth of ErbB2 over-expressing variants. Molecular investigations revealed that TNFα down-regulated the transforming oncogene ErbB2 at both the mRNA and protein levels. This was accompanied by the down-regulations of p21 and p53 at the mRNA level.

In summary, while differentiation does not coincide with loss of proliferative potential, certain pharmacological interventions can selectively inhibit the growth of transformed cells. Such effects can be accompanied by the down-regulation of transformation-associated genes. The in vitro model of breast cancer established in the present study provides a useful tool for further screening of pharmacological agents and studying the modes of drug actions.
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Chapter 1  Introduction

1.1 Breast Cancer

1.1.1 Epidemiology

Breast cancer is reported to be one of the commonest cancers, accounting for almost 20% of all malignancies worldwide, and over half a million women develop breast cancer every year (Parkin, Laara and Muir 1988). The incidence of and mortality from breast cancer vary greatly around the world. In England and Wales, the recorded age standardised incidence of breast cancer in 1992 was 102 per 100,000, indicating almost a 40% increase since 1979 following the introduction of the breast screening programme in 1988 (Quinn and Allen 1995).

Despite an underlying rise in incidence, 5-year survival seems to be improving. Age standardised mortality in the 55-69 age group was 107 per 100,000 in the mid-1980s (Quinn and Allen 1995), but between 1986-1990, the excess mortality is reported to have declined by 11% (Richards et al 2000). Furthermore, for this age group, 5-year survival was 7% higher for women who were diagnosed in 1993 than for those
diagnosed in 1989 (78.3% vs. 71.5%, respectively) (Coleman 2000). However since these findings may be influenced by the effect of accelerated diagnosis consequent of increased awareness as well as the breast screening programme (Stockton et al 1997), further studies on 10 year survival statistics are still required to confirm the true improvement in long term survival (Reynolds and Wierzbicki 2000).

According to the latest figures provided by the Cancer Research Campaign (www.crc.org.uk), the number of deaths due to breast cancer was 10,800 in 1999 and the annual number of new cases is in the region of 38,000. Breast cancer hence accounts for one in four of all female cancers.

1.1.2 Pathology

The National Health Service (NHS) offers mammograms (breast X-rays) to women between the ages of 50-64, every 3 years, as part of the national breast screening programme. Although mammograms can detect cancer before it can be felt as a lump, its impact on survival improvement is considered negligible (Quinn and Allen 1995, Baum 2000, Gotzsche and Olsen 2000). In fact, the vast majority of breast tumours are first detected by women themselves. Symptoms in the breast are change of size or shape, dimpling of the skin and presence of a lump. This may be accompanied by the nipple
becoming inverted or rarely appearing with rash or bloodstained discharge. Some patients also experience swelling in their armpit.

Diagnosis of breast cancer is made using imaging tools such as mammography, magnetic resonance imaging (MRI scan), ultrasound (used to see if a lump is solid or contains fluid) and colour Doppler (shows the blood supply to the lump and may help to distinguish between a cancer and a benign lump), followed by histopathological investigations on needle aspiration, needle biopsy or excision biopsy.

**Staging of breast cancer**

Breast cancers are staged according to the tumour size, lymph node involvement and signs of metastasis. The commonly affected lymph nodes are the axillary nodes and the nodes along the internal thoracic artery. Ultimately, the metastasis may appear in many viscera and in bones. Briefly, in stage I, breast cancers are <2cm with no sign of lymph node involvement or metastasis. Stage II breast cancers are 2-5cm or with lymph node involvement or both, but without signs of metastasis. In stage III, breast cancers are >5cm, and the lymph nodes are usually affected, but there is no sign of metastasis. Stage IV breast cancers are of any tumour size, but the lymph nodes are affected and metastasis has spread to other parts of the body. It is also important to note that the
5-year survival is much poorer for later stage breast cancer: 20% for stage IV at presentation compared with 85% for stage I (Quinn and Allen 1995).

**Grading of breast cancer**

Pathologists further employ grading systems on histopathological samples, in order to predict the prognosis of overall and disease free survival (Sloane, Trott and Lakhani 2001). The most widely applied system in the UK is the Bloom and Richardson system modified and adopted for the European Breast Screening Programmes (Elston and Ellis 1991). The grading (of infiltrating carcinoma) is based on tubule formation, pleomorphism and mitotic counts. Tubule formation is concerned with the proportion of ductal or glandular differentiation, indicated by the presence of clearly defined lumen. More than 75% scores 1, between 10-75% scores 2 and less than 10% scores 3. Spaces formed as a consequence of other mechanisms, such as poor cellular cohesion or cellular necrosis, are excluded. For pleomorphism, score 1 represents small, regular nuclei as in normal breast epithelial cells with uniform chromatin. Score 2 indicates significantly larger nuclei with open vesicles and moderate variability in size and shape. The highest score 3 stands for large, vesicular and prominent nuclei with a marked variation in size and shape and occasional very large and bizarre forms. Mitotic count is
obtained according to the European breast screening programmes standard, and these scores are added together to indicate the Grade 1 (score 3-5), Grade 2 (score 6-7) and Grade 3 (score 8-9).

**The Nottingham Prognostic Index (NPI)**

Out of these prognostic indicators, lymph node status is considered the most important pathological factor (Sloane, Trott and Lakhani 2001). However, it is a time-dependent factor, not taking account of the innate aggressiveness of tumours. Therefore, the NPI is applied to include the three most significant prognostic indicators; tumour size, lymph node status and grade \(0.2 \times \text{size in cm} + \text{stage (lymph node, 1-3 by number of nodes involved; 0 nodes = 1, 1-3 nodes = 2, >3 nodes = 3) + grade}\). According to the NPI, the 5 year survival rate of the poor prognostic group (index > 5.4) is approximately 20%, whilst moderate prognostic group (index 3.41-5.4) 70% and the good prognostic group (index 2.1<3.4) 95%.

**1.1.3 Treatments for breast cancer**

The first line of treatment for breast cancer is surgery. Depending on histopathological diagnosis, surgical procedure can vary between lumpectomy / wide local excision,
segmental excision / quadrantectomy, simple mastectomy (the removal of the breast), radical mastectomy (the additional removal of the muscles on the chest wall) and modified radical mastectomy (the removal of the breast and lymph glands, leaving the chest wall muscles intact). The axillary lymph nodes are usually removed at the same time for the investigation of lymph node metastasis.

In order to reduce the risk of recurrence, radiotherapy may be prescribed following surgery (occasionally only radiotherapy is given without surgery). Radiotherapy aims to destroy remaining cancer cells by using high-energy rays, and the selective use of irradiation in high-risk patients provides both an improvement in local control and an improvement of 8-10% in the survival rate (Taghian and Powell 1999).

Adjuvant therapy consists of drug treatments given after the initial surgery to reduce the risk of recurrence and micrometastasis (Fisher et al 1999). Over the past three decades conventional adjuvant chemotherapy regimens have also been employed pre-operatively (neo-adjuvant or primary chemotherapy) (Smith, Hutcheon and Heys 2000).

The two main types of adjuvant therapy are hormone therapy and chemotherapy. In postmenopausal women with oestrogen receptor (ER) positivity and axillary lymph node involvement, the use of hormone therapy (tamoxifen for up to 5
years) has become standard treatment (Pinedo and Giaccone 1997). However, additional chemotherapy may be used depending on factors such as the tumour size, tumour grade, hormone receptor status and the extent of lymph node involvement. In premenopausal women, chemotherapy is more commonly used, although additional tamoxifen has also been shown to be of benefit (Aebi et al 2000, Sugimachi et al 1999). Anti-cancer (cytotoxic) drugs are prescribed orally or intravenously as a course of treatment, which usually lasts a few days. For the treatment of breast cancer, anthracycline-containing combination chemotherapy is reported to be more effective than other agents without anthracyclines (Pinedo and Giaccone 1997). Additional alternate treatment with paclitaxel as adjuvant therapy seems to further reduce the risk of recurrence and improve survival (Buzdar and Hortobagyi 1999, Ravdin 1997).

1.1.4 Treatments for advanced breast cancer

For advanced breast cancer (recurrence and metastasis), the aim of the drug treatments is to shrink the existing breast cancers, or to decrease the rate at which they grow or spread.
Hormone therapy

The most widely used hormone therapy is Tamoxifen (Nolvadex, or Tamofen) (Fisher et al 1999), and it is effective in reducing the risk of recurrence and death when administered to patients with ER-positive tumours. The benefit of tamoxifen is significant, irrespective of age, menopausal status and whether tamoxifen was given alone or in association with chemotherapy (Buzdar and Hortobagyi 1999). Other selective oestrogen receptor modulators (SERMs) such as Toremifene (Fareston) exist, but their superiority in effectiveness or reduction of side effects is inconclusive at present (Holli et al 2000, Buzdar and Hortobagyi 1998). Anti-oestrogen is the first line of hormone therapy, but if breast cancers do not respond, alternatives are as follows: artificial progesterones such as megastrol acetate (Megace) and medroxyprogesterone acetate (Farlutal, Provera); aromatase inhibitors that block the production of oestrogen in adipose tissues such as anastrozole, letrozole and formestane; pituitary down regulators such as goserelin (Zoladex) that reduce the production of oestrogen-stimulating hormones. For premenopausal women, permanent surgical removal or radiotherapy on the ovaries can also be an effective method for reducing the level of oestrogen in the body, although the reversible hormone therapy has been considered more preferable in recent years.
**Chemotherapy**

For metastasis affecting the liver or lungs, chemotherapy is used as the first treatment. It is also applied if patients do not respond to hormone therapy. For advanced breast cancer, the combination of paclitaxel with doxorubicin appears highly effective, and taxanes have activity in patients who are refractory to anthracyclines (Pinedo and Giaccone 1997). High-dose chemotherapy with haemopoietic-progenitor-cell support is widely used in high-risk breast cancer (Antman et al 1997), although this therapy has a moderate risk of morbidity and mortality, and the superiority of high-dose chemotherapy over conventional chemotherapy has not been established by randomised trials (Buzdar and Hortobagyi 1999, Rodenhuis et al 1998).

*Why new therapy is required*

The currently available treatments are far from sufficient, failing patients particularly with advanced breast cancer (Tannock 1998, Quinn and Allen 1995). Hormone therapy (Tamoxifen) is applicable only to patients with breast cancer that is positive for ER. Chemotherapy is associated with considerable side effects due to the narrow gap between the optimum cytotoxic dosage against breast cancer cells and the concurrent
damage incurred to normal somatic cells of the patients. Furthermore, resistance to
treatment can develop following either of these therapies.

Therefore, for both preventative and therapeutic purposes, new treatments need
to be developed. Properties such as a low level of side effects and selectivity towards
cancer cells compared to normal somatic cells are highly desirable. Potential new
therapeutic candidates include combination therapy, where different treatments may be
applied together with the existing hormone and / or chemotherapeutic agents for
synergistic effects and reduction of side effects (Huang and Oliff 2001).

**Intelligent drug design**

The current best example of intelligent drug design is based on an oncogene human
epidermal growth factor receptor-2 /C-ERBB-2 / HER2 (ErbB2) that is amplified in
approximately 30% of breast cancers. The transforming effect of ErbB2 *in vitro* and the
clinical association with poorer prognosis brought the development of a monoclonal
antibody Herceptin (Trastuzumab) that targets against this specific breast cancer marker.
Herceptin achieves significant improvement in survival of metastatic ErbB2 positive
breast cancer patients when combined with chemotherapeutic agents (for review see
Piccart 2001, Kumar, Mandal and Vadlamudi 2000). Another receptor specific therapy
in development is IRESSA / epidermal growth factor receptor (EGFR-1) tyrosine kinase inhibitor ZD1839 (Arteaga and Johnson 2001, Barker et al 2001, Mendelsohn and Baselga 2000), which is currently undergoing clinical trials.

Whilst the success of the intelligent drug design remains statistically significant yet modest (Piccart 2001, Kumar, Mandal and Vadlamudi 2000), novel genes and proteins are being discovered at an ever-increasing pace. Computer aided differential analysis is yielding hundreds of potential breast cancer markers from the 30-40,000 genes identified as the human genome (Perou et al 2000). However, it is likely that only a few of these will prove therapeutically relevant. To be so, they need to meet two fundamental criteria. First, a novel breast cancer marker must be expressed in a sufficiently large proportion of patients to engage interest in terms of costly and time-consuming drug development. Second, its function must be demonstrated to drive the actual tumour progression. Therefore, for these target specific therapies to be developed, the thorough understanding of human mammary biology plays a crucial role.
1.2 The breast

1.2.1 Anatomy

The breast, also known as the mammary gland, is present in both the male and female, and its structure is essentially similar in both sexes until puberty. It lies on the thoracic wall, attached to the pectoralis muscles by fasciae over the 2\textsuperscript{nd} and 6\textsuperscript{th} ribs. Each fully developed breast in adult consists of 12-20 lobes, subdivided into lobules, which bear numerous secretory acini / alveoli. Acini drain into intralobular ducts which in turn drain into interlobular ducts, then into separate lactiferous sinuses and from there into lactiferous ducts which open onto the nipple. The space between alveoli is filled with interstitial tissue that contains varying amounts of adipose tissue as well as other cell types such as fibroblasts and blood vessels and lymphatics lined by endothelial cells.

1.2.2 Physiology

At birth, the extent of morphological development in human breast is highly variable (Anbazhagan \textit{et al} 1995). During the first few postnatal days, maternal hormones may cause a transient secretory activity in the infant breast, and up to two subsequent months, additional secretory development may occur, with areas of localised hyperplasia.
At puberty in the female, the breast increases in size, mainly due to the deposition of interlobular fat, but further ductal and alveolar development is believed also to take place (Vorherr 1974). By adulthood the ductal architecture is complete, and the breast remains in this mature inactive state with relatively limited menstrual cycle associated changes. During the luteal phase, the luminal epithelial cells become more columnar and cellular proliferation increases. This is followed by abrupt involution and apoptosis shortly before the onset of menstruation (Topper et al 1986, Neville 1983, Fanger and Ree 1974).

During pregnancy, in response to hormones initially from the corpus luteum (oestrogen and progesterone), followed by placental hormones (oestrogen, progesterone and somatotropin), pituitary hormones (prolactin) and adrenocorticoids from the adrenal gland, there is a steep rise in the proliferation rate of the breast epithelial cells (Figure 1.1). This is accompanied by dramatic morphological changes, characterised by glandular-alveolar growth, expansion of acini (Figure 1.2) and polarisation of luminal epithelial cells (Battersby and Anderson 1988, Vorherr 1974).

At parturition, an abrupt drop in blood oestrogen and progesterone level occurs. For the first few days, the breast secretes colostrum, a milky fluid with a low lipid content. Colostrum contains maternal antibodies, conferring passive immune protection
to the infant. Following colostrum, the secretory luminal epithelial cells become fully active, producing large volumes of milk. Milk synthesis is maintained by prolactin, released from the anterior pituitary in response to a suckling stimulus, and the alveolar contents are expressed in response to oxytocin (Vorherr 1974). At the end of the suckling period, lactation ceases. Epithelial cell numbers are reduced through apoptosis, and the breast regresses back to the mature but inactive stage.

With the onset of the menopause, the levels of circulating ovarian hormones fall, and the ductal elements degenerate and dense connective tissue replaces the intralobular loose connective tissue.

1.2.3 Mammary Biology

The inside of the mammary tree is lined with luminal epithelial cells, which are in direct contact with another epithelial population called myoepithelial cells (Figure 1.1). They are located between the luminal epithelial cells and basement membrane, which separates the mammary epithelium from the interstitial tissue (Figure 1.2). The phenotypic differences between these two populations can be readily demonstrated by expression of cytokeratin (K) 18, K19, mucin 1 (MUC1) and epithelial membrane antigen (EMA) in luminal epithelial cells and expression of K4, K14, K17, cluster
differentiation antigen (CD) 10 and β4 integrin subunit in myoepithelial cells. The expression of these specific markers is associated with phenotypic differentiation, which means that the cells have reached the lineage maturity. Once phenotypically differentiated, under normal physiology, the expression of these markers is stably maintained, and the phenotypically differentiated cells are destined to carry out functions that are unique to their phenotype.

The function of luminal epithelial cells is to synthesise milk. Therefore, those that are capable of producing milk proteins such as caseins and lactalbumin are considered functionally differentiated. Functional differentiation of luminal epithelial cells takes place during pregnancy and lactation. Myoepithelial cells are contractile, and they are responsible for the ejection of milk in response to oxytocin.
Figure 1.1

Quadruple multi-immunofluorescence labelling on the human breast tissue obtained at the 15th week of pregnancy. During pregnancy, a greater proportion of breast epithelial cells are in active proliferation compared to the non-pregnant stage. The increase in the number of luminal epithelial cells results in the expansion of alveoli and is followed by the induction of milk synthesis. Luminal epithelial cells are labelled for cytokeratin 19 (blue), myoepithelial cells for cytokeratin 14 (red) and fibroblasts for vimentin (green). Hoechst stained nuclei are shown in blue. Cells in active proliferation are labelled for Ki67 (white).

Figure 1.2

Triple multi-immunofluorescence labelling on the human breast tissue obtained at the 18th week of pregnancy. The extensively dilated alveoli contain milk proteins and lipid droplets. The surrounding myoepithelial cells and the basement membrane become highly stretched. Collagen IV in the basement membrane is shown in red and collagen I in the interstitial tissue shown in green. Hoechst stained nuclei are shown in blue.
1.3 Differentiation and transformation

1.3.1 Overview

It has been proposed that there is an inverse correlation between differentiation and transformation (Figure 1.3). It is true that some differentiation characteristics can be lost in hyperplasia (inappropriate but self-limited growth in response to a signal), dysplasia (indifference to functional and structural signals leading to a disordered appearance) and neoplasia (genetic instability that leads to a clonal and progressive accumulation of mutations and unlimited growth).

In the context of breast cancer, the inverse correlation between differentiation and transformation has been established from *in vivo* evidence from clinical samples. Infiltrating ductal carcinoma (the commonest form of breast cancer) is assigned to a histologic grade depending on the degree of differentiation of the tumour cells. Differentiation is defined as how closely they recapitulate normal mammary ducts. In a retrospective study of 1,600 invasive breast cancers, the grade of the tumours has been shown to correlate with the stage of the disease, disease-free survival after treatment and ER positivity (Fisher, Redmond and Fisher 1980). The high-grade cytologic characteristics, however, do not necessarily indicate poor prognosis. For example,
medullary carcinoma is composed of very atypical cells, but it is well circumscribed and contains a heavy inflammatory infiltrate. Despite the seemingly high-grade appearance of the tumour cells, medullary carcinoma generally carries a good prognosis even when axillary nodes are involved.

Nonetheless, if it is true that differentiation and malignancy are reciprocal, then this might imply that the induction of differentiation in cancer cells could lead to a reduction in their malignant potential.

1.3.2 Differentiation inducers as potential therapy

Previous development of cytotoxic cancer therapy has been predominantly based on the belief that cancer cells invade the "host" like an autonomous pathogen and that carcinogenesis is an irreversible process (Bodmer and Murday 1995, Rew 1998). However some modern researchers suggest that cancer cells are largely "normal". At the genetic level, with the exception of deletion, the majority of DNA remains intact to the extent that the metabolic machinery of cancer cells have a growth advantage over the "host" (Schipper, Turley and Baum 1996). Furthermore some malignant cells retain the ability to express differentiation characteristics when induced by appropriate stimuli (Anderson, Gage and Weissman 2001, Scott 1997) (Figure 1.3).
Various chemical substances have been found to act as the “appropriate stimuli” and they are referred to as differentiation inducers. Differentiation inducer is a category applicable to substances that stimulate cells to exhibit changes associated with differentiation. Beyond this, they do not share definable characteristics in a biochemical or pharmacological sense. Well-documented differentiation inducers include dietary nutrients such as retinoic acid, synthetic chemical compounds such as dimethyl sulfoxide (DMSO) and hexamethyl bisacetamide (HMBA) and peptide growth factors such as transforming growth factor-β (TGF-β) and heregulin. Studies of these differentiation inducers have been mostly limited to in vitro findings, but the therapeutic potential of retinoids (Camerini et al 2001, Douer et al 2001, Zujewski et al 1999) and HMBA (Andreeff et al 1992) has been investigated in animal models and human clinical trials. Differentiation inducers may be promising therapeutic agents in that they inhibit the growth rate of cancer cells (by induction of both differentiation and apoptosis) and hence delay the recurrence and prolong the overall survival of patients. Potentially, differentiation inducers can reverse, limit or delay the development of carcinogenesis (Scott 1997, Ferrari and Waxman 1994, Beere and Hickman 1993), but the currently available differentiation inducers suffer from drawbacks such as high

To identify novel differentiation inducers for cancer therapy, there requires the establishment of an *in vitro* model that allows screening and examination of underlying biological mechanisms of differentiation.
Figure 1.3 A conceptual diagram demonstrating the inverse correlation between differentiation & transformation and the possible influence of differentiation inducers.
1.4 Primary aims of the present study

The present study aimed to examine the therapeutic relevance of differentiation in context of breast cancer. There are two means by which the induction of differentiation could theoretically treat breast cancer. One is to achieve total growth arrest in breast cancer cells, and the other is to interfere with malignant properties that drive the progression of breast cancer cells. These two concepts were investigated \textit{in vivo} and \textit{in vitro} respectively.

1.4.1 \textit{In vivo}

\textit{Background of immunohistochemistry}

Histological and immunohistochemical comparisons between normal (non-transformed) breast epithelial cells and cancer (transformed) cells are possible using clinical samples. Normal breast epithelial cells can be examined in the breast tissues surrounding the primary breast tumours as well as in breast tissues obtained from cosmetic reduction mammoplasty, and cancer cells in primary breast tumours, invaded lymph nodes and sites of metastasis such as the liver.
In contrast, there has been very little progress on the subject of functional
differentiation of breast epithelial cells. The main reason for this is the scarce
availability of functionally differentiated breast tissues, i.e. those from pregnant and
lactating women. The lack of understanding on functional differentiation leaves the
investigation on the inverse correlation between differentiation and transformation
incomplete. The problem is deepened by the fact that functional differentiation of
human breast epithelial cells has never been successfully modelled \textit{in vitro}. The
currently available models thus are largely rodent models both \textit{in vivo} (Pepper \textit{et al}
1995, Franke and Keenan 1979) and \textit{in vitro} (Neville, Morton and Umemura 2001,
Neville 1983, Smith and Vonderhaar 1981), with one exception of a human breast tissue
xenograft model in pregnant nude mice (Dubois \textit{et al} 1987).

The only direct immunohistochemical study reported on human breast tissues
from pregnant women was conducted on archival materials (Battersby and Anderson
1988). This study provided detailed description on morphological development, the
proliferation index of breast epithelial cells and functional differentiation indicated by
labelling for $\alpha$-lactalbumin. However, archival samples are normally fixed in formaline
and embedded in paraffin. While they are excellent for morphological integrity, for
immunohistochemical labelling they require a procedure to retrieve the antigen.
In contrast, freshly frozen tissue samples do not require this procedure since they are unfixed and cut as frozen sections. Hence for multi-immunofluorescence technique, which labels for more than one antigens at a time, freshly frozen tissue samples can examine a greater range of antigens than archival materials. One of the main problems with freshly frozen tissue samples at present, however, is probably its inaccessibility. For systematic studies, there requires a panel of high quality samples that have been snap frozen in liquid nitrogen as soon as they have been resected from patient’s body. This needs a great deal of collaboration with medical staff as well as well-maintained storage system for such precious samples. These factors, at least partially, explain why there have been very few studies on functional differentiation of human breast up until now.

**Background of differentiation and proliferative potential**

Some researchers believe that the breast epithelium consists of undifferentiated stem cells and differentiated epithelial cells, and the latter is considered to have little or no ability to proliferate (Anderson and Clarke 1999, Chepko and Smith 1999, Li et al 1998). For example, Vorherr (1974) writes in The Breast ‘proliferating mammary tissues cannot exert specialised functions and conversely differentiating mammary
epithelium cannot effectively proliferate'. However, it is important to point out that in human breast biology the inverse correlation between differentiation and proliferative potential is supported by little evidence. In fact, the existing evidence is by and large limited to findings on the rodent mammary glands. The idea that induction of differentiation may achieve total growth arrest of breast cancer cells is based on a conventional theory that differentiation coincides with loss of proliferative potential. It has been hypothesised that if differentiation was induced in breast cancer cells, it might concurrently deprive them of the ability to proliferate, thereby preventing further growth of breast cancer. Should this hypothesis be true, pregnancy induced hormonal interventions such as prolactin or differentiation inducers like heregulin that stimulates milk synthesis will have direct relevance to breast cancer therapy.

**Approaches of the current study**

To examine whether induction of differentiation could indeed result in the reciprocal growth arrest, the present study conducted multi-immunofluorescence labelling on human breast tissues obtained from pregnant women. Therefore, the primary aim of the
in vivo studies was to determine whether luminal epithelial cells, once differentiated, irreversibly lose or continue to retain their proliferative potential.

With recent advances in cancer therapy, it is now possible to surgically resect primary breast tumours from pregnant women followed by chemotherapy without significant damage to the foetus. Such operations inevitably remove histologically normal human breast tissues adjacent to the tumours, and this provides an excellent opportunity to obtain freshly frozen tissue samples for studying functional differentiation of human breast.

In the present study, a panel of human breast epithelial tissues from pregnant women was investigated with multiple-immunofluorescence labelling. Markers of proliferation, phenotypic differentiation and functional differentiation were studied for their expression patterns. In addition, integrins and extracellular matrix (ECM) components were examined, since they have been shown to exert regulatory influences on mammary differentiation and morphogenesis in vitro and in the rodent mammary gland (For review, see Petersen et al 1998).
1.4.2 *In vitro*

This section focuses on the *in vitro* application of human breast cancer cells and their normal counterpart, human mammary luminal epithelial cells (HMLEC). Both of these can be cultured either as primary cell populations or as cell lines. The word ‘primary’ in this context means that the cells have been obtained directly from patient’s tissue samples, irrespective of site of origin. While primary normal adult human cells have a limited *in vitro* life-span, cell lines have accumulated or been engineered to express the genetic changes necessary for indefinite growth *in vitro*. Hence the latter are often described as ‘established’ or ‘immortalised’.

**Background of primary breast cancer cell culture**

When cultured *in vitro*, the normal breast epithelial cells readily attach to a plastic substratum, spread outwards and proliferate. In contrast, malignant breast epithelial cells often remain non-adherent and grow slowly, if at all.

This presents the apparent paradox that while *in vivo* breast cancer cells grow and invade over the surrounding breast tissue (Hanahan and Weinberg 2000), *in vitro* they generally exhibit less growth capacity than normal cells (Masters 2000, O’Hare 1991). It may be that the *in vitro* culture lacks specific factors on which breast cancer
cells have been dependent for growth in the patient’s body. However, an alternative explanation is that the *in vitro* growth exhibited by the normal cells is a physiologically intrinsic wound healing response that differs from the malignant growth of breast cancer cells. The latter line of reasoning has two implications. First, most primary breast cancer cells seem to have lost this wound healing response. Second, manipulating primary breast cancer cells to grow rapidly *in vitro* may, therefore, result in an inappropriate model, divergent from true tumour biology.

**Background of breast cancer cell lines**

As an alternative, most researchers use breast cancer cell lines. The obvious advantage of cell lines, if handled correctly (UK Co-ordinating Committee on Cancer Research Guidelines), is that *in vitro* experiments are readily reproducible, and some breast cancer cell lines can retain many important features of the original tumours (Wistuba *et al* 1998).

Nevertheless, there remain problems associated with the use of breast cancer cell lines. The first is that they can hardly be considered as representative of most breast cancer cells *in vivo*. This is evident from the fact that very few primary breast cancer cells establish as cell lines. Furthermore, many of the more commonly used lines were
derived from late stage breast cancer i.e. pleural effusions or ascites (Sutherland et al 1999, Cailleau et al 1974). Secondly, despite the expanding number of breast cancer cell lines available, the majority of in vitro research papers are still based on a small number of cell lines (Figure 1.4). Obviously, such a limited range is even less likely to represent the majority of clinical breast cancers and may seriously bias the scientific conclusions.
Figure 1.4

Numbers of publications in which breast cell lines were mentioned by name in either title or abstract were searched via MedLine for years 1966-2000. A total of 26 cell lines were investigated, and the top ten are shown.
Background of primary normal breast epithelial cell culture

The commonest sources of primary normal breast epithelial cells is breast tissue obtained from reduction mammoplasty, which can yield a substantial number of primary breast epithelial cells after removing stromal components through a series of mechanical and enzymatic processes (Easty et al 1980).

An important but frequently overlooked fact is that such primary cultures contain two phenotypically and functionally distinct epithelial cell types, the luminal epithelial population and the myoepithelial population (Figure 1.5). Based on cytokeratin and membrane antigen profiles, the majority of breast cancers have been shown to possess a luminal epithelial phenotype (Taylor-Papadimitriou, Wetzels and Ramaekers 1992, Taylor-Papadimitriou et al 1989, Guelstein et al 1988). Therefore, to obtain their normal counterpart, primary cultures of breast epithelial cells need to undergo a cell sorting process such as Fluorescence-Activated Cell sorting (FACS) (O'Hare 1991) or equivalent immunomagnetic separation systems (Gomm et al 1995, Clarke et al 1994). Without sorting, primary cultures consist of a mixture of cell types at earlier passages and predominantly of myoepithelially-derived cells with a basal phenotype at later passages, because the latter have a greater proliferative life span in vitro.
Distinction between a sorted population, referred to as HMLEC (human mammary luminal epithelial cells) and unpurified cells, HMEC (human mammary epithelial cells), is becoming increasingly important in the interpretation of differential gene and protein analysis. Recently, the global patterns of protein expression in freshly isolated normal luminal and myoepithelial cells have been analysed by proteomics (Page et al 1999), demonstrating that about 10\% of all proteins are expressed at significantly different levels in the two cell types. Comparisons between breast cancer cells and the unpurified HMEC therefore tend to identify lineage specific differences in cytokeratins and membrane antigens rather than transformation specific changes (Nacht et al 1999, Trask et al 1990).

Normal primary HMLEC have a relatively short in vitro life span of no more than 20 doublings (O'Hare 1991). Hence, as with breast cancers, immortalised breast epithelial cell lines are often employed as an alternative.
Figure 1.5

Immunofluorescence images showing the expression of the intermediate filaments vimentin (green), K14 (red), K18 (purple) and K19 (blue), in primary culture of normal breast epithelial cells, before (left column) and after immunomagnetic sorting for luminal epithelial cells (centre column) and myoepithelial cells (right column).
Background of normal breast epithelial cell lines

To provide the ‘normal’ counterparts of breast cancer cell lines, breast epithelial cell lines should be non-transformed and have a luminal epithelial phenotype. There are now about 20 non-malignant breast epithelial cell lines. While some of these lines were apparently immortalised spontaneously (Soule et al 1990, Briand, Petersen and Van Deurs 1987), others were established by artificial means such as exposure to chemical mutagens (Stampfer and Bartley 1985) or by use of specific viral genes (Stamps et al 1994, Band et al 1990). The advantage of viral proteins (e.g. SV40 large T antigen, HPV E6/E7) compared to spontaneous or chemical immortalisation is that the mechanism is specifically defined. Immortalisation with viral genes does interfere with normal growth regulatory molecules such as p53 and Rb (Band et al 1990). However, this problem can be reduced by the use of a temperature-sensitive mutant of the SV40 large T antigen that allows conditional immortalisation, where cells grow at 33°C but arrest at 39.5°C (Stamps et al 1994).

Background of in vitro models of breast cancer

In general, in vitro studies based on a single cell line should be interpreted with caution, since many signalling pathways and ligand responses that influence the transformed
phenotype are cell type or cell line specific (Keane et al 1999). The use of multiple cell lines is therefore preferable, and this can be applied as either horizontal or longitudinal models.

In a horizontal model of transformation, a number of cell lines from different individuals are compared with each other. There are, therefore, pre-existing variables such as the age of donors and grade/stage of tumours as well as patterns of genetic abnormalities in the different lines, which could influence their response to a specific culture environment.

To overcome the inherent heterogeneity of horizontal models, longitudinal models of transformation were developed in the 1990’s. In a longitudinal model, the starting point is a single parental cell line. This line is then modified to give rise to variant cell lines by, for example, long term culture under selective conditions (Briand et al 1996), exposure to a chemical carcinogen or insertion of specific genes (Harris et al 1999).

Longitudinal models are particularly useful for determining the functional importance of a specific gene. Although these longitudinal models allow direct cause and effect observations, ideally, not a single but a panel of longitudinal models should be used, in order to take into account possible variability originating from the unique
nature of each different parental cell line. However, such extensive studies are rare at present. Bearing the possibility of developing an extensive panel in the future, the current study investigated the applicability of one longitudinal model of transformation (LMT) for the study of differentiation and transformation.

**Approaches of the current study**

The inverse correlation between differentiation and transformation implies that the induction of differentiation in breast cancer cells may reduce their transformed phenotype, resulting in less malignant or so-called less 'aggressive' disease. Once again, however, the evidence supporting such concept is limited at present. As discussed earlier, in histopathology, morphologically less differentiated breast cancers are associated with poorer prognosis. Nonetheless, there are currently no molecular markers generally applicable to indicate the level of differentiation or transformation as such. *In vitro*, both differentiation and transformation are subjectively defined concepts, and the inverse correlation between the two are loosely assumed rather than proved.

In order to investigate whether the induction of differentiation can bring a therapeutically favourable outcome in breast cancer, there is a requirement for an *in vitro* model that represents a scale of transformation and differentiation. Many of such
existing models are horizontal in that a number of cell lines from various individuals are compared with each other. However, the present study concentrated on the development and use of a LMT, so that direct comparisons between normal HMLEC and transformed HMLEC could be made.

The LMT of breast cancer

The in-house LMT consists of HB4a and its existing variant cell lines. HB4a was derived from HMLEC prepared by FACS and immortalised (but not transformed) by the temperature sensitive A58-U19 mutant of the SV40 polyoma large T antigen (Stamps et al 1994). Two HB4a variants, C3.6 and C5.2, were engineered by DNA plasmid transfection to over-express the breast cancer-associated proto-oncogene ErbB2. FACS analysis was used to demonstrate the different ErbB2 expression levels on the surface of C3.6 and C5.2, which were lower than and equivalent to the level found in an ErbB2 over-expressing breast cancer cell line SKBR-3, respectively. Another line, H4.1, was generated concurrently as a transfection control (Harris et al 1999). Another HB4a variant, R4.2, was subsequently engineered to express mutant active Ha-(Val12)-Ras to represent an example of a cell line whose transformed growth properties are not dependent on ErbB2.
Collectively, the panel of HB4a, H4.1, C3.6, C5.2 and R4.2 was applied as an experimental LMT of breast cancer. The main aims of the current study were 1) to characterise the cell lines of the LMT by immunocytochemistry, 2) to determine whether the LMT can screen for pharmacological selectivity against the transformed cells and 3) to demonstrate potentials of the LMT for studying modes of drug actions at molecular levels.

Characterisation of the LMT

Although cell lines are convenient alternatives to primary cells for in vitro experiments, utmost care should be taken to monitor their phenotypes. Breast cancer cell lines can be susceptible to phenotypic drifts due to their genetic instability. Even normal breast epithelial cell lines may lose their original phenotype at both genetic and epigenetic levels. In order to ascertain that the cell lines utilised in the LMT are close and stable representatives of primary counterparts, their phenotypes were characterised in detail by immunofluorescence labelling for a selected range of molecular markers. One of the most important points to be addressed in the present study was the non-transformed and luminal epithelial phenotype of HB4a, since it is the parental line of the LMT. The variant lines of HB4a were also examined to demonstrate the varying degrees of alterations in the expressions of these markers.
Growth assays of the LMT

The ability to specifically attack cancer cells while sparing non-transformed cells is of immense therapeutic value. To address whether the LMT can screen for pharmacological selectivity against the transformed cells, three pharmacological agents were selected and their effects on growth rates were studied. They were Tumour Necrosis Factor alpha (TNFα), Tumour Necrosis Factor-related apoptosis-inducing ligand (TRAIL) and Dimethyl Sulphoxide (DMSO). The growth inhibitory effects of TNFα and TRAIL have been previously reported to be selective against the transformed phenotype, while DMSO is expected to act non-selectively.

Molecular investigation of the LMT

Once therapeutic candidates with appropriate selectivity are identified, their modes of actions as well as potentials in combination therapy must be investigated. To address the third point i.e. whether the LMT can be used for studying modes of drug actions, RT-PCR and Western blotting were applied to C3.6 populations. Differential regulation of key molecules was examined at mRNA and protein levels respectively. Three types of growth inhibited populations (growth arrested at the non-permissive temperature 39.5°C, growth inhibited by DMSO and growth inhibited by TNFα) were compared with the cell population in growth phase and also with one another.
Chapter 2
General Methods

2.1 Immunohistochemistry

Sources of reagents, consumables and computer programs are listed as the appendices, unless otherwise stated.

2.1.1 Tissue details

Three human breast tissue samples were obtained at the 12th, 15th (age 30) and 18th (age 31) weeks of pregnancy from patients undergoing surgery for breast cancers. The samples representing the 12th and 15th weeks of pregnancy were acquired from the same patient who underwent sequential resections. Four quiescent human breast tissue samples (age 30 parity 0; 32 parous but parity unknown; 40 parity 0; 43 parity 2) were obtained with consent from non-pregnant non-lactating pre-menopausal women undergoing cosmetic reduction mammoplasty. Subsequently, the human breast tissue samples obtained from pregnant women and non-pregnant women are referred to as the pregnant breast and the non-pregnant breast respectively.
All the samples were snap-frozen in liquid nitrogen and stored at -70°C. Frozen sections (5µm) were collected onto Vectabond treated glass slides, air-dried for two hours at room temperature and stored at -70°C. The sections were brought to room temperature, fixed with 1:1 mixture of chloroform and acetone (-20°C) for 5 minutes and washed in running tap water. Representative sections were stained with haematoxylin and eosin (H&E) to define the overall morphology and to select areas for immunohistochemical labelling. The sections were dipped into Harris type haematoxylin for 3 seconds, rinsed under running tap water for 3 minutes, dipped into eosin for 1 minute and rinsed under running tap water for 1 minute.

2.1.2 Labelling

The following procedures were carried out at room temperature. The vehicle for all immunolabelling incubations was 0.5% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS), and antibodies were applied at a total of 100µl per section. Incubation was carried out on a shaker, and in between each step, slides were washed three times in PBS. Sources of primary antibodies are provided in Table 2.1.

With minor changes to a method described previously (Atherton et al 1994) immunoperoxidase labelling was carried out to examine the labelling patterns of
individual primary antibodies on all the samples. The sections were first blocked with 100μl of 0.5% (w/v) BSA in PBS for 30 minutes. Primary antibody was applied for 1 hour, followed by the corresponding secondary antibody for 1 hour (Table 2.2). The slides were dipped in freshly prepared diaminobenzidine tetrahydrochloride, 3-3’ diaminobenzidine (DAB) solution containing 5 mg DAB and 0.1ml of 30% hydrogen peroxide per 100 ml of PBS for 5 minutes, rinsed in running tap water for 3 minutes, counterstained in Harris’ haematoxylin for 20 seconds and rinsed in running tap water for 3 minutes. The slides were finally dehydrated by 1 minute exposure to 70% ethanol, 2 sets of 100% ethanol and 2 sets of xylene. Glass covers were placed unto the sample sections using DPX mountant.

Multiple immunofluorescence labelling was carried out using combinations of primary mouse monoclonal antibodies of different immunoglobulin classes and subclasses together with appropriate class and subclass-specific anti-mouse secondary antibodies, as well as combinations of primary antibodies of different species (rabbit) together with species-specific secondary antibodies. The sections were first blocked with 100μl of 0.5% (w/v) BSA in PBS for 30 minutes. A mixture of primary antibodies was applied for 1 hour. After washing, the corresponding mixture of secondary fluorophore-conjugated antibodies was applied for 1 hour (the combinations of primary
and secondary antibodies for double labelling are shown in Table 2.3. and for quadruple labelling in Table 2.4.) For quadruple labelling, one of the secondary antibodies was biotinylated, and hence streptavidin-7-amino-4-methyl-3-coumarinylacetic acid (AMCA) conjugate was further applied for 30 minutes. After washing, all the sections were finally incubated in 2μg/ml bisbenzimide trihydrochloride (Hoechst 33258) in autoclaved distilled water for two minutes to stain nuclei (Rost 1995). The sections were mounted using 1:1 mixture of Hydromount and Citifluor antifadant AF1. The control for double labelling was the absence of primary antibodies. Control sections for quadruple labelling were carried out in the absence of all the primary antibodies or omitting the rabbit anti-Ki67 primary antibody only or in the absence of mouse primary antibodies. No cross-reaction was detected between any of the rabbit primary antibodies and anti-mouse secondary antibodies and *vice versa*.
<table>
<thead>
<tr>
<th>Target antigen</th>
<th>Antibody and dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>rabbit affinity isolated A0047 (Gerdes et al 1991) 1:50</td>
<td>DAKO (Ely, Cambridgeshire, UK)</td>
</tr>
<tr>
<td>Ki67</td>
<td>mouse monoclonal IgGl M0722 (Gerdes et al 1984) 1:100</td>
<td>DAKO</td>
</tr>
<tr>
<td>κ-casein</td>
<td>mouse monoclonal LICR-LON 14.1 IgGl (Earl and McIlhinney 1985) 1:1</td>
<td>LICR, London Branch</td>
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<td>mouse monoclonal LICR-LON 32.2 IgGl (Earl and McIlhinney 1985) 1:1</td>
<td>LICR, London Branch</td>
</tr>
<tr>
<td>Cytokeratin 14</td>
<td>mouse monoclonal IgG2a LL001 (Lane et al 1985) 1:5</td>
<td>Prof. E.B. Lane (University of Dundee, UK)</td>
</tr>
<tr>
<td>Cytokeratin 19</td>
<td>mouse monoclonal IgG2b LP2K 1 (Lane et al 1985) 1:5</td>
<td>Prof. E.B. Lane</td>
</tr>
</tbody>
</table>

Table 2.1

Immunohistochemistry in vivo: sources of primary antibodies

Target antigen | Antibody and dilution | Source |
<table>
<thead>
<tr>
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<tr>
<td>Ki67</td>
<td>rabbit affinity isolated A0047 (Gerdes et al 1991) 1:50</td>
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<tr>
<td>Cytokeratin 14</td>
<td>mouse monoclonal IgG2a LL001 (Lane et al 1985) 1:5</td>
<td>Prof. E.B. Lane (University of Dundee, UK)</td>
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<td>Cytokeratin 19</td>
<td>mouse monoclonal IgG2b LP2K 1 (Lane et al 1985) 1:5</td>
<td>Prof. E.B. Lane</td>
</tr>
<tr>
<td>Integrin subunit</td>
<td>Monoclonal Ab</td>
<td>Source and Reference</td>
</tr>
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<td>------------------</td>
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<td>----------------------</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Mouse monoclonal IgM VIM 13.2</td>
<td>Sigma, Poole, Dorset, UK (Adams and Watt 1988) 1:50</td>
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<tr>
<td>Integrin subunit</td>
<td>Mouse monoclonal IgG2a 5E8D9 (Arroyo et al 1992) 1:100</td>
<td>Upstate Biotechnology</td>
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<tr>
<td>α1</td>
<td>Mouse monoclonal IgG1 HAS4 (Tenchini et al 1993) 1:100</td>
<td>Dr. F. Watt (Imperial Cancer Research, London UK)</td>
</tr>
<tr>
<td>Integrin subunit</td>
<td>Mouse monoclonal IgG1 MAB 2257</td>
<td>Chemicon</td>
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<tr>
<td>α3</td>
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<td>International (Cardinal Way, Harrow, UK)</td>
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<tr>
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<tr>
<td>α6</td>
<td>Mouse monoclonal IgG1 3E1 (Humphries 1990) 1:10</td>
<td>International</td>
</tr>
<tr>
<td>Integrin subunit</td>
<td>Mouse monoclonal IgG1 3E1 (Humphries 1990) 1:50</td>
<td>Life Technologies</td>
</tr>
</tbody>
</table>
Collagen I  rabbit polyclonal HP/9 (Warburton et al 1987) 1:100  Dr. M. Warburton

Collagen IV  rabbit polyclonal COLL M4-B6 (Liotta et al 1979) 1:50  Dr. M. Warburton

Collagen IV  mouse monoclonal IgG1 M0785 (Odermatt et al 1984) 1:50  DAKO

Laminin  rabbit polyclonal LAM2 (Timpl et al 1979) 1:50  Dr. M. Warburton

Fibronectin  guinea pig polyclonal FN-1 (Mautner and Hynes 1977) 1:50  Dr. M. Warburton
### Table 2.2

**Immunohistochemistry: Primary and secondary antibodies used for immunoperoxidase labelling**

<table>
<thead>
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<th>Primary antibody</th>
<th>Secondary Antibody</th>
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</thead>
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<td>FN1-1</td>
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</tr>
<tr>
<td>LAM2, COLL M4-B6, HP1/9</td>
<td>Sheep anti-rabbit Ig horseradish peroxidase linked whole antibody</td>
</tr>
<tr>
<td>Clone 3E1, Clone P4C10, MAB2257, HAS4</td>
<td>Sheep anti-mouse Ig horseradish peroxidase linked whole antibody</td>
</tr>
<tr>
<td>Clone 5E8D9, CIV22 M0785, LICR-LON</td>
<td></td>
</tr>
<tr>
<td>14.1, LICO-LON 32.2</td>
<td></td>
</tr>
</tbody>
</table>

* for the sources of the primary antibodies, see Table 2.1

** all the secondary antibodies were from Amersham (Little Chalfont, Buckinghamshire, UK)
Table 2.3

Immunohistochemistry: Combinations of primary and secondary antibodies for double immunofluorescence labelling

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Primary antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67 &amp; κ-casein</td>
<td>A0047 *</td>
</tr>
<tr>
<td></td>
<td>LICR-LON 14.1 **</td>
</tr>
<tr>
<td></td>
<td>and</td>
</tr>
<tr>
<td>Ki67 &amp; β-casein</td>
<td>A0047 *</td>
</tr>
<tr>
<td></td>
<td>LICR-LON 32.2 **</td>
</tr>
<tr>
<td></td>
<td>and</td>
</tr>
<tr>
<td>Collagen I &amp; Collagen IV</td>
<td>HP1/9 *</td>
</tr>
<tr>
<td></td>
<td>and M0785 **</td>
</tr>
<tr>
<td>Ki67 &amp; α2 integrin subunit</td>
<td>A0047 *</td>
</tr>
<tr>
<td></td>
<td>HAS 4 **</td>
</tr>
<tr>
<td></td>
<td>and</td>
</tr>
</tbody>
</table>

* Goat anti-rabbit IgG (whole molecule) FITC conjugate (Sigma) was applied as the secondary antibody.

** Goat anti-mouse IgG1 TRITC human absorbed (Southern Biotechnology Associates, via Cambridge BioScience Ltd, Cambridge, Cambridgeshire, UK) was applied as the secondary antibody.
Table 2.4

**Immunohistochemistry: Combination of primary and secondary antibodies for quadruple immunofluorescence labelling**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin 19</td>
<td>LP2K Mouse IgG2b</td>
<td>Goat anti-mouse IgG2b BIOT human absorbed (Southern Biotechnology Associates) followed by AMCA fluorophore conjugated streptavidin (Jackson Immunoresearch Laboratories, via Stratech Scientific, Luton, Bedfordshire, UK)</td>
</tr>
<tr>
<td>Cytokeratin 14</td>
<td>LL001 Mouse IgG2a</td>
<td>Goat anti-mouse IgG2a TRITC human absorbed (Southern Biotechnology Associates)</td>
</tr>
<tr>
<td>Vimentin</td>
<td>VIM 13.2 Mouse IgM</td>
<td>Goat anti-mouse IgM FITC human absorbed (Southern Biotechnology Associates)</td>
</tr>
</tbody>
</table>
Ki 67  A0047 Rabbit polyclonal  Donkey anti-rabbit IgG (H+L)

Cy5-conjugated affiniPure (Jackson Immunoresearch Laboratories)
2.1.3 Visualisation

Image data files describing H&E and immunoperoxidase labelled specimens were collected with a high resolution Leaf camera mounted over an Axiophot microscope fitted with either a x10 NA 0.3 or a x20 NA 0.5 objective as described elsewhere (Entwistle 1998, Entwistle 2001). The 36 bit data (3 channels of 12 bits each) generated by the sensor of the camera was truncated to the most significant 8 bits for each channel and transferred to the computer through a SCSI interface using proprietary software supplied with the camera which ran as a TWAIN 32 plugin with Adobe Photoshop. Following calibration, the software was used to collect image data sets using a routine that ensured that the brightest pixels were not quite saturated without generating conflict with the balance of colours. Where required, correction of any spatial shading effects was made after two data sets had been collected, one describing a region of interest and the other a nearby empty field of view. Correction of spatial shading was implemented by dividing each pixel value in the empty field of view and multiplying the product by 250, for each of the three channels, using Image-Pro Plus. The resulting data set was then subjected to contrast stretching and saved as the 24 bit RGB TIFF files. The immunoperoxidase labelled sections were evaluated by three individuals, and consensus agreement was reached following further review.
Image data files depicting immunofluorescence staining were collected as a matrix of 1024 x 1024 elements, each with a bit depth of 12, on a Coolview 12 cooled Charge Coupled Device (CCD) camera mounted over an Axiophot microscope fitted with either a x20 NA 0.5 or a x40 NA 0.75 objective. The readout from the CCD camera was transferred to a Snapper frame grabber card mounted in a personal computer running under Windows 95 version 4.0 by proprietary software (Photonic Science) controlled by Image Pro Plus v 3.01. The fluorophores were visualised with various filter combinations, or modes, as follows: AMCA and Hoechst 33258 using a blue bandpass emission filter and UV illumination, fluorescein isothiocyanate (FITC) using a green bandpass emission filter and blue illumination, tetramethylrhodamine isothiocyanate (TRITC) using an orange bandpass emission filter and green illumination, and CY5 with a red / near infrared bandpass emission filter and red illumination. The emission from each fluorophore into the other modes, cross talk, was insignificant, < 0.1% (Entwistle, personal communication). Images depicting double immunofluorescence labelling with additional Hoechst 33258 staining were generated by assigning green to the emissions from FITC, red to emissions from TRITC and blue to Hoechst 33258. Images displaying quadruple immunofluorescent labelling with additional Hoechst labelling were made by assigning the emissions from: both AMCA
(exclusively cytoplasmic) and Hoechst (exclusively nuclear) to blue, red to TRITC, white to FITC, and green to CY5. The contrast in the colour data sets was adjusted so that the information that could be perceived in the final images corresponded as closely as possible to the data sets presented on a computer monitor screen that had been balanced against a colour test card.

2.1.4 Proliferation index (PI)

To determine the numbers of proliferating cells, sections were labelled with the A0047 antibody against Ki67 followed by goat anti-rabbit IgG (whole molecule) FITC conjugate and the nuclei were stained with Hoechst 33258. Areas containing epithelium were randomly selected by Hoechst 33258 labelling. At high magnification (x40/0.75 lens), image of Hoechst 33258 labelling were captured through the UV<sub>ex</sub>/blue<sub>em</sub> filters and then images of Ki67 labelling of the same areas through the blue<sub>ex</sub>/green<sub>em</sub> filters. From each sample of the non-pregnant breast tissues, five images were taken. Each image contained approximately 100 nuclei of epithelial cells. The mean of five Ki67-positive epithelial fractions was used to represent the average PI of the mammary epithelium from each donor. The same procedure was applied to the pregnant breast except that the average PI was obtained from nine (12th week) or ten (15th and 18th
weeks) images per sample (over 1,000 epithelial cells per sample were examined). To examine differences between the PIs, non-parametric statistical methods were applied (a Mann-Whitney U test for the increased PI in the pregnant breast compared to the non-pregnant breasts), since a normal distribution of the samples could not be assumed. A Wilcoxon Signed Ranks test was employed to compare the PI in the 15th week to the 12th week of pregnancy, as these samples were from the same patient (SPSS version 7.5).

2.2 Cell culture

HB4a, H4.1, C3.6, C5.2 and R4.2 were obtained from the originators (Harris et al 1999, Stamps et al 1994). MDA-MB-231, a TRAIL sensitive human breast cancer cell line, was obtained from the American Type Culture Collection (ATCC). The cells were cultured in RPMI 1640 medium containing 10% (v/v) foetal calf serum (FCS), 2mM L-glutamine, 100U/ml penicillin, 100U/ml streptomycin, 1μg/ml hydrocortisone and 5μg/ml insulin, at 37°C in an atmosphere of 5% (v/v) carbon dioxide in air in a humidified incubator.
2.2.1 Immunocytochemistry

Cells were plated onto sterile cover slips (13mm) at 4x10⁴/ml in medium. When they reached 80% confluency, the cells were washed twice with PBS, fixed with cold methanol (-20°C) for 7 minutes, washed three times with PBS and stored in PBS at 4°C.

Immunofluorescence labelling was carried out at room temperature on a shaker, and every incubation step was followed by five rinses in PBS. The cells were first blocked with 500µl of 0.5% (w/v) BSA in PBS for 30 minutes. Primary antibody (Table 2.5) was applied on its own or as a mixture for 1 hour, and corresponding secondary fluorophore-conjugated antibodies were applied for 1 hour. The cells were finally exposed to Hoechst 33258 for two minutes to stain nuclei. The cover slips were mounted using 1:1 mixture of Hydromount and Citifluor antifadant AF1. Image data sets depicting immunofluorescence labelling were collected and processed as described in 2.1.3.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary antibody (dilution)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin 18</td>
<td>LE65 mouse IgG (1:10)</td>
<td>Dr. E.B. Lane</td>
</tr>
<tr>
<td>Cytokeratin 19</td>
<td>LP2K mouse IgG2b (1:10)</td>
<td>Dr. E.B. Lane</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>C20820 mouse IgG2a (1:100)</td>
<td>Transduction Laboratories</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(via Affiniti, Exeter, Devonshire, UK)</td>
</tr>
<tr>
<td>Epithelial</td>
<td>Ber-EP4 mouse IgG1 (1:20)</td>
<td>DAKO</td>
</tr>
<tr>
<td>Membrane Antigen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>Vim 13.2 mouse IgM (1:100)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cytokeratin 14</td>
<td>LL002 mouse IgG31 (1:10)</td>
<td>Dr. E.B. Lane</td>
</tr>
<tr>
<td>ErbB2</td>
<td>c-neu (Ab-3) IgG (1:100)</td>
<td>Calbiochem (via CN Biosciences, Beeston, Nottingham, UK)</td>
</tr>
</tbody>
</table>

- All the secondary antibodies were from Southern Biotechnology Associates
2.2.2 Anchorage-dependent growth assays

Methylene blue staining

The methylene blue growth assay was conducted with minor changes to a previously described protocol (Oliver et al 1989). Cells were plated in medium at 5x10^4 cells/well in 24 well plates. After 24 hours, the cells were treated in quadruplicate as follows: control (medium alone); 1, 2 and 3% (v/v) DMSO; 1, 10, 20, 30 and 100 ng/ml TNFα; 0.1, 1, 10 and 100 ng/ml TRAIL. TRAIL was used with and without TRAIL enhancer 2 mg/ml (Schneider et al 1998, Schneider et al 1997). TRAIL enhancer is a monoclonal antibody to an unspecified antigen, and it has been shown to enhance the action of TRAIL, and it is accompanied by commercially available TRAIL from Alexis. Previously, it has been shown that the effect of TRAIL can be enhanced from an undetectable to significant level by TRAIL enhancer (Schneider et al 1998, Schneider et al 1997). Additional controls with TRAIL enhancer in the absence of TRAIL were carried out on MDA-MB-231 and HB4a. TRAIL enhancer was shown to have no effect. At the times indicated in the Results, the cells were washed with PBS twice, fixed with 4% (v/v) formaldehyde in PBS and stored at 4°C overnight. The cells were stained with 1% (w/v) methylene blue in borate buffer 0.01 M pH 8.5 for 30 minutes and rinsed five
times with 0.01M borate buffer pH8.5. Methylene blue that remained within the cells after washing was eluted by adding 1ml of 1:1 (v/v) ethanol : hydrochloric acid (HCl) 0.1M. The eluted dye solution from each well was transferred in triplicate into a 96 well plate (200μl/well) and the absorption was measured at 650nm wavelength on a MRX micro plate reader. The differences in growth between the controls and cells to which the differentiation inducers had been added were compared using the Kruskal-Wallis test (SPSS version 7.5).

**MTT growth assay**

Cells were plated at 1x10⁴ cells/well in a total volume of 100μl medium in 96 well plates in triplicates. After 24 hours, the cells were treated in triplicate as follows: control (medium alone); 1.5, 3.1, 6.2, 12.5, 25, 50 and 100ng/ml TNFα. At the times indicated in the result, 20μl (10% of total volume) MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (5mg/ml in dH2O) was added to each well, and the cells were incubated for 3 hours. Medium was gently removed by aspiration and the dye solubilised in 50μl acidified propan-2-ol. MTT is a viable dye that is taken up and metabolised by viable cells. Cell viability was estimated
by uptake of the dye, quantified by measurement of absorbance at 570nm, using a MRX multiwell colorimetric plate reader and Revelation software.

**Haemocytometer counting**

For confirming the differential effect of TNFα on the growth between the cell lines, cells were plated in medium in 25cm² flasks at $10^5$ cells. After 24 hours of incubation, the medium was replaced in triplicate with fresh medium or 1% (v/v) DMSO or 10ng/ml TNFα. After 5 days, cells were washed with 0.02% w/v ethylenediaminetetraacetic acid in Ca²⁺ and Mg²⁺ free Dulbecco’s PBS (EDTA), treated with 1mg/ml (w/v) type III trypsin in EDTA at 37°C and re-suspended in 4ml of medium. Cells were counted using a haemocytometer. A minimum of 200 cells was counted for each flask.

**2.2.3 Apoptosis Assays**

Staurosporine (10mM stock prepared in DMSO) was used as a positive control for the induction of apoptosis. It is a cell-permeable broad spectrum inhibitor of protein kinases. It is known to induce apoptosis in human malignant glioma cell lines (Couldwell et al
1994) and arrests normal cells at the G1 checkpoint of the cell cycle (Chen, Lowe and Keyomarsi 1999).

**Hoescht 33342 staining**

Cells were plated in medium at 5x10^4 cells/well in 24 well plates. After 24 hours, the cells were treated as follows: control (medium alone); 1% (v/v) DMSO; 10ng/ml TNFα; 10ng/ml TRAIL and 10mM staurosporine. Cells were treated for 5 days, gently washed with PBS, stained with Hoescht 33342, rinsed with PBS and visualised under 20x objective, for 300-600 milliseconds exposure using an Axiovert microscope and AxioVision (version 3.0.6) software. Digital images of three visual fields were taken, and from each image the apoptotic body was counted. The mean and standard deviation of the three visual fields were used to represent each experimental condition.

**Caspase Assay**

Cells were plated at 1x10^4 cells/well in a total volume of 100μl medium in a black walled 96 well microtitre plate. The following day the cells were treated in triplicate with either 1% (v/v) DMSO, 10mM Staurosporine, 10 ng/ml TNF-α, 10 ng/ml TRAIL (plus 2μg/ml enhancer), in a final volume of 200μl/well medium. Control samples were
treated with an additional 100μl medium alone. Cells were then cultured for 5 days. On day 5, apoptosis was analysed using a Fluorimetric Homogenous Caspases Kit, according to manufacturer’s instructions. Briefly, 100μl of lysis buffer containing a caspase substrate were added to each well, and following a 90 minute incubation at 37°C, caspase activity was estimated by quantifying cleaved substrate through the measurement of a fluorimetric change produced by excitation at 470-500 nm and emission at 500-561 nm. Measurements were made using the Cytofluor II fluorescence multiwell plate reader. The quantity of cleaved substrate was determined using a standard curve consisting of a serial dilution of the fluorophore Rhodamine-110. While caspase activity was used to estimate cell death via apoptosis, in parallel, cell viability was measured by performing a MTT assay, using a MRX multiwell colorimetric plate reader and Revelation (version 4.02) software.

2.3 Experimental conditions for molecular investigation

One x10^6 (C3.6) cells were plated as single cell suspension in 50ml of medium in a 175cm^2 flask and incubated at 37°C. After 24 hours of incubation, the medium was discarded and the four experimental conditions were introduced as follows. For growth phase, fresh medium was added and the cells were incubated at 37°C. For growth arrest
by temperature 39.5°C, fresh medium was added and the cells were incubated at 39.5°C.

For growth inhibition by DMSO, fresh medium containing 1% (v/v) DMSO was added and the cells were incubated at 37°C. For growth inhibition by TNFα, fresh medium containing 10 ng/ml TNFα was added, and the cells were incubated at 37°C. After 72 hours of further incubation, the cells were lysed for molecular analysis.

2.3.1 mRNA analysis by reverse transcriptase polymerase chain reaction (RT-PCR)

Cell lysis

The cells were washed twice with PBS and lysed in 4ml of freshly prepared lysis buffer containing 4M guanidinium, 25mM sodium citrate, 0.5% sarcosyl, 0.1M β-mercaptoethanol on ice. The lysed cells were transferred into a 15ml tube, and 0.4ml of 2M sodium acetate pH4, 4ml of phenol and 0.8ml of chloroform-isoamyl alcohol mixture (29:1) were added. The final suspension was shaken vigorously for 10 seconds and incubated on ice for 15 minutes. Samples were centrifuged at 5000rpm for 20 minutes at 4°C. The aqueous phase was transferred to a fresh 15ml tube, mixed with 4ml of isopropanol and stored at 4°C.
Estimation of RNA concentration

The RNA pellet was recovered by centrifuging at 5000rpm for 20 minutes at 4°C, air-dried and dissolved in 100μl of 0.1% (v/v) diethyl pyrocarbonate in DEPC water. The concentration of RNA was determined by measuring the OD260nm of 1:100 aliquot (5μl sample in 500μl DEPC water) of the preparation. A solution of RNA whose OD260nm = 1 contains approximately 40μg/ml RNA. All RNA samples were adjusted to 2.0mg/ml for future analysis. One μl of RNase inhibitor was added to each sample, and the samples were stored at -70°C.

RT-PCR

A mixture of 10μl of RNA sample and 3μl of primers was freshly prepared, heated at 70°C for 10 minutes and placed on ice. To this mixture, 4μl of 5xbuffer, 2μl of 0.1M DTT, 1μl 10mM dNTPs and 1μl autoclaved distilled water were added followed by heating at 42°C for 10 minutes. Keeping at the heated temperature, 1μl Superscript II reverse transcriptase was added. The samples were heated at 42°C for further 50 minutes, then heated at 70°C for 10 minutes and placed on ice. The samples were diluted at 1:5 in double distilled water and stored at -20°C.
**PCR reaction**

One μl of cDNA was mixed with 2μl of 10×buffer, 1μl 10mM sense primer, 1μl 10mM antisense primer (Table 2.6), 0.5μl of Amplitaq and 12.5μl of water (HiPerSolv for HPLC). Each sample was mixed gently and a drop of mineral oil was used to overlay the surface. The samples were incubated at 94°C for 2 minutes, and then optimised (Table 2.6) cycles of PCR conditions were conducted. Ten μl of the amplified sample was analysed using agarose gel electrophoresis (all the reagents from Life Technologies).
Table 2.6

Primer sequences of markers examined by RT-PCR

Each reaction contained 2μl x10 buffer, 2μl 2μM dNTP, 0.4μl 10μM Primer, 2μl RT, 0.2μl TAQ and 13.4μl water.

<table>
<thead>
<tr>
<th>Oligoname</th>
<th>Sequence</th>
<th>Length</th>
<th>Temp</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPD Sense</td>
<td>Cggagtcacggatggtcg</td>
<td>21</td>
<td>65</td>
<td>30</td>
</tr>
<tr>
<td>GPD Antisense</td>
<td>Tggagagtgggtctg</td>
<td>20</td>
<td>65</td>
<td>30</td>
</tr>
<tr>
<td>Actin Sense</td>
<td>Cgtgagcactggagggccg</td>
<td>21</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>Actin Antisense</td>
<td>Gccgagcgaaaatcgtcg</td>
<td>22</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>MUC1 Sense</td>
<td>Aaggagacctggctacccag</td>
<td>21</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>MUC1 Antisense</td>
<td>Aaatggcactcacaagc</td>
<td>20</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>K9 Sense</td>
<td>Gctagcggcggtctcctg</td>
<td>18</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>K9 Antisense</td>
<td>Ctttccgttctcgtcgc</td>
<td>19</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>ER Sense</td>
<td>Ggagcacgagagcgtgtccaa</td>
<td>21</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>ER Antisense</td>
<td>Ccagcaagctctcgaagc</td>
<td>21</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>ErbB2 Sense</td>
<td>Tgaaacctgtctctcctacatg</td>
<td>23</td>
<td>65</td>
<td>40</td>
</tr>
<tr>
<td>ErbB2Antisense</td>
<td>Tgtagagggatcccaagaccc</td>
<td>23</td>
<td>65</td>
<td>40</td>
</tr>
<tr>
<td>ErbB3Sense</td>
<td>Ccttggagtctaggtgccca</td>
<td>21</td>
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<tr>
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<td>Acggccagtgtgagaaaaagtgcc</td>
<td>22</td>
<td>65</td>
<td>40</td>
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<tr>
<td>P21Sense</td>
<td>Tgacctgaagctgagccagc</td>
<td>22</td>
<td>60</td>
<td>30</td>
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<tr>
<td>P21Antisense</td>
<td>Gccgagaacagagtccagcc</td>
<td>22</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>P53Sense</td>
<td>Cacatgcggagtcgtgacacg</td>
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<td>40</td>
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<tr>
<td>P53Antisense</td>
<td>Atgggtgtatctcagagc</td>
<td>20</td>
<td>65</td>
<td>40</td>
</tr>
</tbody>
</table>
2.3.2 Immunoblotting

Immunoblotting was based on a method described previously (Harris et al 1999) with minor modifications.

Cell lysis

The cells were washed with PBS containing 1 mM sodium orthovanadate and the cells lysed in 2 ml of RIPA buffer containing 150 mM sodium chloride, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS), 50 mM trishydroxymethyl-methylamine (Tris)-HCl pH 8.0 and tyrosine phosphatase inhibitors (1 mM sodium orthovanadate and 10 mM benzamidine hydrochloride) for five minutes on ice (Epstein et al 1992). Cells were scraped from the surface of the flask using a cell lifter and the lysates were collected and transferred to 2 ml safe-lock tubes, which had been pre-cooled on ice. Lysates were subjected to centrifugation at 14000 rpm for 10 minutes at 4°C to pellet any cell debris. Lysates were then transferred to fresh tubes and stored at -70°C.

Estimation of protein concentration

Protein concentration was estimated using the bicinchoninic acid (BCA) based system (Smith et al 1985), which employs the biuret reaction of protein reducing Cu^{++} in an
alkaline medium to produce Cu\(^+\). The colour change produced by this reaction can be quantitated spectrophotometrically by measurement of optical density at 560 nm (OD\(_{560}\)). By comparing the OD\(_{560}\) values produced by samples to those obtained from known BSA standards, protein concentrations can be calculated. All samples were diluted to a concentration of 1mg/ml and stored in aliquots at -80°C.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

Protein samples were separated by SDS-PAGE (Harlow and Lane 1988). Prior to loading, 1/3 volume of 3 times concentration load buffer was added to each sample. To make 10ml of this concentrated load buffer, 0.188ml 1M tris pH6.8, 4.5ml glycerol, 0.6g SDS, 1.5ml β-mercaptoethanol, 3.8ml water and approximately 0.001% (v/v) bromophenol blue solution were mixed. The samples were denatured by heating at 95°C for 5 minutes. SDS-PAGE was performed using the Hoefer SE 400 electrophoresis unit which holds polyacrylamide gels 18 x 16 cm in size. All analyses in this study used resolving gels of 7.5% (w/v) acrylamide, 0.18% (w/v) bis-acrylamide, 0.1% (w/v) SDS, 375mM Tris-HCl pH 8.7 and stacking gels of 3% (w/v) acrylamide:bis (29:1), 0.1% (w/v) SDS, 125 mM Tris-HCl pH 6.8.
Samples were loaded into the wells of the stacking gel using a Hamilton syringe, alongside "Rainbow" coloured protein molecular weight markers, and electrophoresed at 25 mA, 70 V, overnight, in Western run buffer containing 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS. Electrophoresis was allowed to proceed until the bromophenol blue dye of the load buffer had just run off the bottom of the gel.

**Blocking and probing of Western blots**

Following electrophoresis, proteins were transferred to Immobilon-P membranes by conventional wet Western blotting. The probing of Western blots was conducted at room temperature on a shaker. The membrane was first incubated for 30 minutes in 2% (w/v) BSA and 0.1% (v/v) Tween-20, in PBS pH7. After blocking, the membrane were probed with a specific antibody against ErbB2 c-neu (Ab-3) IgG (1:100) (Calbiochem, via CN Biosciences, Beeston, Nottingham, UK) for 1 hour and washed 6 times for 5 minutes each wash in PBS. The membranes were then incubated with secondary antibody sheep anti-mouse Ig horseradish peroxidase linked whole antibody (Amersham, Little Chalfont, Buckinghamshire, UK) for 1 hour and washed as above. Protein bands were visualised by enhanced chemical luminescence (ECL) plus reagents according to manufacturer's instructions. The membranes were soaked for 2 minutes in a fresh
mixture of 10ml of reagent A and 10ml of reagent B on a shaker, after which 50ml of distilled water was added to deactivate the reagents. The membranes were drained by gently touching tissue paper at the edges, wrapped with cling film and placed in an autoradiography cassette. In a dark room, 10 second exposure of the membrane was made using ECL-Hyperfilm and developed as autoradiographs.
Chapter 3

Differentiation *in vivo*

3.1 Introduction

This study utilised histologically normal human breast tissues obtained at the 12th, 15th and 18th weeks of pregnancy. Freshly frozen tissues allow multiple immunofluorescence labelling for a greater range of antigens than paraffin embedded archival materials, and this technique is particularly useful when applied to specimens of limited availability.

To determine if differentiated breast epithelial cells can proliferate, the relationship between differentiation and proliferation was investigated using Ki67, cytokeratins and milk proteins β and κ caseins. Ki67 is a nuclear antigen, which is present in all the phases of cell cycle except G0. Hence it distinguishes between cycling and non-cycling (G0) cells (Gerdes *et al* 1991). Differentiation was sub-divided into phenotypic and functional differentiation. As markers of phenotypic differentiation, K19 and K14 were used for luminal epithelial cells and myoepithelial cells respectively. When functionally differentiated, luminal epithelial cells secrete milk proteins.
Therefore, the milk proteins β and κ caseins were employed as markers of functional differentiation.

Expression patterns of extracellular matrix (ECM) components (fibronectin, laminin, collagen I and collagen IV) and their receptor integrin subunits (α1, α2, α3, α6, β1 and β4) were also studied, since they have been shown to play active roles in growth, morphogenesis, functional differentiation and malignant transformation of human, as well as rodent, mammary luminal epithelial cells in vitro (for review see Streuli 1999, Streuli and Edwards 1998, Petersen et al 1998).

3.2 Results

3.2.1 Histological morphology

When reviewed by an experienced breast pathologist (Dr. Sunil R. Lakhani, Department of Histopathology, Royal Free and University College Medical School, Rockefeller Building, University Street, London), the non-pregnant human breast samples were typical of the age group studied: two were histologically normal (age 30), one contained apocrine cysts (age 32), one showed atrophy, fibrocystic change and apocrine metaplasia (age 40) and one had focal fibrocystic changes (age 43). The pregnant
human breast samples selected from the normal tissues surrounding the primary
tumours were free of malignant invasion.

Compared to the non-pregnant breast, the pregnant breast contained abundant
numbers of acini, and dilation of the alveoli progressed with duration of pregnancy
(Figure 3.1.A-D). The extent of the progression was, however, notably asynchronous in
that the same sections contained clusters of extensively dilated acini together with
clusters of smaller less-developed acini. Some of the acini contained highly polarised
columnar luminal epithelial cells (Figure 3.2.) with myoepithelial cells extensively
stretched around their basal aspects. Functional differentiation of luminal epithelial cells,
in the pregnant breast, especially at the 15th and 18th weeks, was evident by the presence
of lipid droplets and was later confirmed by labelling for the milk proteins.
Figure 3.1

Immunoperoxidase labelling for collagen IV (shown in brown) on the non-pregnant human breast (A) and the human breast at the 12\textsuperscript{th} (B), 15\textsuperscript{th} (C), 18\textsuperscript{th} (D) weeks of pregnancy. Nuclei were counterstained with haematoxylin and shown in blue. Bar: 70\textmu m

This series of figures shows gradual alveolar expansion during pregnancy as well as the more defined and increased labelling for collagen IV that surrounds each alveolus.
Figure 3.2

The polarised luminal epithelial cells of the human breast tissue obtained at the 15th week of pregnancy. Bar: 20μm
3.2.2 Proliferation

The proliferation index (PI) for the non-pregnant breast was 2.69 ± 1.21 (mean ± standard deviation), and there was a significant increase in the PI (P< 0.05) for the 3 samples of the pregnant breast (Table 3.1.). There was also a significant difference (P<0.01) between the pregnant breast samples obtained from the same patient at the 12th (6.1 ± 3.8) and 15th weeks (17.6 ± 3.9).

In the pregnant breast, Ki67 positive luminal epithelial cells were uniformly co-labelled for K19 (Figure 3.3).

None of the myoepithelial cells in the non-pregnant breast was labelled for Ki67. In the pregnant breast, however, approximately 2% of myoepithelial cells were labelled with Ki67 (This figure was obtained by counting 200 myoepithelial cells that were positively labelled for K14. Four such cells out of 200 were co-labelled for K14 and Ki67 in the breast tissue at the 15th week of pregnancy.) The differentiated phenotype of myoepithelial cells was confirmed by the presence of K14 (Figure 3.3).

3.2.3 Milk proteins

Neither β-casein nor κ-casein was detectable in the non-pregnant breast or the pregnant breast at the 12th week (Figure 3.4.AB). At the 15th week, some luminal epithelial cells
were labelled for β-casein (Figure 3.4.C), while κ-casein was found in a smaller proportion of luminal epithelial cells (Figure 3.4.D). Finally at the 18th week β-casein was observed in almost all luminal epithelial cells (Figure 3.4.E). In contrast κ-casein was found in many but not all luminal epithelial cells (Figure 3.4.F).

3.2.4 Proliferative potential in functionally differentiated luminal epithelial cells

When the antibody for Ki67 was simultaneously applied with that for β-casein or κ-casein to the sections of the 15th and 18th week pregnant breast, occasional cells were seen to be positive for both Ki67 and either casein. Thus, luminal epithelial cells were shown to be able to synthesise milk proteins at the same time as undergoing proliferation. This was best demonstrated in the sample representing the 15th week of pregnancy where milk synthesis was still intermittent (Figure 3.5.AB), and at the 18th week of pregnancy, such co-labelling continued to be observed within acini that were abundantly positive for β-casein (Figure 3.5.C).
Table 3.1

Table of proliferative index (PI) values on human breast tissues from pregnant women and non-pregnant women

<table>
<thead>
<tr>
<th></th>
<th>12\textsuperscript{th} week</th>
<th>15\textsuperscript{th} week</th>
<th>18\textsuperscript{th} week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant Breast</td>
<td>6.1 ± 3.8*</td>
<td>17.6 ± 3.9</td>
<td>12.5 ± 1.5</td>
</tr>
<tr>
<td>Non-pregnant Breast</td>
<td>1.6 ± 2.2</td>
<td>2.6 ± 2.6</td>
<td>3.9 ± 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.4 ± 2.6</td>
<td></td>
</tr>
</tbody>
</table>

* SD = Standard Deviation
Supplementary figures for Table 3.1

Labelling for Ki67 (green nuclei), K19 (blue) and K14 (red) on human breast tissues obtained from a non-pregnant woman (A) and obtained at the 12th (B), 15th (C) and 18th (D) weeks of pregnancy. Bar: 80μm
Figure 3.3

Quadruple immunofluorescence labelling on the human breast at the 12th (A) and 15th (B) weeks of pregnancy for K19 in luminal epithelial cells (blue), K14 in myoepithelial cells (red), vimentin in mesenchymal cells (green) and Ki67 in nuclei of actively proliferating cells (white). Hoechst stained nuclei are shown in the same blue as K19. The arrowheads point to the proliferating myoepithelial cells labelled for Ki67 in the nuclei and for K14 in the cytoplasm. Bar: 45µm
Figure 3.4

Immunoperoxidase labelling on the human breast at the 12$^{\text{th}}$ week of pregnancy for $\beta$-casein (A) and $\kappa$-casein (B) (shown in brown), at the 15$^{\text{th}}$ week of pregnancy in the same order (C, D) and at the 18$^{\text{th}}$ week of pregnancy in the same order (E, F). Nuclei were counterstained with haematoxylin and shown in blue. Bar: 25$\mu$m
Figure 3.5

Double immunofluorescence labelling for β-casein (A) and κ-casein (B) shown in red, Ki67 shown in green and Hoechst labelled nuclei shown in blue on the human breast at the 15th week of pregnancy. In order to show the co-labelling clearly, each fluorescence channel has been enhanced by adjusting the input levels. White lines outline luminal epithelial cells co-labelled for Ki67 and Hoechst exclusively in the nuclei and milk proteins within the cytoplasm and at the apical borders. Bar: 45μm

Double immunofluorescence labelling for β-casein (C), shown as above, at the 18th week of pregnancy. Bar: 45μm
3.2.5 Integrins

Cells require appropriate anchorage and polarity to conduct many of their physiological functions, and integrins are the primary cellular receptors for extracellular matrix components such as laminin. Not only do integrins provide such structural support for the cell-matrix adhesion, but also they have been shown to play an active role in morphogenesis and differentiation in the mammary system. Since the panel of breast tissues examined in the present study exhibited considerable expansion of alveoli and also gradual induction of milk protein synthesis, the expression patterns of integrin subunits were investigated for possible differential regulations.

The labelling of integrin subunit α1 was found on myoepithelial cells, luminal epithelial cells and some stromal cells albeit at low levels. While in the alveoli of the non-pregnant breast, most luminal epithelial cells showed very faint labelling in the entire cell circumference (Figure 3.6A), there was an additional sub-population of luminal epithelial cells in the alveoli (Figure 3.6B) and ducts (Figure 3.6C) of the pregnant breast with noticeably stronger overall labelling with distinct cap-like apical labelling (Figure 3.6B). The cap like apical labelling was evident where luminal epithelial cells were polarised, showing clear luminal aspects of each cell (see arrow Figure 3.6DE). It was also seen in polarised luminal epithelial cells of the mammary
ducts of the non-pregnant breast. Although, the presence of milk and mucins in the lumen can result in non-specific labelling, this cap like apical labelling was specific to α1 and was not observed in the labelling for other integrin subunits.

The labelling for integrin subunit α2 was uniformly and strongly positive on myoepithelial cells in both the non-pregnant (Figure 3.7A) and pregnant breasts (Figure 3.7B-D), but it was comparatively more defined and intense in the latter. In the non-pregnant breast, luminal epithelial cells showed weak labelling in the entire cell circumference and, in addition, stronger baso-lateral labelling. However, in the pregnant breast representing the 15th (Figure 3.7C) and 18th (Figure 3.7D) weeks, the α2 labelling on luminal epithelial cells was markedly diminished in some areas. This decrease was rather subtle in immunoperoxidase labelling but easier to observe in immunofluorescence labelling (Figure 3.7EF). The decreased expression of α2 has been demonstrated and illustrated by double immunofluorescence labelling and correlated with Ki67 positivity. There was no labelling for α2 on stromal cells.

Integrin subunit α3 was uniformly but weakly positive on myoepithelial cells and diffusely and very weakly positive on luminal epithelial cells and some stromal cells (Figure 3.8AB). The labelling intensity on luminal epithelial cells was extremely variable with some acini having no positive cells and others having a few positive cells.
but never all of the luminal epithelial cells were positive. Where $\alpha_3$ was positive, the labelling intensity varied from weak to strong even within the same acini. There was no obvious difference between the non-pregnant and pregnant breasts. Characterisation of the antibody against $\alpha_3$ had not been previously published. With the weak and variable patterns of labelling found in the present study, it was not practically possible to examine changes (if any) of this integrin subunit.

The labelling for integrin subunit $\alpha_6$ was strong on all myoepithelial cells, weak and diffuse on luminal epithelial cells and strong on some stromal cells. Compared to the non-pregnant breast (Figure 3.8C), the labelling on myoepithelial cells was noticeably greater in intensity and more defined in the pregnant breast (Figure 3.8D). The positive baso-lateral labelling was found in some but not all luminal epithelial cells.

In both the non-pregnant (Figure 3.8E) and pregnant (Figure 3.8F) breasts, all myoepithelial cells were labelled for integrin subunit $\beta_4$. In the non-pregnant breast, the intensity appeared relatively greater in the ducts than in the alveoli (Figure 3.8G). When the alveoli of the non-pregnant and pregnant breasts were compared, the overall labelling on myoepithelial cells was considerably more intense and defined in the latter. No labelling was detected on luminal epithelial cells, while some stromal cells were weakly positive.
3.2.6 ECM components

Collagen I labelling (Figure 3.9 A-D) was found in the interstitial space. There was no difference in the intensity or pattern of labelling between the pregnant and non-pregnant breasts. However, with the duration of pregnancy, alveolar expiation increased, and this was accompanied by relative decrease in the interstitial space.

The labelling for Collagen IV (Figure 3.9 E-H) was found in the basement membrane around each alveolus as well as around some stromal cells that appear to represent endothelial cells of capillaries (no co-labelling with an endothelial marker was conducted to prove their identity, however). With the duration of pregnancy, the overall impression of labelling for collagen IV increased. This was partially due to the fact that in later stages of pregnancy, especially at the 18th week, the breast tissue was highly vascular with abundant capillaries between alveoli (Figure 3.9H). In addition, some alveoli of the pregnant breast also exhibited more defined and more intense pattern of labelling than the non-pregnant breast (Figure 3.9GH). Relative increase in the endothelial cells and in the basement membrane could be determined in finer details if multiple immunofluorescence labelling was conducted with relevant markers. However, due to a limited supply of the tissue samples, this was not possible.
In the pregnant breast, larger and more dilated acini were observed with collagen I reaching the area of basement membrane (Figure 3.10A) while smaller and less developed acini (Figure 3.10B) showed the similar labelling pattern to the non-pregnant breast. In fact, a double labelling showed partial co-localisation of collagen I and collagen IV, a marker of the basement membrane in the areas where alveoli are extensively dilated in the pregnant breast, while in the non-pregnant breast, collagen I was seen around the delimiting fibroblasts and below, but not on the basement membrane.

Immunoperoxidase labelling was also conducted for fibronectin (Figure 3.11A, B) and laminin (Figure 3.11C, D). Fibronectin was found in the interstitial tissue, and laminin in the basement membrane. There was no significant difference in the labelling patterns between the pregnant and non-pregnant breasts.

In the absence of primary antibodies, the negative control sections produced no labelling on (Figure 3.12AB).
Figure 3.6

Weak immunoperoxidase labelling for integrin subunit α1 (shown in brown) on the alveoli of non-pregnant human breast tissue (A) and on the alveoli (B) and duct (C) of 15th week pregnant human breast tissue. Nuclei were counterstained with haematoxylin and are shown in blue. Bar: 50μm

Myoepithelial cells (see red dots), luminal epithelial cells and some stromal cells (see red circles) were labelled at low levels. In addition to the faint labelling around the entire cell circumference, some luminal epithelial cells showed stronger labelling and distinct cap-like apical labelling (see red arrows) as shown in the enlarged image of B (D) and of C (E). Bar: 25μm
Figure 3.6 ABCDE Upper Half. Figure 3.7 ABCD Lower Half.
Figure 3.7

Labelling for integrin subunit α2 (brown) on the alveoli of non-pregnant human breast tissue (A) and on the alveoli of 12\textsuperscript{th} (B), 15\textsuperscript{th} (C) and 18\textsuperscript{th} (D) weeks pregnant human breast tissue. Nuclei were counterstained with haematoxylin and are shown in blue. Bar: 50µm

Double-immunofluorescence labelling for integrin subunit α2 (red) and Ki67 (green) on the 18\textsuperscript{th} week pregnant breast tissue (E) and non-pregnant breast tissue (F). In the pregnant breast tissue, there are patches where the labelling for α2 was considerably decreased on luminal epithelial cells. Such areas were found to contain actively proliferating luminal epithelial cells. Bar: 50µm
Figure 3.8

Weak immunoperoxidase labelling for integrin subunit α3 (brown) on the alveoli of non-pregnant human breast tissue (A) and on the alveoli of 15th week pregnant human breast tissue (B). Immunoperoxidase labelling for integrin subunit α6 in the same order (C, D). Bar: 100μm

Immunoperoxidase labelling for integrin subunit β4 on the alveoli of non-pregnant human breast tissue (E), on the alveoli of 15th week pregnant human breast tissue (F) and on the duct of the non-pregnant human breast tissue (G). Bar: 50μm
Figure 3.9

Immunoperoxidase labelling (shown in brown) for collagen I on the non-pregnant human breast (A) and the human breast at the 12th (B), 15th (C), 18th (D) weeks of pregnancy. Collagen I was found in the interstitial space. With the duration of pregnancy, alveolar expansion increased, and this was accompanied by relative decrease in the interstitial space.

Immunoperoxidase labelling for collagen IV in the same order (E, F, G, H). The labelling for collagen IV was found in the basement membrane around each alveolus as well as around some stromal cells that appear to represent endothelial cells of capillaries (although no co-labelling with an endothelial marker was conducted to prove their identity). With the duration of pregnancy, the overall impression of labelling for collagen IV increased. There were two contributing factors for this increase. First, in later stages of pregnancy, especially at the 18th week, the breast tissue was highly vascular with abundant capillaries between alveoli. Second, some alveoli of the pregnant breast exhibited more defined and more intense pattern of labelling than the non-pregnant breast.

Nuclei were counterstained with haematoxylin and are shown in blue. Bar: 70μm
Figure 3.10

Double immunofluorescence labelling on the human breast at the 15th week of pregnancy for collagen I in red and collagen IV in green and Hoechst stained nuclei in blue. In the extensively dilated acini (A), collagen IV in the basement membrane was highly stretched and co-localised with collagen I. In the less developed acini (B) collagen I was seen around the delimiting fibroblasts and below, but not on the basement membrane. Bar: 30μm
Figure 3.11

Immunoperoxidase (shown in brown) labelling for fibronectin on the non-pregnant human breast (A) and the human breast at the 15th (B) weeks of pregnancy. Immunoperoxidase labelling for laminin in the same order (C, D). Nuclei were counterstained with haematoxylin and are shown in blue. Bar: 70μm

Fibronectin was found in the interstitial tissue, and laminin in the basement membrane. There was no significant difference in the labelling patterns between the pregnant and non-pregnant breasts.
Figure 3.11
Figure 3.12

Images of the negative control for the immunoperoxidase antibodies using the 15\textsuperscript{th} week pregnant human breast tissue. In the absence of primary antibodies, the secondary anti-mouse (A) and anti-rabbit antibodies (B) produced no labelling.
3.3 Discussion

The present study investigated the human mammary functional differentiation in vivo for which the currently available literature is notably restricted. The findings were summarised in Table 3.2. The breast tissues utilised here were essentially normal by careful histological examination, and the morphological development observed in the pregnant breast samples was consistent with previous reports using paraffin-fixed embedded tissues (Battersby and Anderson 1988).

In the pregnant breast, proliferating luminal epithelial cells (Ki67 positive) were uniformly labelled for K19 and occasionally labelled for β-casein and κ-casein. These cells thus represent direct in vivo evidence of what Potten and Loeffler (1990) describe as a dividing transit population in that simultaneously they display signs of proliferation, phenotypic differentiation and functional differentiation. The ability of secretory luminal epithelial cells to proliferate has been previously observed in the rat mammary gland by electron microscopy (Franke and Keenan 1979). Contrary to the conventional model that suggests an inverse correlation between differentiation and proliferation, at the cellular level these events are not mutually exclusive in rats as clearly demonstrated by Franke and Keenan (1979) and also in humans as shown in the present study. However, the simultaneous presence of Ki67 and the milk proteins within
a single cell does not exclude the possibility that the proliferative state might still shut down the expression of milk proteins at the RNA level, if only temporarily.

β-casein and κ-casein have been previously applied as markers of terminal differentiation (Ronen et al 1990, Wilde, Hasan and Mayer 1984). However, such application is inappropriate, since some mammary luminal epithelial cells display the ability to synthesis these proteins during active proliferation. They are, nevertheless, specific markers of functional differentiation, since neither type of casein was detected in the non-pregnant breast, as shown in previous (Earl and McIlhinney 1985) and present studies. Furthermore, in this study, their labelling patterns were shown with a gradual induction of synthesis with the duration of pregnancy.

Active proliferation of myoepithelial cells in the pregnant human breast is a novel observation. The approximate frequency of proliferating myoepithelial cells (2%) in the pregnant breast is higher than that previously determined in the non-pregnant breast (0.2-0.3%) by thymidine labelling and ultra structural studies (Joshi et al 1986). The proliferative potential of myoepithelial cells has been difficult to determine in the past due to their rarity in the non-pregnant breast and the possibility that the proliferating cells were of basal clear cell phenotype (Joshi et al 1986). In the current study, proliferation of phenotypically differentiated myoepithelial cells was
unequivocally demonstrated by concurrent immunolabelling for Ki67 and K14 in the pregnant breast (basal clear cells are largely devoid of cytokeratin filaments) (Smith, Monaghan and Neville 1984).

The examination on pregnant breast tissue cast extra insight into the possible mechanism behind \( \alpha_2 \) down-regulation. Pignatelli, Hanby and Stamp (1991) reported that the expression of \( \alpha_2 \) was significantly reduced (\( P<0.005 \)) in the poorly differentiated grade III ductal breast carcinomas compared to the grade I and II, suggesting its possible role in invasion and undifferentiated morphology. It is worth pointing out, however, that the tumour grading applies the count of mitotic figures, which indicates the proliferation rate of breast cancer cells (Pignatelli, Hanby and Stamp 1991). In the present study, the decreased \( \alpha_2 \) expression was associated with higher proliferation rate of breast epithelial cells demonstrated by Ki67 labelling index during pregnancy. The relative decrease in the expression of integrins has been also observed at the growing terminal end buds of human infant breasts (Anbazhagan et al 1995). Furthermore, in the lactating rodent mammary gland, the level of \( \alpha_2 \) has been reported to decrease at both the protein and mRNA levels (J Oliver and CH Streuli, personal communication, Keely et al 1995). It is therefore possible that the \( \alpha_2 \)
down-regulation in carcinomas is a physiological reflection of increased cellular proliferation, rather than a sign of transformation or poor differentiation *per se*.

The labelling intensity for \( \alpha_2, \alpha_6 \) and \( \beta_4 \) on myoepithelial cells was noticeably greater in the pregnant breast. These findings correspond to the increased intensity and more defined appearance of extracellular matrix (ECM) components, which are in direct contact. In the pregnant breast, myoepithelial cells are highly stretched around the expanding alveoli, and they may require additional mechanical support by the strengthened adhesion between myoepithelial cells and the ECM.

In contrast, the increased expressions of integrins in myoepithelial cells were not accompanied by changes in luminal epithelial cells. The labelling for integrin subunits (\( \alpha_1, \alpha_3, \alpha_6 \)) did not appear to alter noticeably during the examined period of pregnancy (12th, 15th and 18th weeks). This is in agreement with the previous findings in the pregnant mammary gland in rodents (Drs. J Oliver and CH Streuli, personal communication). Therefore, the overall patterns of integrin subunits existing in the quiescent breast prior to pregnancy seem to support adequately the expansion of acini, polarisation of luminal epithelial cells and induction of milk protein synthesis, although there may be functional changes such as ligand bound status that the reagents used in this and other studies do not detect.
The multiple immunofluorescence labelling technique allowed cell-by-cell correlation of various molecular markers on this limited resource, and it has provided \textit{in vivo} evidence of the ability of differentiated luminal epithelial cells to remain in the cell cycle. As well as their importance in understanding human breast biology, the findings reported here are relevant to understanding breast cancer. Many breast tumour cells phenotypic markers of differentiation, such as CK18 and CK19 (Taylor-Papadimitriou \textit{et al} 1989). A few also express the milk proteins (Bartkova \textit{et al} 1987, Simickova \textit{et al} 1989), and a number of human breast cancer cell lines have been shown to synthesis the milk proteins \textit{in vitro} (Ronen \textit{et al} 1990). The ability of breast cancer cells to express signs of differentiation without losing proliferative potential is, therefore, not an abnormality unique to carcinogenic transformation but may reflect the inherent nature of mammary luminal epithelial cells from which the majority of breast cancer cells derive.

A further implication of this finding is that since induction of differentiation (phenotypic or functional) does not coincide with irreversible loss of proliferative potential, it is unlikely that differentiation inducers can achieve complete growth arrest of breast cancer cells. Instead, they may influence malignant potential by affecting
specific characteristics or key genes of transformation. In order to examine such effects on transformation, *in vitro* models of transformation are required.
Table 3.2

Summary Table: Immunohistochemistry on human breast tissues obtained from non-pregnant and 12, 15 and 18 weeks pregnant donors.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ki67 (proliferation)</strong></td>
<td>All luminal epithelial cells in the active cell cycle (Ki67 positive) were phenotypically differentiated (K19 positive) and some were also functionally differentiated (casein positive). Proliferative Index of luminal epithelial cells was in the descending order as follows: 15th week of pregnancy &gt; 18th week &gt; 12th week &gt; non-pregnant. Approximately 2% of myoepithelial cells (K14 positive) were in the active cell cycle.</td>
</tr>
<tr>
<td><strong>β-casein and κ-casein</strong></td>
<td>β-casein and κ-casein were proven as good functional markers since they were undetectable in any of the non-pregnant breast samples, and their labelling gradually increased at the 15th and 18th week of pregnancy.</td>
</tr>
<tr>
<td><strong>Integrin subunit α1</strong></td>
<td><strong>Luminal</strong></td>
</tr>
<tr>
<td></td>
<td>Weakly detectable.</td>
</tr>
<tr>
<td>Integrin subunit $\alpha_2$</td>
<td>Detectable.</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Partial decrease in the pregnant breast.</td>
</tr>
<tr>
<td></td>
<td>Possible inverse correlation with Ki67.</td>
</tr>
<tr>
<td>Integrin subunit $\alpha_3$</td>
<td>Diffuse, weak and variable.</td>
</tr>
<tr>
<td>Integrin subunit $\alpha_6$</td>
<td>Diffuse and weak</td>
</tr>
<tr>
<td>Integrin subunit $\beta_4$</td>
<td>Undetectable.</td>
</tr>
<tr>
<td></td>
<td>Increase in the intensity in the pregnant breast.</td>
</tr>
<tr>
<td>Collagen I</td>
<td>Detectable in the interstitial tissue. Gradual increase in the alveolar expansion and relative decrease in the interstitial</td>
</tr>
</tbody>
</table>

135
space with the duration of pregnancy.

<table>
<thead>
<tr>
<th>Collagen IV</th>
<th>Detectable in the basement membrane around each alveolus and strongly detectable around some stromal cells (likely to be endothelial cells of capillaries). Increase in the overall labelling in the pregnant breast.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>Detectable in the interstitial tissue.</td>
</tr>
<tr>
<td>Laminin</td>
<td>Detectable in the basement membrane.</td>
</tr>
</tbody>
</table>
Chapter 4
The Longitudinal Model of Transformation (LMT) of breast cancer \textit{in vitro} - immunocytochemistry

4.1 Introduction

Up until now, experimental comparisons between differentiation and transformation have been examined largely by horizontal comparisons between cell lines obtained from various individuals. In breast cancer investigation, such methods can be confounded by variables resulting from differences in patient background, specimen sites (from primary site to pleural effusion), tumourigenicity and expression levels of critical growth factor and hormone receptors (Sutherland \textit{et al} 1999). Ideally primary human mammary luminal epithelial cells (HMLEC) should be compared directly with primary breast cancer cells from the same individuals. However, since generation of such primary cells is laborious, and only limited materials are available from each donor, the corresponding longitudinal model of transformation (LMT) offers practical alternative for \textit{in vitro} studies.
In order to address these points, the present study utilised HB4a, H4.1, C3.6, C5.2 and R4.2 as a panel of cell lines that represent a LMT of breast cancer. HB4a is a normal HMLEC line immortalised but not transformed by temperature sensitive large T antigen SV40 (Stamps et al 1994). It was derived from a pure population of non-pathological HMLEC and retains the anchorage dependence, growth factor requirements and morphological characteristics of primary HMLEC in vitro (Stamp et al 1994). From HB4a, Harris et al (1999) derived transformed variants C3.6, C5.2 and R4.2. C3.6 and C5.2 which were engineered to over-express a breast cancer oncogene ErbB2, while H4.1 was concurrently generated as the transfectant control lacking over-expressed ErbB2 (Harris et al 1999). FACS analysis (Harris et al 1999) previously demonstrated the difference between C3.6 and C5.2 as the levels of ErbB2 over-expression being lower and higher respectively compared to a commonly used ErbB2-overexpressing breast cancer cell lines SKBR-3. In comparison with HB4a and H4.1, the transformed phenotypes of C3.6 and C5.2 have been demonstrated as the increase in monolayer growth, anchorage independent growth, reduced growth factor requirement and loss of contact inhibition (Harris et al 1999).

R4.2 was subsequently engineered to express mutant Ha-(Val12)ras. The Ha-(Val12)-Ras expressing line (R4.2), is an important component of the existing LMT,
as it provides an example of a cell line transformed by means independent of ErbB2 over-expression. Ras activates signalling pathways down-stream from the ErbB2 receptor protein tyrosine kinase, involving the family of GTP-binding proteins, Rho, Rac, Cdc42, TC10 and RhoG (Hall 1994, Ridley and Hall 1992). While Ras mutation is rare in breast cancer, high levels of protein expression have been detected, which may mimic the effect of the mutant allele (Pethe and Shekhar 1999). This mutant cell line R4.2 represents a highly transformed phenotype, scoring higher in both anchorage dependent and independent growth assays than either C3.6 or C5.2 (Harris et al 2001), and it has the ability to produce tumours in vivo (Dr H Paterson, personal communication).

Using the anchorage dependent and independent growth assays, the level of transformation has been previously demonstrated in the following order: the highest with the Ha-(Val12)-Ras expressing line (R4.2), followed by the higher ErbB2 over-expressing line (C5.2) and lastly the lower ErbB2 over-expressing line (C3.6), as compared with HB4a the parental line and H4.1 the transfectant control (Harris et al 2001).
In order to confirm that these cell lines retain the presumed phenotypes, detailed immunocytochemical analysis was conducted examining the expressions of K14, K17, K18, K19, Epithelial Antigen, E-cadherin, ErbB2 and vimentin.
4.2 Results

The findings of the immunocytochemistry investigation are summarized in Table 4.1. The parental line HB4a was positive for all the phenotypic markers of HMLEC; K18, K19 (Figure 4.1A), E-cadherin and Epithelial Antigen. While all the cells of HB4a appeared to express these markers, the immunofluorescence labelling intensity varied from extremely thick and bright in some areas to faint and sparse in occasional cells. The moderate ErbB2 over-expressing variant (C3.6) showed a similar positive labelling for these markers to HB4a (Table 4.1).

In contrast, the high ErbB2 over-expressing variant (C5.2) showed a distinct partial labelling. For example, in the C5.2 population, K19 was strongly positive in about a half of the population, while the labelling on the other half of the population was only weakly detectable (Figure 4.1B). Further investigation showed that in C5.2 the same partial labelling pattern was found with Epithelial Antigen (Figure 4.2A) and K18 (Figure 4.2B). Under the phase contrast microscopy, C5.2 comprised of two morphologically distinct populations: islands of round epithelial cells and streams of fibro-epithelial cells (Figure 4.3A). Double immunofluorescence labelling on C5.2 showed that while the former was positive for E-cadherin, the latter was positive for vimentin (normally a marker of cells of mesenchymal origin), and the labelling patterns
for these antigens were mutually exclusive (Figure 4.3B). In C5.2, the ratio of E-cadherin and vimentin labelling was approximately 1:1.

When C3.6 (Figure 4.4A) and HB4a were examined, they both contained occasional cells labelled for vimentin, but the majority of the population was positive for E-cadherin. Further immunofluorescence investigation revealed that the vimentin negative cell populations were labelled for Epithelial Antigen in both C3.6 (Figure 4.4B) and HB4a (Figure 4.4C). The labelling pattern of the transfectant control variant (H4.1) was, however, more like C5.2, in that the epithelial population (labelled for Epithelial Antigen) and the fibroépithelial population (labelled for vimentin) fell into a ratio of approximately 1:1 (Figure 4.5AB). When labelled for K19, the half of H4.1 cell population was positive, while the other half was negative (Figure 4.5C). When the Ha-(Val12)-Ras expressing line (R4.2) was investigated by the same double labelling techniques, all the cells were positive for vimentin, and there was no detectable positivity for E-cadherin or Epithelial Antigen (Figure 4.6).

Although small numbers of HB4a and H4.1 did contain K14 (Table 4.1), which is expressed by myoepithelial cells in vitro (O’Hare 1991), these cells were also positive for the luminal markers K18 and K19. ErbB2 labelling was very strong on C5.2 (Figure 4.7A), positive on C3.6 (Figure 4.7B) and weak/undetectable on HB4a, H4.1 and R4.2.
(Table 4.1). The expression of ErbB2 in C5.2 was strongly detectable in both the epithelial and fibroepithelial populations. All the C5.2 cells were positive for ErbB2, but in some areas the fluorescence labelling was stronger than the rest of the population. Indeed, the labelling intensity in such areas was so strong that the detection was saturated. These cells were typically of the epithelial population, but not all epithelial cells were found with the same level of intensity. In C3.6, all the cells were positive for ErbB2, and there were also some cells with noticeably increased intensity of labelling than the rest of the epithelial population. These findings indicate that the expression of ErbB2 was ubiquitously maintained in both of the lines with a degree of variation in the intensity of labelling within the populations.
Figure 4.1

Immunofluorescence labelling on HB4a (A) and C5.2 (B) for K19 shown in green. Hoechst stained nuclei are shown in blue. HB4a cells had an epithelial morphology and were constitutively positive for K19. C5.2 consisted of two morphologically distinct populations: epithelial and fibro-epithelial. K19 labelling was clearly positive in the epithelial population of C5.2, whereas the labelling in the fibro-epithelial population was extremely faint. Bar: 20\mu m

![Figure 4.1](image)
Figure 4.2

Immunofluorescence labelling on C5.2 for Epithelial Antigen (A) and K18 (B). Hoechst stained nuclei are shown in blue. Only the epithelial population of C5.2 showed clear positive labelling for epithelial antigen and K18. Amongst the fibro-epithelial cells, there were occasional cells labelled for K18, but the labelling on the majority was extremely faint. Bar: 40μm
Figure 4.3

Phase contrast image of C5.2 (A). A monolayer of C5.2 contained the ‘islands’ of cobblestone shaped epithelial cells and the ‘streams’ of spindle shaped fibro-epithelial cells.

Double immunofluorescence labelling on C5.2 for E-cadherin (shown in green) and vimentin (shown in red). Hoechst stained nuclei are shown in blue. The epithelial population was positive for E-cadherin and negative for vimentin. The fibro-epithelial population was positive for vimentin and negative for E-cadherin. Bar: 20μm
Figure 4.4

Double immunofluorescence labelling on C3.6 for E-cadherin (shown in green) and vimentin (shown in red) (A). Double immunofluorescence labelling on C3.6 (B) and HB4a (C) for Epithelial Antigen (shown in green) and vimentin (shown in red).

Hoechst stained nuclei are shown in blue. In C3.6, the labelling for vimentin was found in occasional cells. Bar: 20µm (A), 45µm (B)
Figure 4.5

Double immunofluorescence labelling on H4.1 for Epithelial Antigen (shown in green) and vimentin (shown in red). Hoechst stained nuclei are shown in blue. Like C5.2, H4.1 contained two morphologically distinct epithelial and fibro-epithelial populations. The former was positive for Epithelial Antigen and negative for vimentin. The latter was positive for vimentin and negative for Epithelial Antigen. Bar: 90μm (A), Bar: 45μm (B)
Figure 4.6

Double immunofluorescence labelling on R4.2 for E-cadherin and vimentin (shown in red). Hoechst stained nuclei are shown in blue. The morphology of R4.2 was predominantly fibroblast like with transformed characteristics. The spindle shaped cells overlap each other, forming irregular patches of cells. They were uniformly positive for vimentin and negative for E-cadherin. Bar: 45µm
Figure 4.7

Immunofluorescence labelling for ErbB2 on C3.6 (A) and C5.2 (B). Hoechst stained nuclei are shown in blue. In both lines, the intensity of labelling varied from saturatingly strong in some areas to weakly detectable on other areas. The strongly stained cells were typically of the epithelial population, but not all epithelial cells were found with the same level of intensity of labelling.
Figure 4.7
4.3 Discussion

Some breast epithelial cell lines express characteristics inappropriate to their primary counterparts (as discussed in the Introduction). Therefore, in the present study the LMT was phenotypically validated by extensive immunocytochemical analysis, and the findings were summarised in Table 4.1. As expected from its origin, HB4a showed the expression of the luminal epithelial phenotypic markers (K18, K19, E-cadherin, Epithelial Antigen) and is thus fully representative of the primary HMLEC. These phenotypic markers were aberrantly expressed to varying degrees amongst the variant lines of HB4a, and the most noticeable change was the presence of vimentin positive E-cadherin negative sub-population in C5.2 and H4.1.

Although *in vitro*, the vimentin positive breast cancer cell lines are associated with greater invasive ability and motility (Sommers *et al* 1994a, Sommers *et al* 1994b, Thompson *et al* 1992), *in vitro* the over-expression of vimentin by transfection in MCF7 does not result in enhanced metastatic ability in immunodeficient mice (Hendrix *et al* 1997). In an immunohistochemical study on archival breast tumour samples, the vimentin positivity has been significantly correlated with high-grade tumours, absence of hormone receptors, increased p53 expression and high tumour proliferation fraction (Seshadri *et al* 1996). However, despite such associations, vimentin was not correlated...
with tumour size, axillary lymph node involvement, increase in risk of relapse or death from breast cancer (Seshadri et al 1996). More recently, Thomas et al (1999) observed that if vimentin was expressed concurrently with cytokeratins in breast tumour samples, it indicated poorer prognosis than vimentin positive cytokeratins negative or vimentin negative cytokeratins positive breast tumour samples. In another immunohistochemical study, Dandachi et al (2001) found that the expression of vimentin was significantly associated with the expression of an extracellular matrix glycoprotein tenascin-C. However, it was tenascin C, not vimentin that was positively associated with markers of poor prognosis such as ErbB2 over-expression and ER negative status.

In case of the LMT established in this study, the transfectant control H4.1 that contains vimentin positive fibro-epithelial population remains otherwise relatively similar to the parental HB4a in the basic growth rate, insensitivity to TNFα and unamplified status of ErbB2 expression. While previously some researchers have suggested the gain of vimentin expression as one of the characteristics of transformed phenotype (Bichat et al 1997, Sommers et al 1989), H4.1 offers an alternative explanation that vimentin positivity may be a property of culture adaptation or clonal selection rather than a direct consequence of transformation.
An emerging picture is that in both \textit{in vitro} breast cancer cell lines and \textit{in vivo} breast cancer cells, the gain of vimentin expression is associated with theoretical characteristics of ‘aggressiveness’, but by itself vimentin does not determine the actual clinical outcome of the disease in contrast to oncogenes such as ErbB2 (Harris \textit{et al} 2001).

The G5.2 cell line was derived through the combination of dilution and ring cloning from a FACS sorted ErbB-2 over-expressing population. However, as the line proliferates towards confluence the clone displays a mixture of 2 distinct morphologies, being split between epithelial and more fibroepithelial in appearance. During the initial characterisation of the line, attempts were made to separate these apparently different populations using sequential rounds of differential trypsinisation. This technique is based on the observation that fibroepithelial cells are more sensitive to trypsinisation than epithelial cells. A brief incubation in trypsin allows the fibroepithelial cells to be removed by washing while leaving the epithelial cells attached to the tissue culture surface. Fibroepithelial cells and epithelial cells were then cultured separately. Although, three rounds of differential trypsinisation were performed, both of the cultures continued to produce a mixed morphology (Harris, unpublished data).
Table 4.1

Expression patterns of phenotypic markers on HB4a, H4.1, C3.6, C5.2 and R.2 examined by immunocytochemistry

+++++ = very strongly and uniformly positive
++++ = uniformly positive
+++ = positive throughout but intensity of labelling varies
++ = partially positive
+ = occasional single cells positive
- = undetectable

<table>
<thead>
<tr>
<th>Antigen</th>
<th>HB4a</th>
<th>H4.1</th>
<th>C3.6</th>
<th>C5.2</th>
<th>R4.2</th>
<th>Primary antibody (dilution)</th>
</tr>
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<tr>
<td>Cytokeratin 18</td>
<td>++++</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
<td>-</td>
<td>LE65 mouse IgG (1:10)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cytokeratin 19</td>
<td>++++</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
<td>-</td>
<td>LP2K mouse IgG2b (1:10)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Antibody</td>
<td>Strength</td>
<td>Source</td>
<td>Notes</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-cadherin</td>
<td>++++</td>
<td>C20820 mouse</td>
<td>IgG2a (1:100)²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial Antigen</td>
<td>++++</td>
<td>Ber-EP4 mouse</td>
<td>IgG1 (1:20)³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>+</td>
<td>Vim 13.2 mouse IgM</td>
<td>(1:100)⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokeratin 14</td>
<td>+</td>
<td>LL002 mouse</td>
<td>IgG31 (1:10)¹</td>
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<tr>
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<td>-</td>
<td>c-neu (Ab-3)</td>
<td>IgG (1:100)⁵</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- All the secondary antibodies were from Southern Biotechnology Associates, Cambridge, UK

- Sources of the primary antibodies
  1 Dr. E.B. Lane University of Dundee, Dundee, UK
  2 Transduction Laboratories via Affiniti, Exeter, UK
  3 DAKO, High Wycombe, UK
4 Sigma-Aldrich, Poole, UK

5 Calbiochem via CN Biosciences, Beeston, UK
Chapter 5
Pharmacological selectivity demonstrated by the LMT of breast cancer

5.1 Introduction

Pharmacological agents that work specifically against transformed cells while sparing non-transformed cells are of immense potential therapeutic value. Such selectivity is associated with greater efficacy and reduced toxicity compared to the existing broad cytotoxic agents. In the treatment of breast cancer, selective agents like Herceptin can yield significant benefit when combined with conventional chemotherapy (Piccart 2001, Kumar, Mandal and Vadlamudi 2000), and identification of further selective agents would generate future therapeutic candidates.

However, at present there are only a few established LMT systems of breast cancer. One of the most recent examples was the application of primary human mammary epithelial cells (HMEC) populations transfected to over-express ErbB2. When Orr, O’Connor and Kohn (2000) compared the ErbB2 over-expressing HMEC and the transfectant control HMEC, none of the examined chemotherapeutic agents
showed selectivity. It was hence concluded that for ErbB2 over-expressing cells to become more chemo-sensitive than the normal counterparts, there requires additional genetic alterations. However, primary HMEC as normally generated consist mainly of basal / myoepithelial cells (Page et al 1999), and thus they are phenotypically distinct from HMLEC that are a normal counterpart of most breast cancers (Taylor-Papadimitriou et al 1989). This may possibly have influenced the findings of Orr, O'Connor and Kohn (2000). In addition, pharmacological selectivity is more likely to be found with agents whose actions are receptor or pathway specific than broad cytotoxic agents.

Chapter 4 verified the cell lines of the LMT as an appropriate in vitro model of breast cancers from the following points of view. First, HB4a was observed to express the selected phenotypic markers of primary luminal epithelial cells as expected from its origin. This is a crucial point for the LMT, since HB4a was used to represent 'normal HMLEC'. Without such immunocytochemical characterisation, uncertainties remain about its phenotype, in particular in respect to possible contamination and overgrowth by myoepithelial cells or fibroblasts. Second, C3.6 and C5.2 expressed ErbB2 as expected from its transfection history (mimicking approximately 30% of primary breast tumours). Third, the variants (C3.6, C5.2, R4.2 and H4.1) of HB4a expressed varying
degrees of the phenotypic markers of luminal epithelial cells and of mesenchymal cells
as found in true breast cancer samples (Seshadri et al 1996, Thomas et al 1999,
Dandachi et al 2001). For instance, a subset of clinical breast cancer samples consists of
a mixture of epithelial (vimentin negative, E-cadherin and Epithelial Antigen positive)
and fibroepithelial (vimentin positive) cell populations. This was the case in H4.1 and
C5.2. In other breast cancer samples, the majority of cells are found to contain epithelial
population, and this was certainly the case in HB4a and C3.6. In some breast cancers,
the majority of cells are of fibroepithelial phenotype, as was the case in R4.2. These
immunocytochemical studies have allowed the fundamental understanding in respect to
the phenotypic characteristics of the cell lines, the presence of fibroepithelial population
and how that is not a direct consequence of the cells being transformed. Therefore, the
immunocytochemical profiling in Chapter 4 demonstrated the presently utilised LMT
reflects the in vivo observations commonly found in breast cancer.

The present chapter examined whether the LMT can be utilised to analyse the
selectivity of pharmacological agents. The effects of TNFα, TRAIL and DMSO were
investigated. Both TRAIL and TNFα have been believed to exert selectivity on the
transformed cells (for review see Bonavida et al 1999, Mueller 1998) while DMSO is
associated with potent growth inhibition and differentiation effects on both transformed
and non-transformed cell types (Neulieb and Neulieb 1990). TNFα has been reported to induce differentiation in a number of cell types such as haematopoietic cells, human rectal adenocarcinoma cell line (DiFi) (Novotny-Smith et al 1993), myeloblastic cell line (ML1) (Sakai et al 1993) and keratinocytes (Wang and Jensen 1998). In contrast, the majority of publications to date on TRAIL have explored its selective action as an apoptosis inducer. Therefore, at present, it is unknown whether TRAIL also induces differentiation in these cell types. The present study is the first investigation of these pharmacological agents using a LMT, in the context of human breast cancers.

Differentiation is partly estimated by growth arrest, which in turn is measured as a reduction in cell numbers over a time-course, compared to an untreated control. The reduction in cell numbers is influenced by both cell proliferation and cell death. Cell death is divided into two physiologically distinct phenomena: necrosis and apoptosis.

Apoptosis is a form of programmed cell death and is a normal and fundamental event in many biological processes, including involution of the breast (Groner and Hennighausen 2002, Anderson 1999). The balance of the steroid hormones progesterone and oestrogen determines the rate of cell growth and cell death by apoptosis in normal breast cells and many breast cancers (Shi et al 1994). The acquisitions of mechanisms that allow the evasion of death by apoptosis, such as the
overexpression of the bcl-2 proto-oncogene, are acknowledged as an important step in the development and progression of malignancy (Jager, Jansen and Arends 2002).

The ligands of the ErbB receptor protein tyrosine kinase family, epidermal growth factor and heregulin have both been shown to act as survival factors (Merlo et al 1995, Pinkas-Kramarski et al 1994), while overexpression of ErbB-2 has been shown to be inversely correlated with resistance to apoptosis induced by therapeutic agents such as cisplatin and radiotherapy (Langton-Webster et al 1994, Merlo et al 1995). Many current therapeutics such as tamoxifen cause tumour regression through the induction of apoptosis (Mandlekar and Kong 2001).

Several molecular and morphological events have been described as characteristic of apoptosis. The most common characteristics include the fragmentation of DNA and condensation of chromatin, the activation of proteases called caspases that act on components of the DNA repair, and the redistribution of phosphatidylserine from the cell interior to the cell surface. These characteristics represent late, mid and early events in the apoptotic process (for reviews, see Wajant 2002, Kanduc et al 2002, Saikumar et al 1999).

Binding of death ligands (FasL/CD95L, TRAIL/APO-2L, APO-3L and TNF) induces trimerization of their receptors (Fas/CD95, DR4/DR5, DR3, and TNFR), which
then recruit adaptors, namely FADD (Fas-associated death domain protein) and TRADD (TNFR-associated death domain protein), and activate caspases. Some caspases (e.g., caspase 8 and 10) are involved in the initiation of apoptosis and others (caspase 3, 6, and 7) execute the death order by destroying essential proteins in the cell. Binding of membrane bound or soluble TNF which leads to trimerization of TNFR1 is believed to activate two major pathways: a caspase cascade leading to cell death and a kinase cascade leading to the activation of the NF-kB and JNK pathways. These two pathways are believed to diverge at the level of an adaptor molecule TRADD. In the first pathway, the proenzymatic form of caspase 8, formerly known as FLICE, MACH or Mch5, binds to the complex by interaction between the death effector domains (DED) of the FADD and the caspase 8 molecule. Binding results in the activation of caspase 8 which then triggers the caspase cascade. This pathway can be blocked by FLIP but not by Bcl-2 (for reviews, see MacEwan 2002, Sartorius, Schmitz and Krammer 2001, Daniel et al 2001).

The second pathway involves the accessory proteins TRADD, RIP (receptor-interacting protein) and TRAF2 (TNF receptor-associated factor 2). Binding of these proteins to oligomerized TNFR1 can either lead to apoptosis by induction of the caspase cascade or formation of the second messenger ceramide or to survival by
NF-κB activation and the subsequent expression of survival factors. TRAF2 activates IκB kinase (IKK). The active IKK activates NF-κB by degrading the inhibitor protein IκB. Activation of NF-κB triggers the degradation of IκB through the ubiquitin system, where the target molecule is masked by a chain of ubiquitins for degradation by the 26S proteosome. The free NF-κB can then translocate to the nucleus and activate transcription (for reviews, see Almond and Cohen 2002, Ben-Neriah 2002).

5.2 Results

The effects of TNFα, TRAIL and DMSO on cell growth were examined using methylene blue growth assay, haemocytometer counting and MTT assay. MTT was conducted to investigate dosage and time dependent effects of these compounds on cell growth, whereas the former two methods were applied to confirm the differential responses to specified dosages between the cell lines. A minimum of three separate methylene blue growth assays was carried out for each pharmacological agent, and the selectivity of TNFα was further confirmed by haemocytometer counting.

Methylene blue growth assays demonstrated that TNFα (10ng/ml) decreased the 5th day final cell numbers of the ErbB2 over-expressing lines, C3.6 (P<0.02) and C5.2 (P<0.02), but not of their non-transformed counterparts (HB4a, H4.1) or the
Ha-(Val12)-Ras expressing line (R4.2) (Figure 5.1). The level of this effect by TNFα correlated with the level of ErbB2 over-expression, as both were higher in C5.2. Preliminary results of serial dilutions showed that TNFα at 1ng/ml had no effect (data not shown). TNFα at 20, 30 or 100ng/ml exerted no greater growth inhibitory effect than at 10ng/ml (data not shown).

Using a MTT assay, it was confirmed that both C3.6 and C5.2 were more sensitive to TNFα than HB4a and R4.2 (Figure 5.2). The dosage required for the differential growth inhibition in the MTT assay was at 50-100ng/ml. The TNFα sensitivity of C3.6 and C5.2 were statistically significant in comparison to HB4a (P value = 0.016 and 0.021 respectively). In contrast, R4.2 was as resistant to TNFα as HB4a, and there was no statistically significant difference between these lines. When the effect of TNFα was examined at three time points (day 1, day 3 and day 5) (Figure 5.3), there was no significant difference between the lines on day 1 or day 3. The sensitivity difference, however, became obvious on day 5. Interestingly, according to the MTT assay, C3.6 showed the greatest sensitivity to TNFα and C5.2 was the second most sensitive, whereas according to the methylene blue growth assay C5.2 was more sensitive than C3.6. Direct cell counting by a haemocytometer demonstrated that C5.2
was more sensitive to TNFα than C3.6, confirming the result of the methylene growth assay.

In the methylene blue assay, DMSO induced the same dose-dependent decrease in the 5th day final cell numbers (P<0.02) compared to untreated controls on all the cell lines used in this study, with no apparent selectivity (Figure 5.1). TRAIL (100ng/ml) had no effect in the absence of TRAIL enhancer (data not shown), and TRAIL enhancer (2μg/ml) on its own did not affect HB4a or MDA-MB-231, which was used as a positive control (Schneider et al 1997, Schneider et al 1998) (data not shown). However, when TRAIL (100ng/ml) and TRAIL enhancer (2μg/ml) were combined, they decreased the 5th day final cell numbers of all the lines significantly (P<0.02) including MDA-MB-231 (Figure 5.1). The ErbB2 over-expressing lines (C3.6, C5.2) were no more sensitive to the combination of TRAIL and TRAIL enhancer than the non-transformed lines (HB4a, H4.1), while the Ha-(Val12)-Ras expressing line (R4.2) showed the greatest sensitivity to this treatment.

Next, the cell lines were examined for possible induction of apoptosis. Alongside with the positive control staurosporine, the cell lines were treated with TNFα, TRAIL or DMSO for 5 days. They were stained for caspase expression or with Hoechst for identifying the number of apoptotic bodies.
In the Hoechst labelling assay, the apoptotic cells were manually counted from digital images of three separate visual fields (Figure 5.4). The mean and standard deviation of the three counting results were used to represent each experimental condition. The numbers of apoptotic cells were significantly increased (P<0.002) when HB4a and R4.2 were treated with staurosporine. However, other experimental conditions produced no significant effect in these lines. In C3.6, the numbers of apoptotic cells were slightly lower (P<0.02) in the populations that were treated with DMSO, staurosporine or TNFα than the untreated population or population treated with TRAIL. C5.2 showed no significant difference between the treated and untreated populations. It was surprising that in C3.6 and C5.2, there were no significant increase in the number of apoptotic bodies, because when the treated populations were examined under microscope, there were some floating cells in the medium. For the Hoechst labelling, the medium was discarded and the monolayer of cells gently rinsed. Thus, this procedure might have removed the existing cells that were apoptotic.

Therefore, the present study also employed the caspase assay. In the cascades of apoptotic events, the induction of caspases is an early stage event in comparison to the appearance of apoptotic bodies (identified by the Hoechst labelling). The caspase assay showed (Figure 5.5) that TNFα produced as high an induction of caspase as the positive
control compound staurosporine. In contrast, the inductions of caspase in C3.6 by TNFα or TRAIL were not as marked as staurosporine, and they were not significantly different when compared to the control population. The induction of caspase was noticeably low in R4.2 population treated with staurosporine. However, it should be pointed out that in the presence of this compound there were very few adherent cells left with the majority of the cells floating as cell debris. An additional MTT assay was conducted to confirm these points (Figure 5.6). The findings of the present study hence indicate that the low level of caspase induction in R4.2 by staurosporine simply reflects the low cell number. In contrast, when the R4.2 population was treated with TNFα, the induction of caspases was significantly increased (Figure 5.5), while the overall cell numbers measured by the MTT assay was equivalent to that of the untreated population (Figure 5.6).
Figure 5.1

Methylene Blue Growth Assays: cells were treated with the experimental conditions (untreated, 1% DMSO, 10ng/ml TNFα or 10ng/ml TRAIL) for 5 days. Y axis represents the 5th day final cell number. Cell viability was estimated by uptake of the methylene blue dye, quantified by measurement of absorbance at 650nm wavelength. Y axis shows the % cells at day 5 in comparison to the untreated control. The decrease in the 5th day final cell numbers in comparison to the untreated control was statistically significant (P<0.05) in all conditions except the HB4a and H4.1 treated by TNFα.
Figure 5.2

MTT growth assay: cells were treated with 50ng/ml TNFα for 1, 3, and 5 days. Cell viability was estimated by uptake of the dye, quantified by measurement of absorbance at 570nm. X axis shows days and Y axis shows the % cells in comparison to the untreated control. Error bars represent standard deviations. No difference in sensitivity was observed on day 1 or 3. However, on day 5, the ErbB2 variants (C3.6 and C5.2) showed significantly greater sensitivity to TNFα than HB4a and R4.2.
Figure 5.3

MTT growth assay: cells were treated with TNFα at the indicated levels in ng/ml (X axis) for 5 days. Y axis shows the % cells in comparison to the untreated control. Error bars represent standard deviations. The ErbB2 expressing variants (C3.6 and C5.2) were significantly more growth inhibited than HB4a and R4.2 by TNFα at 50 and 100ng/ml.

According to the unpaired T test, the TNFα sensitivity of C3.6 and C5.2 were statistically significant in comparison to HB4a (P value = 0.016 and 0.021 respectively).

In contrast, R4.2 was as resistant to TNFα as HB4a, and there was no statistically significant difference between these lines.
Figure 5.3

![Graph showing TNFalpha ng/ml vs. % of Control for HB4a, C3.6, C5.2, and R4.2](image-url)
Figure 5.4

Hoechst labelling assay: the apoptotic cells were manually counted from digital images of three separate visual fields. The mean and standard deviation of the three counting results were used to represent each experimental condition (Y axis). Error bars represent standard deviations. The numbers of apoptotic bodies were significantly increased (P<0.02, marked with upward black arrow) when HB4a and R4.2 were treated with Staurosporine. However, other experimental conditions produced no significant effect in these lines. In C3.6, the numbers of apoptotic cells were slightly lower (P<0.02, marked with downward black arrows) in the populations that were treated with DMSO, Staurosporine or TNFα than the untreated population or population treated with TRAIL. C5.2 showed no significant difference between the treated and untreated populations.
Figure 5.5

Caspase assay; Cells were plated at $1 \times 10^4$ cells/well in a black walled 96 well microtitre plate and treated, after overnight incubation, with 1% DMSO, 10mM Staurosporine, 10 ng/ml TNF-α, 10 ng/ml TRAIL (plus 2µg/ml enhancer). On day 5, apoptosis was analysed using a Fluorimetric Homogenous Caspases Kit. Y axis represents the extent of caspase induction quantified by cleaved substrate measurement of a fluorimetric change produced by excitation at 470-500 nm and emission at 500-561 nm. Error bars represent standard deviations. When treated with each compound, HB4a showed statistically significant decrease in the caspase induction in comparison with the untreated population ($P<0.02$, marked with ▼). In comparison with the untreated population, C3.6 showed statistically significant decrease when treated with DMSO ($P<0.02$, marked with ▼) and significant increase when treated with Staurosporine ($P<0.02$, marked with ▲). In comparison with the untreated population, C5.2 showed statistically significant decrease when treated with DMSO ($P<0.02$, marked with ▼) and statistically significant increase when treated with Staurosporine and TNFα ($P<0.02$, marked with ▲). In comparison with the untreated population, R4.2 showed significant decrease when treated with Staurosporine ($P<0.02$, marked with ▼) and significant increase when treated with TNFα ($P<0.02$, marked with ▲).
Figure 5.5

[Graph showing caspase induction for different treatments: Untreated, DMSO, Staurosporine, TNFα, and TRAIL. The treatments are represented by different colors and symbols: HB4a, C3.6, C5.2, and R4.2.]

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Figure 5.6

MTT growth assay: R4.2 cells were treated with the experimental conditions (untreated, 1% DMSO, 10mM Staurosporine which was used as the positive control for the apoptosis assays, 10ng/ml TNFα) for 5 days. Cell viability was estimated by uptake of the dye, quantified by measurement of absorbance at 570nm (Y axis). In comparison with the untreated population, the 5th day final cell number was decreased significantly when treated with Staurosporine and with DMSO (P<0.02, marked with ▼) but not with TNFα. The cell viability was the lowest in the presence of Staurosporine.

* Statistically significant decrease compared to the untreated population
5.3 Discussion

The present study established a LMT for breast cancer and demonstrated its potential value for screening for pharmacological selectivity. The LMT expressing individual oncogenes, such as ErbB2 or Ha-(Val12)-Ras as in the present study, can provide an important tool for the investigation of the relationship between chemotherapeutic agents and specific oncogenes. To examine whether the LMT was able to identify pharmacological selectivity against the transformed cells, the present study employed two agents previously reported to be selective, TNFα and TRAIL, and one non-selective agent, DMSO.

The growth assays employed in the present study did not produce totally equivalent results. Such differences may be due to the fact that each growth assay measures a different biological aspect. The methylene blue dye is absorbed by macromolecules including proteins and nucleotides. This growth assay measurement included any cells that were adherent to the substratum after the monolayer had been washed gently with PBS twice before fixation. Haemocytometer is the most direct method of counting actual cell numbers. However, this assay also counted any cells that were adherent after the monolayer had been washed gently with EDTA twice before trypsinisation. It is therefore reasonable that the two counting methods yielded
compatible results. The MTT growth assay in contrast measures the mitochondrial activity as a surrogate marker of cell viability. It is a convenient way of measuring cell growth, and it is particularly suited to high throughput screening of chemical libraries. The dye absorbance of the MTT growth assay can be influenced by changes in the mitochondrial activity, and hence such high throughput results generally require further confirmation by direct methods of cell number counting such as haemocytometer and Coulter Counter. In the MTT growth assay, the supernatants were aspirated, but the monolayers were not washed. Although the absence of washing steps might have left more cells adherent to the substratum, the dye would have been absorbed only by viable cells.

Despite some differences observed in these growth assays, the enhanced TNFα sensitivity of the ErbB2 over-expressing variants was a consistent finding. In both of the assays, C3.6 and C5.2 were more TNFα-sensitive than HB4a and R4.2, and the decreases in the 5th day cell numbers were statistically significant. It is unclear however why according to the MTT growth assay C3.6 was slightly more sensitive than C5.2, whilst in the methylene blue growth assay C5.2 was more sensitive than C3.6. In the methylene blue growth assay, the decreases in the 5th day cell numbers were statistically significant at 10ng/ml, whilst in the MTT growth assay, they were observed at higher
concentrations (25, 50 and 100ng/ml). The difference in the concentrations may be due to the fact that TNFα stock was prepared at two separate occasions. For the methylene blue growth assay, the stock was prepared fresh (30 minutes before it was diluted and applied to the cells). In contrast, for the MTT growth assay, the stock was prepared a week in advance, although this was within the recommended storage period according to the product data sheet. Ideally, these growth assays were conducted at the same time from the same TNFα stock. However, this was not possible due to the time and reagent limitations.

The interpretation of the apoptosis assays also requires further considerations as to what each assay measures. The caspase induction is an earlier event than the appearance of apoptotic bodies detected by Hoechst labelling. It is also possible that these assays would have detected different levels of apoptosis if they had been conducted on earlier time points. The induction of caspase assay indicated that the decrease in the 5th day cell number of C5.2 in the presence of TNFα may be due, at least partially, to apoptosis. In C3.6, the induction of apoptosis was not detectable by the Hoechst labelling or the induction of caspases. Nevertheless, it is possible that TNFα induced apoptosis to some extent, some floating cells were observed under microscope
on day 5. By the time the apoptotic cells become detached from the substratum, the apoptotic assays utilised in the present study are unable to detect them.

**TNFα**

TNFα is an extracellular multi-functional cytokine with cytotoxic, cytostatic, immunomodulatory and differentiating activities (Sidhu and Bollon 1993). The apoptosis-inducing effect of TNFα has been studied in great details. The cognate receptor of TNFα, TNF Receptor (TNFR) contains the cytoplasmic ‘death domain’ which binds to an adaptor protein FADD through TRADD (Boldin *et al* 1995, Hsu *et al* 1996). FADD in turn activates the ced-3-related protease MACHα/FLICE (caspase 8), thereby initiating a series of caspase-dependent events that lead to cell death (Boldin *et al* 1996).

While chemotherapeutic drugs and radiotherapy normally require functional p53 tumour suppressor gene, cancer cells often acquire inactivating p53 mutations and become resistant to therapy. In contrast, TNFα induces apoptosis independent of p53 in many tumour cells. TNFα was thus once a much-hoped-for therapeutic candidate for cancer treatment (Sidhu and Bollon 1993). However, TNFα activates the proinflammatory transcription factor NF-κβ in vascular endothelial cells and
macrophages, leading to a lethal inflammatory response (Tartaglia and Goeddel 1992). This severe toxic side effect precluded TNFα from its use in systemic anti-cancer therapy. Nevertheless, more recent pre-clinical and clinical studies have revived TNFα for its low dose application in combination therapy with cytotoxic drugs for the treatment of solid tumours (van Der Veen et al 2000).

In the present study, TNFα exerted selective decrease in the 5th day final cell numbers on the ErbB2 over-expressing variants, whilst having no effect on the non-transformed cells or the ras-expressing variant. This selectivity towards the ErbB2 over-expressing phenotype is a finding in favour of TNFα as a future therapeutic agent for breast cancer.

Although there have been a number of reports on ErbB2 over-expressing cell lines being TNFα resistant, most of these studies were based on other systems such as mouse fibroblast cell line (Hudziak et al 1988) and human ovarian cancer cells (Lichtenstein et al 1990). In the context of human breast cancer, the association between ErbB2 over-expression and TNFα resistance is less clear. While a breast cancer cell line SKBR-3 that over-expresses ErbB2 is TNFα resistant (Lichtenstein et al 1990), so is another breast cancer cell line MDA-MB-231 that does not over-express ErbB2 (Sutherland et al 1999). Furthermore, when a non-ErbB2 over-expressing breast cancer
cell line MCF7 was transfected to over-express ErbB2, it did not become resistant to TNFα (Egeblad and Jaattela 2000). When Lichtenstein et al (1990) examined a human cell line HBL100 that does not over-express ErbB2, the cell line was resistant to TNFα, as the present study found with the non-transformed HMLEC lines, HB4a and H4.1. Our findings suggest that the TNFα resistance in these cell lines may be due to their non-transformed phenotype, since the growth of primary HMLEC is also unaffected by this agent (data not shown). The ErbB2 over-expressing lines (C3.6, C5.2) applied in the current LMT are unique in that their transformed phenotypes are the result of a single oncogene, ErbB2 over-expression. In contrast, SKBR-3 and MDA-MB-361 (another ErbB2 over-expressing breast cancer cell line) were derived from breast cancer cells obtained as a pleural effusion or as a brain metastasis respectively (Sutherland et al 1999). They are thus likely to contain other genetic changes that could influence TNFα sensitivity.
TRAIL was discovered because of its sequence homology to TNFα. It triggers apoptosis in a number of tumour cell lines (Pitti et al 1996, Wiley et al 1995). Although most reports on TRAIL to date have been based on its apoptosis-inducing effect, TRAIL is expected to be multi-functional like TNFα, and its receptor Death Receptor (DR) 4 (Pan et al 1997) and DR5 (Sheridan et al 1997) belong to the TNFR gene superfamily.

In contrast to TNFα, TRAIL mRNA is expressed constitutively in many human tissues, which indicates the physiological mechanisms that protect these tissues from TRAIL-induced apoptosis (Pitti et al 1996, Wiley et al 1995). One potential mechanism has been proposed to involve expression of antagonistic decoy receptors (Decoy Receptor (DcR) 1, DcR2) that can compete with DR4 and DR5 for ligand binding (for review see Marsters et al 1999).

Recent enthusiasm for the therapeutic application of TRAIL is based on two factors. First, unlike TNFα, the toxicity of TRAIL is very low in rodents and primates (Ashkenazi et al 1999). Second, TRAIL has been widely reported to induce apoptosis selectively against transformed cells while sparing non-transformed cells (Marsters et al 1999, Ashkenazi et al 1999). However in breast cancer, induction of apoptosis by TRAIL has been shown to be not so selective. When the cytotoxic effect of TRAIL was
examined in 8 breast cancer cell lines and 8 normal breast epithelial cell populations, 2 of them, MCF10 and MDA-MB-231, were growth inhibited by TRAIL (Keane et al 1999). Since MCF10 (Soule et al 1990) is considered a normal breast epithelial cell line and MDA-MB-231 (Cailleau et al 1974) a breast cancer cell line, the effect of TRAIL was concluded not to be specific to transformed phenotype. Furthermore, in this panel, there was no correlation between the expression levels of DR4, DR5, DcR1 and DcR2 and the TRAIL sensitivity or resistance.

The findings of the present study are in agreement with Keane et al (1999), in that TRAIL exhibited growth inhibitory effects on both the transformed and non-transformed phenotypes. These findings represent a good example of how phenomena such as pharmacological selectivity require system specific examinations.
**DMSO**

The differentiation inducers mentioned above are receptor specific and resistance can develop through either down-regulation of their receptors or up-regulation of inhibitory binding proteins, whereas synthetic compounds such as DMSO act independently of any known receptors. Since DMSO is relatively small and bipolar, its potential effects on cellular biology are numerous and complex (Yu and Quinn 1998).

DMSO has been shown to stabilise the phospholipid membrane by decreasing its fluidity (Lyinan and Preisler 1976), and this effect is accompanied by changes in lectin agglutinability, protein expression, DNA structure and DNA methylation (Hunt and Marshall 1979, Eisen et al 1977, Neulieb and Neulieb 1990) as well as a transient increases in the intracellular calcium ion concentration (Morley and Whitfield 1993). DMSO has been reported to induce both differentiation and growth arrest in many cell types, and administration of 10% DMSO during human bone marrow transplantation has been proposed to enhance the success rate achieved in various malignancies by exerting its differentiating effect on metastatic cells (Toren and Rechavi 1993).

As expected, DMSO 1% (v/v) decreased the 5th day final numbers (P<0.02) of both the transformed and non-transformed lines to equivalent extents. The lack of
selectivity in DMSO shows that selective effects of other pharmacological agents is not
due to general properties of the cell lines such as different growth rates.

Discussion

The exact mechanism behind the TNFα selectivity on the transformed cells, in
particular on the ErbB2 over-expressing variants, is yet to be investigated. The only
other agents that have been shown to induce apoptosis and growth inhibition
specifically in ErbB2 over-expressing cell lines (e.g. SKBR-3) is neu differentiation
factor (NDF) – a member of the epidermal growth factor related peptide family (Daly et
the NDF receptors, erbB3 and erbB4, and causes down-regulation of ErbB2. NDF
stimulates phosphatidylinositol 3-kinase (PI3K) pathway and the induction of apoptosis
is dependent on p38 MAPK (Daly et al 1997). In the area of TNF cytokine receptor
family, ErbB2 has been shown to associate with Fas in a breast epithelial cell line
MCF-10AT, and anti-Fas treatment results in significant decrease in this Fas-ErbB2
association as well as the induction of apoptosis (Shen and Novak 1997). TNFα has
been reported to decrease the expression of ErbB2 mRNA and protein in pancreatic
tumour cells (Kalthoff et al 1993) and in an ErbB2 over-expressing breast cancer cell

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line SKBR-3 (Kumar and Mendelsohn 1994). It is possible that the TNFα sensitivity observed in the ErbB2 over-expressing lines (C3.6, C5.2) results from ErbB2 down-regulation and a consequent reduction in the growth promoting effect of receptor over-expression (Harris et al 1999). The exact mechanism behind the selectivity of TNFα on the ErbB2 over-expressing lines (C3.6, C5.2) has yet to be established.

The LMT introduced in the present study provides a platform for three types of investigations. First is to study the molecular mechanism of TNFα and ErbB2 interactions. Second is to explore the potential of a combination therapy where TNFα at low concentrations may work synergistically with Herceptin without inducing severe side effects. Such combination therapy may help to avoid the reported side effects of TNFα and may be applicable for, in particular, those patients who develop a resistance to Herceptin despite continued ErbB2 over-expression. Third is to screen agents that are potential equivalents with high sequence identity such as TNFα (Nedospasov et al 1986), TNFC (Nedwin et al 1985), LIGHT (herpes virus entry mediator) (Mauri et al 1998) and vascular endothelial cell growth inhibitor (Tan et al 1997).
Chapter 6

The LMT of breast cancer

6.1 Introduction

The growth assays in the Chapter 4 demonstrated that the LMT system is a robust model for the screening of pharmacological selectivity. The current chapter describes a series of experiments that employ biochemical techniques to characterise changes in the expression of molecules that may be used as markers in the treatment of breast cancer.

The principle aim of the chapter was to demonstrate the utility of the LMT system rather than exploration of any mechanism. For the RT-PCR and Western blotting studies, the moderate ErbB2 expressing variant C3.6 was chosen. C3.6 is sensitive to TNFα (Chapter 5), and it consists largely of an epithelial population (Chapter 4). It was therefore anticipated that any changes in the expression level of epithelial markers such as K19 and MUC1 in the present study could be associated with the entire C3.6 population. In contrast, in C5.2 interpretation of such changes is complicated by potential proportional changes between epithelial and fibroepithelial populations.
The moderate ErbB2 over-expressing cell line C3.6 was cultured for 72 hours in 4 experimental conditions: (1) at the permissive growth temperature (36.5°C), (2) at the non-permissive temperature (39.5°C), (3) in the presence of DMSO at the permissive temperature and (4) in the presence of TNFα at the permissive temperature. By exposing cells to the above conditions, samples were obtained to allow the comparison between the exponential growth, SV40 large T antigen-dependent growth arrest, growth inhibition by a non-selective agent and growth inhibition by a selective agent, respectively.

SV40 polyoma large T antigen binds to p53 (Stamps et al 1994). However, since the immortalising viral protein in C3.6 is a temperature sensitive mutant A58-U19, at 39.5°C it becomes inactivated and releases p53. P53 arrests the cell cycle and directly induces the expression of many genes involved in cell cycle regulation including p21. P21 is transcriptionally activated by wild type p53 and inhibits G1 associated cyclines. In primary breast tumours, the expressions of p21 and p53 are positively correlated (Thor et al 2000).

DMSO increases the integrity of the phospholipid membrane, and this effect is followed by a large scale of changes in gene transcription, protein expression and post-translational modification (Neulieb and Neulieb 1990). In contrast to DMSO, the
growth inhibitory effect of TNFα has been shown to involve various receptor and pathway specific mechanisms (for review see Bonavida et al 1999, Mueller 1998). Of particular interest to the current study are the reports linking TNFα to the down regulation of ErbB2 expression in the breast cancer cell line SKBR-3 and in pancreatic tumour cell lines (Kumar and Mendelsohn 1994) (Kalthoff et al 1993).

ErbB2 belongs to the EGFR family. Another member of the family ErbB3 is also amplified in breast cancer (in 20%), associated with lymph node metastasis (Lemoine et al 1992) and poorer prognosis (Naidu et al 1998). In vitro, ErbB3 heterodimerises with ErbB2 and becomes tyrosine-phosphorylated upon interaction with heregulin. Dimerisation of ErbB2 and ErbB3 is hence believed to be important in breast cancer (Sliwkowski et al 1994).

In primary breast tumours, ErbB2 is inversely correlated with ER, but this correlation is not absolute in that some breast tumours express both receptors (Dittadi et al 1992, Cuny et al 1994). By and large, ErbB2 and ER are associated with oestrogen insensitive clinically aggressive phenotype and oestrogen sensitive ‘less transformed’ phenotype respectively. Other phenotypic markers of differentiation in breast luminal epithelial cells include K19 and MUC1. In the developmental biology of the human mammary gland, K19 is considered a marker of full phenotypic differentiation for
luminal epithelial cells (Bartek, Bartkova and Taylor-Papadimitriou 1990), while the other marker of epithelial phenotype mucin 1 (MUC1) is a class of high molecular weight glycoproteins found in the cell membranes of human epithelial cells. Since both DMSO and TNFα have been reported to have differentiating as well as growth inhibitory effects, these markers were also examined for possible induction of phenotypic differentiation.

C3.6 was the cell line of choice for these studies since it allows the examination of possible ErbB2 regulations. Moreover, unlike C5.2, the population of C3.6 consists largely of epithelial population. Any changes in the expression level of epithelial markers such as K19 and MUC1 could therefore be associated with the entire C3.6 population, rather than proportional changes between epithelial and fibroepithelial populations as in C5.2.

Expression of selected molecules was screened at the level of mRNA by RT-PCR, and the primary oncoprotein ErbB2 was further examined at the protein level by Western blotting.
6.2 Result

6.2.1. Markers that showed no change in the mRNA expression

First, the mRNA levels of GapDH and β-actin (Figure 6.1) were examined. In all the samples, both of these markers showed equal levels of expression. Next, the markers of differentiation, MUC1, K19 and ER (Figure 6.2) were examined. None of these showed noticeable differences between the growth phase and any of the experimental conditions.

6.2.2 Markers that showed change in the mRNA expression

RT-PCR analysis showed that, in comparison to growth phase, ErbB2 mRNA was unchanged in the population growth arrested at 39.5°C. However, ErbB2 mRNA was decreased in the population growth inhibited by DMSO and also by TNFα (Figure 6.3). The differential expression of ErbB3 was different from ErbB2 in that, in comparison to growth phase, it was decreased in the population growth arrested at 39°C as well as the populations growth inhibited by DMSO or by TNFα (Figure 6.3). P21 (Figure 6.4) showed the same regulatory pattern in the mRNA expression as ErbB2. It was unchanged in the population growth arrested at 39.5°C but decreased by DMSO and by
TNFα. P53 (Figure 6.4) was then examined, and the expression pattern was the same as ErbB2 and p21.

6.2.3 ErbB2 expression at the protein level

The differential expression of ErbB2 was further investigated at the protein level by Western blotting. ErbB2 protein showed the same regulatory pattern as mRNA, being unchanged at 39.5°C but decreased by DMSO and by TNFα (Figure 6.5).

6.2.4 Growth inhibitory effect of incubation temperature

C3.6 was derived from the human mammary luminal epithelial cells that had been immortalised by the temperature sensitive mutant of SV40 large T antigen (HB4a). This mutant can be controlled by incubation temperature in that at 33.5-36.5°C, the mutant is permissive and allows active proliferation of the cells, whereas at 39.5°C, the mutant is non-permissive and hence cells become non-proliferative. Haemocytometer growth assay was conducted in order to ascertain that the proliferation of C3.6 is regulated by the incubation temperature (Figure 6.6). The cells were incubated for 2, 4, 6 and 8 days at 33.5°C, 36.5°C and 39.5°C. Each condition was represented in triplicates (T25 x 3) and a minimum of 200 cells were counted per flask. As expected the growth of C3.6
cells were active at the permissive temperature (33.5°C) and the semi-permissive temperature (36.5°C), whilst it was clearly inhibited at the non-permissive temperature (39.5°C). When the growth curve at 33.5°C was compared with that at 36.5°C, the cells grew faster at 36.5°C than at 33.5°C. This is presumably due to the fact that at 33.5°C the general metabolism was slower than at 36.5°C, although appropriate metabolic assays must be conducted to prove such an assumption.
Figure 6.1

RT-PCR on C3.6. The lanes represent the growth phase (70% confluence) (I), growth arrest at the non-permissive temperature 39°C (II), growth inhibition by DMSO 1% (III), growth inhibition by TNFα 10ng/ml (IV), no sample loaded (V) and the pooled (of all the four experimental conditions) sample (VI). There was no differential regulation in the mRNA expression of GDH (A) or β-actin (B).
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Figure 6.2

RT-PCR on C3.6. The lanes represent the growth phase (70% confluence), growth arrest at the non-permissive temperature 39°C, growth inhibition by DMSO1%, growth inhibition by TNFα 10ng/ml, no sample loaded and the pooled (of all the four experimental conditions) sample.

There was no differential regulation in the mRNA expression of MUC1 (A), K19 (B) or ER (pan) (C).
Figure 6.3

RT-PCR on C3.6. The lanes represent the growth phase (70% confluence), growth arrest at the non-permissive temperature 39°C, growth inhibition by DMSO1%, growth inhibition by TNFα 10ng/ml, no sample loaded and the pooled (of all the four experimental conditions) sample. The mRNA of ErbB2 (A) showed down-regulation by DMSO and by TNFα. The mRNA of ErbB3 was (B) decreased by temperature 39°C as well as by DMSO and by TNFα.
Figure 6.3

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![Image of gel electrophoresis patterns for Figure 6.3](image-url)
Figure 6.4

RT-PCR on C3.6. The lanes represent the growth phase (70% confluence), growth arrest at the non-permissive temperature 39°C, growth inhibition by DMSO1%, growth inhibition by TNFα 10ng/ml, no sample loaded and the pooled (of all the four experimental conditions) sample.

The mRNA of both P21 (A) and P53 (B) showed down-regulation by DMSO and by TNFα.
Figure 6.4

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Figure 6.5

Western blot analysis for the expression of ErbB2 on the ErbB2 over-expressing line C3.6. Each protein sample was quantified spectrophotometrically by measurement of optical density at 560nm and was diluted to a concentration of 1mg/ml. Equal quantities of cell protein samples were hence separated by SDS-PAGE before immuno-blotting. Compared to the control (Lane 1), the population that was growth arrested at temperature 39°C showed no or a slightly increased expression (Lane 2). In contrast, the expression level on the population growth inhibited by DMSO 1% (Lane 3) and the expression level on the population growth inhibited by TNFα (Lane 4) were both markedly decreased.
Figure 6.5

Molecular Weight Markers (M_r)

220,000
98,000
66,000
46,000
30,000
21,500
14,300

Enlarged
Figure 6.6

The cells were incubated for 2, 4, 6 and 8 days at 33.5°C, 36.5°C and 39.5°C. Each condition was represented in triplicates (T25 x 3) and a minimum of 200 cells were counted per flask using a haemocytometer. Error bars represent standard deviations. As expected the growth of C3.6 cells were active observed at the permissive temperature (33.5°C) and semi-permissive temperature (36.5°C), whilst it was clearly inhibited at the non-permissive temperature (39.5°C).
6.3 Discussion

The primary aim of the molecular analysis conducted for the present study was to determine the applicability of the LMT for studying the mode of actions of new therapeutic candidates, and this potential was positively confirmed by a number of differential regulations.

In the present study, a panel of molecular markers associated with transformation, differentiation and cell cycle were examined. It should be pointed out that RT-PCR is a non-quantitative method, and hence the mRNA findings described in this chapter are preliminary.

6.3.1 Markers of differentiation

As a differentiation inducer, TNFα has been shown to up-regulate markers associated with differentiation such as MUC1 and epithelial cell adhesion molecule (Ep-CAM) in a breast cancer cell line CAL51 (Grunberg et al 2000) as well as ICAM-I (CD54) in other breast cancer cell lines T47D, ZR-75-1, MCF7D and HS578T (Hutchins and Steel 1994). TNFα also induces differentiation in peripheral blood monocytes (Lyakh et al 2000), HL60 (Kumakura et al 1996) and thymocyte (Zuniga-Pflucker, Di and Lenardo 1995).
In the current study, however, the mRNA expression of MUC1 was unchanged by TNFα, DMSO or temperature 39.5°C. The immunocytochemical study (Chapter 4) has previously shown that C3.6 is already a phenotypically well-differentiated cell line, being constitutively positive for the phenotypic markers of HMLEC such as K18, 19, E-cadherin and EPITHELIAL ANTIGEN. Hence, any induction of MUC1 or K19 expression, should it occur, would be additional rather than de novo.

6.3.2 Markers of transformation – ErbB2

The present study on C3.6 showed that TNFα decreased the expression level of the transforming oncoprotein ErbB2 at both the mRNA and protein levels. This favourable finding was not specific to TNFα, however. The same regulation was observed with DMSO, and this indicates that the down-regulation of ErbB2, p53 and p21 is consequent to pharmacological growth inhibition, but not to the selective action of TNFα. This effect of TNFα has been observed previously (Kumar and Mendelsohn 1994, Kalthoff et al 1993), and in pancreatic tumour cell lines it is evoked by the stimulation of TNF receptor (TNFR) p55 (Kalthoff et al 1993). Currently, the TNFR status of C3.6 or other cell lines of the LMT is unknown. Although TNFα can function as the ligand of TNFR, in breast cancer TNFα sensitivity and the expression level of
TNFR have shown no correlation in an extensive panel of breast cancer cell lines (Keane et al 1996). The growth inhibitory effect of TNFα on C3.6 may be attributed to the down-regulation of ErbB2, although at this stage the exact mechanism behind this regulation remains unclear. The down-regulatory effect of DMSO on ErbB2 is a novel finding. While this may be a consequence of the generally decreased cellular activity, the expression of other markers (such as GDH, actin, K19, MUC1 and ER) showed no change at mRNA level.

6.6.3 Markers of cell cycle

The down-regulation of ErbB2 was accompanied by the decrease in the mRNA of p21 and p53. The tumour suppressor p53 is barely detectable in the nucleus of normal cells. However, upon cellular stress, particularly that induced by DNA damage, p53 is up-regulated and can arrest cell cycle progression. Mutated p53 loses this function, and leads to the genetic instability. Although in vivo, positive immuno-labelling is considered indicative of p53 mutation, in vitro it is important to distinguish between the up-regulation of wild type p53 and that of mutated and non-functional p53. In vitro, p53 may be expressed temporarily as a normal physiological response of the wild type to DNA damage or permanently as a consequence of mutation. In C3.6, p53 is SV40
bound but wild type (Stamps *et al* 1994). This implies that the treatment by DMSO or by TNFα caused no DNA damage to the growth inhibited C3.6 population (no increase in p53 expression), and instead they brought p53 expression to a barely detectable level, as in normal breast epithelial cells. The concurrent down-regulation of p21 was expected since p21 is transcriptionally activated by wild type p53 and inhibits G1 associated cyclines. Furthermore, in primary breast tumours, the expressions of p21 and p53 are positively correlated (Thor *et al* 2000).

### 6.3.4 Conclusion

Therapeutically, the ability of TNFα and of DMSO to inhibit the growth rate and the expression of the oncoprotein ErbB2 as well as of p53 and p21 appears favourable. While the current study investigated the expression of ErbB2 at both mRNA and protein levels, the RT-PCR findings on p53 and p21 require further follow-up to determine the protein expression levels.

The TNFα sensitivity has been associated with the expression of molecules such as an antioxidant enzyme NAD (P) H:(quinone acceptor) oxidoreductase (NQO1) (Siemankowski *et al* 2000), PI3K, Akt (Burow *et al* 2000), an IFN-inducible protein p202 (Wen *et al* 2000) among others. It is worth pointing out that most of the previous
studies on the TNFα sensitivity have been based on a single breast cancer cell line MCF7. However, cultures of MCF7 from different laboratories have been shown to vary considerably in both the TNFα sensitivity and the expression levels of these molecules (Burow et al 1998). Compared to MCF7, the LMT of breast cancer established in the present study provides a superior experimental model for dissecting the receptor and signal pathway specific molecular interactions. For studying modes of drug actions at molecular level, the LMT of breast cancer has considerable versatility in that experimental comparisons are possible not only between the cell lines (e.g. non-transformed vs. transformed, transformed by ErbB2 vs. transformed by ras etc.) but also different modes of growth arrest (e.g. non-permissive temperature vs. pharmacologically induced cytostasis, selective growth inhibition vs. non-selective growth inhibition).
Chapter 7
Final discussion and conclusions

7.1 Introduction

Currently, adjuvant therapy of breast cancer employs three types of approach: 1) tamoxifen and other hormone therapy, 2) chemotherapy and 3) intelligent drug design such as Herceptin targeting ErB2. The present study investigated the potential of the fourth therapeutic approach, the induction of differentiation.

Using the Medline Database, the search term "differentiation" identifies 131,282 papers published since 1966, and the definition of differentiation varies immensely among these publications. In general, the word differentiation is associated with loss of proliferative potential, the ability to respond to surrounding structures, functional competence and genetic stability.

In the present study, the conventional assumption of the inverse correlation between differentiation and transformation was re-examined in context of breast cancer. There were two means by which the induction of differentiation could theoretically be
beneficial for patients with breast cancer. One was to deprive cancer cells of proliferative potential, and the other was to affect the level of transformation.

### 7.1.1 Differentiation and proliferation

The concept of achieving total growth arrest by the induction of differentiation in cancer cells was based on a theory that differentiation coincides with loss of proliferative potential. It was hypothesised that if differentiation was induced in breast cancer cells, it may concurrently deprive them of the ability to proliferate, thereby stopping further growth of breast cancer. However, the conventional theory upon which this hypothesis has been built was developed for non-mammary systems such as haemopoiesis, and as far as the mammary gland was concerned the supportive evidence was limited largely to rodent models. Therefore, the present study examined the relationship between differentiation and proliferative potential in human breast epithelial cells, in particular luminal epithelial cells that are the normal counterpart of most breast cancer cells. The question to be addressed was whether differentiated luminal epithelial cells could proliferate. The state of differentiation was examined in both phenotypic and functional developments.
7.1.2 Differentiation and transformation

The other therapeutic potential of differentiation was by decreasing malignant properties of breast cancer cells. If differentiation was inversely correlated with transformation, the induction of differentiation might result in reduction of the transformed phenotype, making breast cancer cells less 'aggressive'. To examine such possibility, the present study established an in vitro model of breast cancer that allows direct comparisons between non-transformed and transformed HMLEC.

Firstly, the cell lines of this model were characterised in detail by immunocytochemistry in order to ensure their appropriate and stable phenotypes in vitro. Secondly, three pharmacological agents with differentiating and growth inhibitory effects were examined for their influence on cellular growth. Two of them, TNFα and TRAIL, had been previously reported to exert growth inhibitory effects selectively on transformed cells, while the third agent DMSO is associated with potent growth inhibitory and differentiation effect to both non-transformed and transformed cells. Lastly, the present study explored the ability of this model to display differential regulations at the mRNA and protein levels for studying the modes of drug actions.
7.2 Summary of results and discussions

7.2.1 Differentiation and proliferation of human breast epithelial cells

In the present study, the multiple immunohistochemical study on human breast tissues from pregnant women demonstrated that differentiation does not coincide with loss of proliferative potential. All the actively proliferating luminal epithelial cells (detected by Ki67 positivity) were co-labelled for K19, showing that they were phenotypically fully differentiated. Furthermore, some of them were found with the concurrent labelling for milk proteins (β and κ caseins), indicating that even when functionally differentiated, they were capable of active proliferation.

The inverse correlation between proliferation and differentiation was originally observed in the haemopoietic system where a gradual progression along a specific lineage is precisely outlined (Bonifer et al 1998). Functional erythrocytes have lost nuclei hence their proliferation potential is zero. This gain of function and concurrent loss of proliferative potential is defined as terminal differentiation. However, in other systems, functionally competent cells are not necessarily terminally differentiated and lineage-specific commitment is less absolute. The ability of hepatocytes to regenerate the liver after partial hepatectomy demonstrates this clearly (Haber et al 1993).
In cancer, functional differentiation is not always absent. Pituitary adenoma is characterised by an excess synthesis of pituitary hormones (Kumar and Clark 1990), and a proportion of primary breast cancers (17% of 47 samples) has been reported to be positive for the milk protein, casein (Monaco et al 1977). The very rare secretory carcinoma of breast is generally found in young women and has relatively better prognosis in patients younger than 20 years of age, although only limited systematic studies have been possible due to its low incidence (Dr. Sunil Lakhani, personal communication, Maitra et al 1999). In some instances, cancer cells gain occult functions that are inappropriate to their parental cell types. For example, ectopic adrenocorticotrophic hormone can be secreted by breast cancer (David, Solimena and De Camilli 1994) and lung oat cell carcinoma (Collichio, Woolf and Brower 1994). In metaplasia, functionally differentiated cells have been observed to re-differentiate and become phenotypically distinct. Apocrine metaplasia (the milk gland of the breast transforms into the sweat gland of the skin) is a common pathologic change in the breast (Bussolati et al 1986). According to these clinical observations, in transformed cells, differentiation and proliferation can concurrently exist.

The findings in the present study have brought additional insights that the concurrent expression of differentiation and active proliferation is not an abnormality
found exclusively in transformed cells. On the contrary, such ability seems to be shared by both transformed and non-transformed HMLEC. Therefore, it was concluded that the induction of differentiation would not necessarily achieve automatic growth arrest and thus is unlikely to stop the growth of breast cancer. This in turn means that differentiation inducers such as prolactin and heregulin have no direct therapeutic value for breast cancer, unless they exert additional effects to decrease specific malignant properties.

7.2.2. Differentiation and transformation – the LMT of breast cancer

To examine whether the induction of differentiation results in a decrease in malignant potentials of breast cancer cells, an in vitro model of differentiation and transformation was required. The in vitro model of breast cancer established in the present study was a longitudinal model of transformation, where non-transformed HMLEC lines were directly comparable with transformed variants. The in-house LMT consisted of HB4a (immortalised but non-transformed HMLEC), H4.1 (a transfection control), C3.6 and C5.2 (transformed variants over-expressing ErbB2), and R4.2 (another transformed variant expressing mutant active Ha-(Val12)-Ras).
**Immunocytochemical characterisation**

The immunocytochemical findings of the present study confirmed that HB4a has maintained its original phenotype that is akin to primary non-transformed HMLEC. This was demonstrated by a panel of antibodies detecting key phenotypic markers.

While there are over 20 breast epithelial cell lines today, HB4a is particularly appropriate to the present study with the unique property of temperature sensitive SV40 T, which allows the effect of immortalisation to be switched on and off depending on incubation temperature. This in turn makes HB4a an ideal parental line for LMTs of breast cancer.

Another significant finding with immunocytochemistry was the gain of vimentin in the variant lines (H4.1, C5.2, R4.2) of HB4a. The expression of vimentin has been previously associated with transformation, invasiveness, poor prognosis and more aggressive phenotypes in general (Hendrix *et al* 1997, Sommers *et al* 1994a, Sommers *et al* 1994b). However, the finding in the present study demonstrates that the expression of vimentin is not exclusive to transformed phenotype, and it could be found in the non-transformed transfectant control variant H4.1 as well as the transformed variants (C5.2 and R4.2). In case of H4.1, the gain of vimentin expression did not affected the growth rate, anchorage independent growth, morphology or the TNFα sensitivity.
observed in the transformed variants, indicating that vimentin does not have a transforming property like ErbB2.

**Pharmacological selectivity**

Having validated the LMT by immunocytochemistry, the present study examined the potential growth inhibitory effects of three pharmacological agents: TNFα and TRAIL, which have been previously reported to work selectively against the transformed cells, and DMSO, a non-selective growth inhibitory and differentiating agent.

The growth assays of the present study using TNFα, TRAIL and DMSO demonstrated that the *in vitro* model of breast cancer was capable of showing both selective (TNFα) and non-selective (TRAIL, DMSO) growth inhibitory regulations. Hence, this model is particularly useful for screening for therapeutic candidates that act specifically on transformed cells whilst sparing non-transformed cells. Having two types of transformation, one by ErbB2 over expression and the other by Ha-(Val12)ras, means that the selectivity of pharmacological agents can be further determined in respect to its possible specificity against transformed phenotype or individual oncogene. As demonstrated in the present study, the action of TNFα was selective only on the ErbB2 over-expressing lines.
Molecular analysis

To date, with respect to cancers arising from epithelial cells, the inverse correlation between differentiation and transformation has been based mostly on histopathological findings from biopsy and surgically resected samples. So far no breast cancer specific molecular marker that can be directly correlated with differentiation markers has been found. Instead, the expression levels of the molecules present in normal breast epithelial cells are observed up or down regulated in breast cancer cells.

The LMT of breast cancer established in the present study, therefore, allows investigations on drug actions where signalling pathways and ligand-receptor interactions can be dissected in greater details. The potential for differential analysis on this model is quite diverse in that direct comparisons are possible between the cell lines (e.g. transformed vs. non-transformed, ErbB2 amplified vs. un-amplified) as well as experimental conditions such as various types of growth arrest. The ability to induce growth arrest at the non-permissive temperature is particularly useful for distinguishing the differences in molecular profiles between growth inhibition and agent specific pharmacological regulations.
The findings of the present study indicate that some differentiation inducers achieve not only growth inhibitory effects but also possible reduction in malignant properties by down-regulating transforming oncogenes and other functionally relevant molecules. Whether the cellular behaviours associated with transformation and clinical aggressiveness are, too, reduced as a consequence requires further investigation.

7.2.3 Next step

How do we study transformation in vitro?

Malignant potentials / aggressiveness of breast cancer cells are complex. Standardised assays have been developed that individually examine a limited aspect of overall in vitro transformation (Table 7.1), and these are often used in combination with each other and with in vivo xenograft assays using immunodeficient rodents.

One of the characteristics of transformed cells in culture is a reduced dependence on serum-derived factors for growth. The minimum serum requirement for growth gives a measure of the autonomous growth of transformed cells; typically this is less than 2% FCS. Another distinguishing feature of “transformed” as opposed to normal cells in culture is the ability to grow in semisolid agarose / soft agar (anchorage independent growth). Immobilisation in agar at low density prevents cell to cell as well
as cell to substratum interactions and tests colony forming efficiency i.e. how readily a 
single cell can grow and form a colony. Normal breast epithelial cells show very limited 
ability to form colonies in soft agar or grow in low serum environment, while some (but 
not all) breast cancer cell lines grow in these stringent conditions.

While these assays are widely applied for determining transformed phenotype of 
cell lines, their use for screening for pharmacological agents is limited. This is because 
most therapeutic candidates have cytostatic or cytotoxic effect. Decreased growth in low 
serum or on soft agar therefore is difficult to measure independently of overall growth 
inhibition.

In contrast, some researchers have proposed that the level of differentiation and 
transformation can be deduced from the 3D morphogenesis on Matrigel (Korah et al 
2000, Sommers et al 1994a, Sommers et al 1994b, Bergstraesser and Weitzman 1993, 
demonstrated that normal breast epithelial cells were capable of forming duct and 
gland-like structures with internal lumina (Figure 7.1). Once they reached size 
equivalent to the in vivo mammary acini, proliferation stopped. This self-regulation of 
growth was absent in 2 out of 3 primary breast cancer cells and 6 out of 6 breast cancer 
cell lines, which continued to grow and formed much larger colonies of tightly fused
cells, without lumina. Other researchers have further defined the morphogenesis of breast cancer cell lines on Matrigel. Briefly, their morphologies can be categorised as follows: well-circumscribed round colonies of tightly fused cells, loose clusters of spherical cells or networks of stellate structures with spiky protruding branches (Figure 7.1). Tight clusters were associated with a relatively differentiated phenotype and expression of adhesion molecules, whereas stellate structures were typically observed in breast cancer cell lines with highly invasive properties in vitro (Sommers et al 1994a, Sommers et al 1994b, Thompson et al 1992, Sommers et al 1991).

The aggressiveness of breast cancer cells has been also examined using cell migration and invasion assays, which attempt to dissect the components of in vivo metastasis. In the simplest form, the transwell migration assay consists of a porous filter separating two chambers (e.g. the Boyden chamber). Cells are plated on the upper surface and the proportion of cells that traverse to the lower surface and to the lower chamber in a given period of time is used to measure the ability to migrate (Sommers et al 1994a, Sommers et al 1994b, Thompson et al 1992, Sommers et al 1991).

The migration assay is often carried out in the presence of a chemoattractant such as serum growth factors (Rajah et al 2001) or cytokines (Arihiro et al 2000) to induce “chemotaxis”. This assay can be modified to measure the invasive ability of
cancer cells *in vitro* by coating the filter with a biomatrix, such as collagen I to substitute for the interstitial tissue and collagen IV or Matrigel for the basement membrane (Sommers *et al* 1994, Thompson *et al* 1992, Sommers *et al* 1991).
**Table 7.1 Profile of *in vitro* characterisation**

<table>
<thead>
<tr>
<th>Morphology</th>
<th>2D morphology on plastic substratum</th>
<th>3D morphology on extracellular matrix (Matrigel, collagen I etc.)</th>
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<tr>
<td>Growth patterns</td>
<td>Doubling time&lt;br&gt; <em>haemocytometer&lt;br&gt;methylene blue assay&lt;br&gt;</em>&lt;br&gt;<em>MTT assay</em></td>
<td>Proliferation&lt;br&gt;<em>3H-thymidine labelling&lt;br&gt;</em>&lt;br&gt;<em>bromodeoxyuridine labelling</em></td>
</tr>
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<td></td>
<td>Death&lt;br&gt;<em>TUNEL assay&lt;br&gt;</em>&lt;br&gt;<em>Annexin V labelling&lt;br&gt;</em>&lt;br&gt;<em>TUNEL assay</em></td>
<td>Apoptosis&lt;br&gt;<em>Annexin V labelling&lt;br&gt;</em>&lt;br&gt;Necrosis</td>
</tr>
<tr>
<td></td>
<td>Minimum serum requirement for growth</td>
<td>Colony forming efficiency in soft agar (anchorage independent growth)</td>
</tr>
<tr>
<td></td>
<td>Hormone and growth factor responsiveness</td>
<td>Motility and Invasion&lt;br&gt;Transwell migration</td>
</tr>
</tbody>
</table>
Figure 7.1

**Duct- and gland-like structures with internal lumina**
- associated with non-transformed phenotype
- formed by primary HMLEC and normal breast epithelial cell lines (HMT-3522, MCF10)

**Round colonies of tightly fused cells**
- associated with relatively differentiated transformed phenotype
- formed by primary breast cancer cells and breast cancer cell lines (MCF7, BT474)
- correlated with the expression of adhesion molecules (E-cadherin, desmoplakin)

**Loose clusters of spherical cells**
- associated with transformed phenotype
- formed by primary breast cancer cell lines (CAMA1, SKBR3)
- correlated with loss of adhesion molecules

**Stellate structures with protruding branches**
- associated with invasive transformed phenotype
- formed by breast cancer cell lines (MDA-MB-436, HS578T)
- correlated with loss of adhesion molecules and the expression of vimentin
Limits of the currently available assays

The existing differentiation inducers, such as DMSO and TNFα, require further investigation using cell culture assays like invasion and migration assay. The LMT system developed in the current study will be a useful tool for such additional studies.

Nevertheless, it is important to point out that there is a potential gap between the present definition of \textit{in vitro} transformation and the actual malignancy as manifested by breast cancer \textit{in vivo}. It is not clear, for example, how many and how often these \textit{in vitro} characteristics of transformation are actually present in primary breast cancer cells, as most observations were made on long-term cultures of established lines. This point is well demonstrated by a subset of breast cancer cell lines that have been identified as ‘highly transformed’. They share certain characteristics such as the expression of vimentin (which is normally present in fibroblasts but not in normal epithelial cells) (Sommers \textit{et al} 1994a, Sommers \textit{et al} 1994b, Thompson \textit{et al} 1992), stellate morphology on Matrigel and high scores in migration and invasion assays. However, these highly transformed properties \textit{in vitro} do not necessarily correlate with the original ‘aggressiveness’ of the breast cancers. For example, both the T47D (Keydar \textit{et al} 1979) and MDA-MB231 (Cailleau \textit{et al} 1974) cell lines were established from pleural effusions obtained from patients whose primary tumours were high-grade

Furthermore at present, most in vitro assays study breast cancer cells in isolation. To study the cancer-host cell interactions or metastasis to distant viscera, the generally utilised model is the xenograft (where breast cancer cell lines are inoculated and grown in vivo inside immunodeficient rodents (Yang, Guzman, Nandi 2000, Walsh et al 2000, Clarke 1996) The development of appropriate in vitro assays has been hindered by the absence of cell lines representing other normal mammary cell types. However, with the recently established methods for reliable immortalisation of adult human cells, a new generation of cell lines is emerging. This includes normal breast fibroblast cell lines, breast microvascular endothelial cell lines (O'Hare et al 2001) and breast epithelial cell lines from specific donors such as patients with hereditary mutations predisposing to breast cancers.

These new cell lines will help develop more relevant in vitro models of breast cancer in the future. The growth, morphogenesis, invasion and migration of transformed cells will be examined in an environment much closer to reality, taking in account
cancer-host cell interactions as well as tumour angiogenesis and breast cancer predisposition.

7.3. Identification of novel therapeutics for breast cancer

7.3.1. Therapeutic potential of differentiation inducers

As pointed out in the Introduction, the word 'differentiation inducers' is loosely applied to agents that induce any sign of differentiation. The present study showed that the state of differentiation does not, however, coincide with the loss of proliferative potential, and the inverse correlation between differentiation and transformation is not absolute. Therefore, this section discusses two main reasons why the induction of differentiation does not, by itself, guarantee a therapeutic response.

Firstly, differentiation is currently measured by the expression of differentiation markers such as K19, MUC1, E-cadherin and so forth that are unique to HMLEC phenotype. It is not clear at this stage what impact the pharmacological induction of expression of such differentiation markers exerts upon the progression of disease. For instance, the loss of adhesion molecules such as E-cadherin or integrins may provide the initial step for breast cancer cells to break off from the mammary parenchyma.
Nevertheless, in metastasis, breast cancer cells require sufficient anchorage to remain and grow in other solid organs such as the liver or the brain. Hence in an advanced stage, the expression of adhesion molecules may aid the progression of breast cancer, rather than prevent it.

Like differentiation, transformation is a phenomenon measurable by the expression levels of oncogenes and other cancer-associated markers. In breast cancer, two schools of thought exist for the significance of these markers. One believes that cancer cells gain the expression of oncogenes along the progression of disease and this gain is a gradual process driven by the evolutorial selection within the microenvironment of breast cancer. The other explanation is that the molecular profile of breast cancer cells is more or less pre-determined at the time that lesions become apparent. A recent immunohistochemical study on the comparison between ductal breast carcinoma in situ (DCIS) and small invasion lesions has demonstrated that cancer-associated markers such as p53, ErbB2, Ki67, ER, PR and bcl-2 correlate with grade but not with invasiveness. Warnberg et al (2001) conclude that well-differentiated DCIS progress to well-differentiated invasive cancer and poorly differentiated DCIS progress to poorly differentiated invasive cancer. Their findings imply that the
pharmacological intervention that down-regulates cancer associated markers may not influence more clinically relevant outcome such as invasiveness or overall survival.

Breast cancers retain many aspects of differentiation. For example, a considerable proportion of breast cancer cells express differentiation markers of HMLEC phenotype, even when they are metastatic. Detection of micrometastatic breast cancer cells therefore utilises some of the phenotypic markers of HMLEC (Pantel et al 1994). Also in the present study, it was demonstrated that the moderate ErbB2 over-expressing variant C3.6 expresses epithelial membrane antigen, K18, K19 and E-cadherin as its parental line HB4a. Yet, C3.6 exhibits a clearly transformed phenotype in vitro. In the cases of breast cancer cells that have maintained the differentiated phenotype of HMLEC, it is unlikely that additional increase in the expression of differentiation markers would bring any therapeutic benefit.

7.3.2. Emergence of new technologies

Diagnostic and prognostic values

In the last few years, researchers endeavoured to improve sample selection, experimental techniques, data analysis and interpretation of emerging techniques such as genomics and proteomics (Mills et al 2001, Schulze and Downward 2001). Recent
publications consequently have started to yield substantial information. The challenge is to determine how these technologies can be applied for the understanding of breast cancer and possible development of new treatments.

For example, a series of cDNA microarrays experiments on 78 primary breast cancer samples have allowed re-classification of breast cancers based on variations in gene expression and significant correlation between these groups and clinical outcomes (Sorlie et al 2001). Molecular profiling of primary samples has demonstrated striking molecular differences between the specimens of what have been histopathologically uniformly classified as ductal carcinoma (Brenton, Aparicio and Caldas 2001). These findings indicate that microarray investigation on clinical samples can be combined with the existing grading and staging systems of breast cancer to bring additional information for diagnosis and prognosis. Prognostic probes are likely be developed in not too distant future, in particular for oligonucleotide microarrays since they are less time consuming to apply than cDNA arrays and permit detection of splice variants (Schulze and Downward 2001, West et al 2001). Combined with laser capture microdissection, microarrays will allow precise molecular profiling of breast cancer and in turn more accurate prognosis on the clinical outcome of each patient.
It is nevertheless important to remember that the pattern of gene activity is not necessarily or directly correlated with the corresponding protein expression. Proteomics should be therefore combined with the use of genomics, if potential significance of amplified genes is to be studied. Harris et al (2001) have examined 29 breast cancer cell lines and breast epithelial cell lines and shown that proteomics can enable clustering of samples according to protein expressions and subsequent correlation with known factors such as morphology in vitro. However, while proteomics offers investigation at the protein level and hence may yield more functionally relevant data, its use on clinical samples is limited today. This is primarily because, without micro-dissection of samples, be they biopsies or surgical resections, they are heavily ‘contaminated’ with serum proteins and extracellular matrix components that mask changes in the tumour cells. As yet the sensitivity of proteomics is insufficient for it to be applied to micro-dissected samples. If proteomics is to be used for prognostic purposes like microarrays, researchers need to improve the sensitivity of the technique. Alternatively, they need to purify the tumour cells (as is being carried out in this laboratory) and/or amplify the limited sample resource by enhancing their growth potential (as has been so far unsuccessful in this laboratory).
Identification of novel breast cancer markers

The approaches described so far make use of large-scale molecular data by clustering computationally recognisable patterns. They are ideally suited for diagnostic and prognostic purposes, but not for identifying therapeutic targets. Out of approximately 30,000 genes expressed by a typical cancer cell, researchers need to distinguish the critical molecules that drive tumour progression from the epiphenomena of genetic instability and abnormalities in DNA repair (Osin et al 1998, Suzuki et al 2001). In the context of identifying a novel breast cancer marker, two fundamental criteria need to be met. First, a novel breast cancer marker must be either expressed in a reasonable proportion of patients or alternatively identify a subset of tumours with particular distinguishing features. Second, when therapeutic targets are considered, its function must be demonstrated to drive actual tumour progression rather than being a consequence of it. While the former can be determined by the histopathological analysis of clinical samples, the latter depends primarily on in vitro studies.

Figure 7.2 presents the current approaches for identifying and characterising novel breast cancer cell markers. It is believed that the optimum direction is to investigate the consequences of expressing candidate genes one at a time in cell lines with appropriate characteristics using a panel of the longitudinal models. Their exact
role in terms of transformation potential and regulatory effects on other molecules can then be analysed. At a later stage, these lines can also be used to screen for new therapeutics that specifically reverse such effects. Although laborious, developments in laboratory technology such as high efficiency methods of introducing foreign genes into cells using a variety of viral vectors (Danthinne and Imperiale 2000, Binley et al 1999, Naldini 1998) and the developments of “switches” that allow such genes to be turned on and off at will should accelerate this process.
Figure 7.2

A schematic diagram to summarise the current approaches to the identification and characterisation of novel breast cancer cell markers
Figure 7.2

Identifying Gene X

Differential Analysis
- Normal vs. Breast Cancer -
  - Genomics
  - Transcriptomics
  - Proteomics
  - Immunomics

Transfecting Gene X

Normal human breast tissue
  - Mechanical & enzymatic digestion

Primary culture of breast epithelial cells
  - Immuno-magnetic purification

HMLEC (human mammary luminal epithelial cells)
  - Conditional Immortalisation

Parental HMLEC Line

Characterising Gene X

Molecular analysis
- How has Gene X affected the expression of other molecules?

In vitro assays
- Has Gene X made the variant lines more transformed than the parental lines?

In vivo tumourigenicity
- Do the variant lines form tumour in rodents?

In vitro selectivity assays
- Which therapeutics can specifically reverse the effects of Gene X in the variant lines?
7.4 Summary

The validity of a potential therapeutic approach that was based on the inverse correlation between differentiation and transformation was examined in respect to breast cancer in this study. The in vivo findings indicated that the induction of differentiation (either phenotypic or functional) does not by itself result in total growth arrest. Nevertheless, the in vitro model of breast cancer established in the present study demonstrated that some differentiation inducers can cause growth inhibition as well as possible reduction in malignant potential of breast cancer cells by down-regulating the expression of transformation associated molecules. The LMT characterised in the present study will be a useful in vitro model of breast cancer to screen for pharmacological selectivity and also for studying modes of drug actions in the future.
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### Appendix I  Sources of reagents

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<td>TRAIL enhancer</td>
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<td>Tween-20 Organisces</td>
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### Appendix II  Sources of consumables

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<td>25cm² flasks</td>
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<td>175cm² flask</td>
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<td>ssImmobilon-P membranes</td>
<td>Millipore, Bedford, MA, USA</td>
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<td>24 well plates</td>
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<td>15ml tube</td>
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### Appendix III  Sources of equipments and computer programs

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<tr>
<td>Adobe Photoshop</td>
<td>v4.0, Adobe Systems Inc., California, USA</td>
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<tr>
<td>Axiophot microscope</td>
<td>Zeiss, Wellyn Garden City, Hertfordshire, UK</td>
</tr>
<tr>
<td>Axiovert microscope</td>
<td>Zeiss, Wellyn Garden City, Hertfordshire, UK</td>
</tr>
<tr>
<td>AxioVision</td>
<td>Zeiss, Wellyn Garden City, Hertfordshire, UK</td>
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<td>Centrifuge 5410 for 5000rpm</td>
<td>Eppendorf, via Merck Eurolab Ltd, Poole, Dorset, UK</td>
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<tr>
<td>Centrifuge IEC Centra MP4R for</td>
<td>Thermo Quest, Basingspole, Hampshire, UK</td>
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<td>14000rpm</td>
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<td>Coolview 12 cooled Charge Coupled</td>
<td>Photonic Science, Newbury, Berkshire, UK</td>
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<td>Device (CCD) camera</td>
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<tr>
<td>Cytofluor II fluorescence multiwell</td>
<td>PerSeptive Biosystems</td>
</tr>
<tr>
<td>Equipment</td>
<td>Supplier</td>
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<tr>
<td>plate reader</td>
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<td>high resolution Leaf camera</td>
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<td>Image-Pro Plus</td>
<td>v 3.01, Media Cybernetics, Baltimore, Maryland, USA</td>
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<td>MRX microplate reader</td>
<td>Dynatech Laboratories, Ashford, Middlesex, UK</td>
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<td>MRX multiwell colorimetric plate reader and Revelation (version 4.02) software</td>
<td>Dynex Technologies</td>
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<td>PU8625 UV/VIS Spectrophotometer</td>
<td>Philips, Cambridge, Cambridgeshire, UK</td>
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<tr>
<td>personal computer</td>
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<tr>
<td>Revelation (version 4.02) software</td>
<td>Dynatech Laboratories, Ashford, Middlesex, UK</td>
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<td>SCSI interface</td>
<td>AHA-2940, Adeptec Inc., California, USA</td>
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<td>Shaker</td>
<td>Genetic Research Instrumentation, Felsted, Essex, UK</td>
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<tr>
<td>Snapper frame grabber card</td>
<td>Action Imaging, Huntingdon,</td>
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<tr>
<td>Software</td>
<td>Location</td>
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<tr>
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<td>---------------------------</td>
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<tr>
<td>SPSS version 7.5</td>
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<tr>
<td>Windows 95 version 4.0</td>
<td>Microsoft, London, East Anglia, UK</td>
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ORIGINAL ARTICLE

Rami Suzuki • Amanda J. Atherton • Michael J. O’Hare
Alan Entwistle • Sunil R. Lakhani • Catherine Clarke

Proliferation and differentiation in the human breast during pregnancy

Accepted in revised form: 24 May 2000

Abstract Using multiple immunofluorescence labelling on human breast tissues obtained and freshly frozen at the 12th, 15th, and 18th weeks of pregnancy, we have shown that markers of mammary functional differentiation, milk proteins (β-casein and κ-casein), are synthesised by actively cycling (Ki67 positive) as well as non-cycling (Ki67 negative) cells. These results demonstrate that functional differentiation/maturation does not coincide with loss of proliferative potential in human mammary luminal epithelial cells. In addition, we have examined expression patterns of integrin subunits (α1, α2, α3, α6, β1, and β4) and extracellular matrix components (laminin, fibronectin, collagen I, and collagen IV), since they have been shown to exert influences on mammary differentiation and morphogenesis in vitro. Compared to human breast tissues obtained from non-pregnant women, a decrease in α2 labelling on luminal epithelial cells was observed, particularly in expanding acini that showed abundant Ki67 positivity. The expression patterns of other integrin subunits, however, did not change, indicating that the expression patterns of most integrins existing prior to pregnancy are sufficient to support the morphological and functional development associated with milk protein synthesis.

Key words breast • pregnancy • proliferation • Ki67 • milk • casein • integrin • ECM

Introduction

The mammary gland is a unique organ in that full differentiation is not attained by adulthood. The mammary gland in non-pregnant women of reproductive age is described as quiescent or resting, although it is subject to menstrual cycle influence in terms of both structure [14] and cellular proliferation [25]. During pregnancy, induction of functional differentiation takes place under the influence of high oestrogen and progesterone levels and increasing prolactin and placental lactogen levels, as a result of which the mammary gland becomes secretory [42]. Whilst it is generally believed that ‘proliferating mammary tissues cannot exert specialised functions and conversely differentiating mammary epithelium cannot effectively proliferate’ [43], there is no in vivo evidence to support or refute this hypothesis in the human breast. This concept is not unexpected since a complete loss of proliferative potential prior to differentiation is a developmental programme commonly found in neurones, skeletal muscle cells, mammalian erythrocyte, and other cell types [2]. In some systems, however, differentiated cells do not irreversibly lose proliferative potential. The ability of hepatocytes to regenerate the liver after partial hepatectomy demonstrates this clearly [18].

In the human breast, the onset of secretory activity is accompanied by dramatic morphological changes, characterised by glandular-alveolar growth, expansion...
of acini, and polarisation of luminal epithelial cells [8]. Functional differentiation of the mammary gland has been studied with rodent models both in vivo [15, 34] and in vitro [24, 38], but in humans, in vitro systems have predominated [25, 29, 39] with one exception of a human breast tissue xenograft model in pregnant nude mice [10]. Direct immunohistochemical studies on human breast tissues from pregnant women have been limited to archival materials [8] since freshly frozen specimens of this type are rarely available.

With recent advances in cancer therapy it is now possible to surgically resect primary breast tumours from pregnant women following chemotherapy without significant damage to the foetus. Such operations inevitably remove histologically normal human breast tissues adjacent to the tumours, and the present study has utilised samples obtained in this manner at the 12th, 15th, and 18th weeks of pregnancy. Freshly frozen tissues allow multiple immunofluorescence labelling for a greater range of antigens than paraﬃn embedded archival materials, and this technique is particularly useful when applied to specimens of limited availability. Allowing simultaneous visualisation of up to four antigens, the relationship between proliferation and differentiation in the human breast was investigated. To study cellular proliferation, Ki67 labelling, which distinguishes between cycling and non-cycling (Go) cells [16, 17], was used. The concept of differentiation was subdivided into phenotypic differentiation and functional differentiation [31]. In the human mammary epithelium, there are two types of phenotypically differentiated populations. Luminal epithelial cells which form the inner lining of the mammary tree express cytokeratin 18 and 19, and myoepithelial cells which are between the luminal epithelial cells and the basement membrane express cytokeratin 14 [26, 40]. In the current study, the two populations were distinguished by the expression of cytokeratin 19 and 14. When functionally differentiated, luminal epithelial cells secrete milk, while myoepithelial cells are contractile and are involved in the expression of milk during lactation [26]. Our primary question was whether differentiated luminal epithelial cells were capable of proliferative activity. Hence, milk proteins (β-casein and κ-casein) were investigated as markers of functional differentiation [13]. Expression patterns of extracellular matrix (ECM) components (ﬁbronectin, laminin, collagen I, and collagen IV) and their receptors (integrin subunits α1, α2, α3, α6, β1, and β4) were also studied, since they have been shown to play active roles in growth, morphogenesis, functional differentiation, and malignant transformation of human, as well as rodent, mammary luminal epithelial cells in vitro (for review see [31]).

**Methods**

**Tissue details**

Three human breast tissue samples were obtained at the 12th, 15th (age 30), and 18th (age 31) weeks of pregnancy from patients undergoing surgery for breast cancers. The samples representing the 12th and 15th weeks of pregnancy were acquired from the same patient who underwent sequential resections. Four quiescent human breast tissue samples (age 30 parity 0; 32 parous; 40 parity 0; 43 parity 2) were obtained with consent from non-pregnant non-lactating pre-menopausal women undergoing cosmetic reduction mammoplasty. In the current report, the human breast tissue samples obtained from pregnant women and non-pregnant women are referred to as the pregnant breast and the non-pregnant breast, respectively. All the samples were snap-frozen and stored in liquid nitrogen.

Frozen sections (5μm) were collected onto Vectabond treated glass slides (Vector Laboratories, Peterborough, UK), air-dried for two hours at room temperature and stored at −70°C. The sections were thawed, fixed with chloroform/acetone 1:1 (−20°C) for 5 min-

<table>
<thead>
<tr>
<th>Table 1 Sources of primary antibodies</th>
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<tr>
<td>Target antigen</td>
</tr>
<tr>
<td>Ki67</td>
</tr>
<tr>
<td>Ki67</td>
</tr>
<tr>
<td>cytokeratin 14</td>
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<tr>
<td>cytokeratin 19</td>
</tr>
<tr>
<td>vimentin</td>
</tr>
<tr>
<td>integrin subunit α1</td>
</tr>
<tr>
<td>integrin subunit α2</td>
</tr>
<tr>
<td>integrin subunit α3</td>
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<td>integrin subunit α6</td>
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<tr>
<td>integrin subunit β1</td>
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<tr>
<td>integrin subunit β4</td>
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<tr>
<td>collagen I</td>
</tr>
<tr>
<td>collagen IV</td>
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<tr>
<td>collagen IV</td>
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<tr>
<td>laminin</td>
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<td>fibronectin</td>
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Table 2 Primary and secondary antibodies used for immunoperoxidase labelling

<table>
<thead>
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<th>Secondary Antibody**</th>
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<tr>
<td>FNI-1</td>
<td>Goat anti-guinea pig IgG horseradish peroxidase linked whole antibody</td>
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<tr>
<td>LAM2, CALL M4-B6, HP1/9</td>
<td>Sheep anti-rabbit Ig horseradish peroxidase linked whole antibody</td>
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<tr>
<td>MAB1972</td>
<td>Sheep anti-rat Ig horseradish peroxidase linked whole antibody</td>
</tr>
<tr>
<td>Clone 3E1, Clone P4C10, MAB2257, HAS4, Clone SE8D9, CIV22 M0785, LIRC-LON 14.1, LICO-LON 32.2</td>
<td>Sheep anti-mouse Ig horseradish peroxidase linked whole antibody</td>
</tr>
</tbody>
</table>

* for the sources of the primary antibodies, see Table 1
** all the secondary antibodies were from Amersham (Little Chalfont, UK)

Immunohistochemistry

The following procedures were carried out at room temperature. The vehicle for all immunolabelling incubations was 0.5% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS), and antibodies were applied at a total of 100 μl per section. In between each step, slides were washed three times in PBS. Sources of primary antibodies are provided in Table 1.

Using a method described previously [6], immunoperoxidase labelling was carried out to examine the labelling patterns of individual primary antibodies on all the samples. The combinations of primary and secondary antibodies are shown in Table 2. Multiple immunofluorescence labelling was carried out using combinations of primary mouse monoclonal antibodies of different classes and subclasses together with appropriate class and subclass-specific anti-mouse secondary antibodies as well as combinations of primary antibodies of different species together with species-specific secondary antibodies. The sections were first blocked with 100 μl of 0.5% (w/v) BSA in PBS for 30 minutes. A mixture of primary antibodies was applied for 1 hour. The corresponding mixture of secondary fluorophore-conjugated antibodies was applied for 1 hour (the combinations of primary and secondary antibodies for double labelling are shown in Table 3 and for quadruple labelling in Table 4). For quadruple labelling, one of the secondary antibodies was biotinylated, hence streptavidin-AMCA conjugate was further applied for 30 minutes. All the sections were finally incubated in Hoechst 33258/bisbenzimide trihydrochloride (2 μg/ml in water) (Sigma, Poole, UK) for two minutes to stain nuclei and the nuclei were stained with Hoechst. Areas containing epithelium were randomly selected by Hoechst labelling. At highmagni-
**Table 4 Combination of primary and secondary antibodies for quadruple immunofluorescence labelling**

<table>
<thead>
<tr>
<th>tigen</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
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</thead>
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<tr>
<td>Cytokeratin 19</td>
<td>LP2K Mouse IgG2b</td>
<td>Goat anti-mouse IgG2b BIOT human absorbed (Southern Biotechnology Associates) followed by AMCA fluorophore conjugated streptavidin (Jackson Immunoresearch Laboratories, via Stratech Scientific, Luton, UK)</td>
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<tr>
<td>Cytokeratin 14</td>
<td>LL001 Mouse IgG2a</td>
<td>Goat anti-mouse IgG2a TRITC human absorbed (Southern Biotechnology Associates)</td>
</tr>
<tr>
<td>Vimentin</td>
<td>VIM 13.2 Mouse IgM</td>
<td>Goat anti-mouse IgM FITC human absorbed (Southern Biotechnology Associates)</td>
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<tr>
<td>Ki 67</td>
<td>A0047 Rabbit polyclonal</td>
<td>Donkey anti-rabbit IgG (H + L) Cy5-conjugated affiniPure (Jackson Immunoresearch Laboratories)</td>
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</tbody>
</table>

**Results**

**Histological morphology**

When reviewed by an experienced breast pathologist (S.L.), the non-pregnant human breast samples were typical of the age group studied: two were histologically normal (age 30), one contained apocrine cysts (age 32), one showed atrophy, fibrocystic change, and apocrine metaplasia (age 40) and one had focal fibrocystic changes (age 43). The pregnant human breast samples selected from the normal tissues surrounding the primary tumours were free of malignant invasion.

Compared to the non-pregnant breast, the pregnant breast contained abundant numbers of acini, and dilatation of the alveoli progressed with duration of pregnancy. The extent of the progression was, however, notably asynchronous in that the same sections contained clusters of extensively dilated acini together with clusters of smaller, less-developed acini. Some of the acini contained highly polarised columnar luminal epithelial cells with myoepithelial cells extensively stretched around their basal aspects. Functional differentiation of luminal epithelial cells in the pregnant breast was evident by presence of lipid droplets and was further confirmed by labelling for the milk proteins.

**Luminal epithelial cells**

Proliferation index (PI) values were presented as the mean and its standard deviation (Table 5). The percentage of cells expressing Ki67 in the pregnant breast, $12.06 \pm 5.75$, was significantly higher than that in the non-pregnant breast, $2.69 \pm 1.21$, ($P > 0.95$). The samples representing the 12th and 15th weeks of pregnancy were obtained from the same patient (see Methods), and the PI of the sample obtained at the 15th week of pregnancy, $17.6 \pm 3.9$, was significantly higher than the PI at the 12th week of pregnancy, $6.1 \pm 3.8$ ($P > 0.995$). At the 18th week, the PI was $12.5 \pm 1.5$. Mean values of the non-pregnant breast samples were $1.6 \pm 2.2$, $2.6 \pm 2.6$, $3.9 \pm 3.0$, and $4.4 \pm 2.6$.

Neither β-casein nor κ-casein was detectable in the non-pregnant breast. β-casein (Fig. 1A) and κ-casein (Fig. 1B) labelling were absent at the 12th week of pregnancy. At the 15th week, some luminal epithelial cells were labelled for β-casein (Fig. 1C), while κ-casein was found in a smaller proportion of luminal epithelial cells (Fig. 1D). Finally at the 18th week β-casein was observed in almost all luminal epithelial cells (Fig. 1E), but in contrast κ-casein was found in most but not all luminal epithelial cells (Fig. 1F).

In the pregnant breast, Ki67 positive luminal epithelial cells were uniformly co-labelled for cytokeratin 19 (Fig. 2). Ki67 labelling was also observed in luminal epithelial cells co-labelled for the milk proteins κ-casein (Fig. 3A) and β-casein (Fig. 3B). This was best demonstrated in the sample representing the 15th week of pregnancy where milk synthesis was

**Table 5 Table of PI values on human breast tissues from pregnant women and non-pregnant women**

<table>
<thead>
<tr>
<th>tigen</th>
<th>12th week</th>
<th>15th week</th>
<th>18th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant Breast</td>
<td>$6.1 \pm 3.8$</td>
<td>$17.6 \pm 3.9$</td>
<td>$12.5 \pm 1.5$</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>$1.6 \pm 2.2$</td>
<td>$2.6 \pm 2.6$</td>
<td>$3.9 \pm 3.0$</td>
</tr>
</tbody>
</table>
Fig. 1 A-F Immunoperoxidase labelling on the human breast at the 12th week of pregnancy for β-casein (A) and κ-casein (B), at the 15th week of pregnancy in the same order (C, D) and at the 18th week of pregnancy in the same order (E, F). Bar: 25 μm.

still intermittent. At the 18th week of pregnancy, such co-labelling continued to be observed within acini that were abundantly positive for β-casein (Fig. 3C). The labelling patterns of Ki67 and the milk proteins were evidently asynchronous. While some luminal epithelial were labelled for both Ki67 and casein, some expressed only Ki67 or milk proteins and the rest were unlabelled by any of the markers.

In the pregnant breast, the integrin subunit α2 labelling on luminal epithelial cells was markedly diminished in some areas, especially in acini containing many Ki67 positive luminal epithelial cells (Fig. 4A). In the non-pregnant breast, luminal epithelial cells showed weak labelling for the integrin subunit α2 in the entire circumference and, in addition, stronger baso-lateral labelling (Fig. 4B). Due to the small size of the samples, exact quantification of α2 by non-immunohistochemical methods was not possible. There was no marked difference in the patterns of expression of the integrin subunits α1, α3, α6, and β1 between the pregnant and non-pregnant breasts. Most luminal epithelial cells were weakly α1 positive in the entire circumference, and some cells (1–8 cells per acinus) showed noticeably stronger overall labelling with additional apical labelling. α3 was weakly positive, and the intensity of the labelling on luminal epithelial cells was variable among and within

Fig. 2 Quadruple immunofluorescence labelling on the human breast at the 15th week of pregnancy for cytokeratin 19 in luminal epithelial cells (blue), cytokeratin 14 in myoepithelial cells (red), vimentin in mesenchymal cells (white) and Ki67 in nuclei of actively proliferating cells (green). Hoechst stained nuclei are shown in the same blue as cytokeratin 19. The yellow bullets point at the proliferating myoepithelial cells labelled for Ki67 in the nuclei and for cytokeratin 14 in the cytoplasm. Bar: 10 μm.
acini, α6 labelling was found in the entire circumference of luminal epithelial cells and was restricted to some acini. β1 was detected in the entire circumference. Luminal epithelial cells showed no expression of β4 in either the pregnant or non-pregnant breasts (data not shown).

Myoepithelial cells

All Ki67 positive epithelial cells in the non-pregnant breast were of luminal epithelial phenotype as evidenced by their location. In the pregnant breast, however, approximately 2% of myoepithelial cells were observed labelled with Ki67. The differentiated phenotype of myoepithelial cells was confirmed by quadruple immunofluorescence labelling in which they were identified by the presence of cytokeratin 14 (Fig. 2).

In the pregnant breast, the intensity of the labelling of the integrin subunits α2, α6, and β4 on myoepithelial cells appeared stronger and more discrete. In the non-pregnant breast, these integrin subunits were uniformly expressed but at different degrees. Between the pregnant and non-pregnant breasts, there were no changes in the labelling of the integrin subunits α1, α3, or β1. In the non-pregnant breast, it was noted that α1 and β4 on

Fig. 3 A-C Double immunofluorescence labelling for κ-casein (A) and β-casein (B) shown in red, Ki67 shown in green and Hoechst labelled nuclei shown in blue on the human breast at the 15th week of pregnancy. The white arrows point at luminal epithelial cells co-labelled for Ki67 and Hoechst exclusively in the nuclei and milk proteins within the cytoplasm and at the apical borders. Bar: 10 μm. Double immunofluorescence labelling for β-casein (C) as shown above, at the 18th week of pregnancy. Bar: 40 μm.
myoepithelial cells were noticeably stronger on ducts than on acini (data not shown).

ECM components

Around extensively dilated acini in the pregnant breast, collagen I partially co-localised with collagen IV, a marker of the basement membrane (Fig. 5A). This contrasted with the non-pregnant breast, where collagen I was detected on the delimiting fibroblasts and below but not on the basement membrane. Around smaller and less developed acini within the same sections of the pregnant breast (Fig. 5B), however, the basement membrane was clearly devoid of collagen I labelling, and its appearance was akin to that of the non-pregnant breast. Both the pregnant and non-pregnant breasts showed labelling for collagen IV, laminin and fibronectin on the basement membrane. Compared to the non-pregnant breast (Fig. 6A), in the pregnant breast, the labelling for these components on the basement membrane formed discrete bands around enlarged acini and appeared distinctly more uniform (Fig. 6B, C and D).

**Discussion**

In the pregnant breast, proliferating luminal epithelial cells (Ki67 positive) were uniformly labelled for cytokeratin 19 and occasionally labelled for k-casein and b-casein. These cells represent direct in vivo evidence of what Potten and Loeffler [31] describe as a dividing transit population in that they display signs of proliferation, phenotypic differentiation, and functional differentiation, simultaneously. The ability of secretory luminal epithelial cells to proliferate has been previously observed in the rat mammary gland by electron microscopy [15]. Contrary to the conventional model that suggests the inverse correlation between differentiation and proliferation, at the unicellular level, these events are not mutually exclusive in rats, as clearly demonstrated by Franke & Keenan [15], nor in humans as shown in the present study.

Neither b-casein nor k-casein should be used as markers of terminal differentiation, although they have been used as such in some earlier studies [34, 46]. They are, nevertheless, specific markers of functional differentiation, since neither type of casein was detected in the non-pregnant breast, as previously reported [11]. Furthermore, in the present study, their labelling patterns exhibited gradual induction of synthesis with the duration of pregnancy.

Active proliferation of myoepithelial cells in the pregnant human breast is a novel observation. The approximate frequency of proliferating myoepithelial cells (2%) in the pregnant breast is higher than that previously determined in the non-pregnant breast (0.2-0.3%) by thymidine labelling and ultrastructural studies [21]. The proliferative potential of myoepithelial cells has been difficult to determine in the past due to their rarity in the non-pregnant breast and the possibility that the proliferating cells were of basal clear cell phenotype [21]. In the current study, proliferation of phenotypically differentiated myoepithelial cells was unequivocally demonstrated by concurrent immunolabelling for Ki67 and cytokeratin 14 in the pregnant breast (basal clear cells are largely devoid of cytokeratin filaments) [37].

The current study on the histologically normal areas of pregnant breast indicates that decreased expression of the integrin subunit α2 may be a normal process in mammalian mammary biology related to levels of proliferation or physiological status rather than malignancy per se. Previous studies have shown diminished levels or absence of α2 in poorly differentiated and invasive breast cancers [4, 30, 47], and in vitro reduced expression of α2 results in disruption of three-dimensional gland-like morphology on collagen I [22]. However, α2 labelling on luminal epithelial cells is diminished in the pregnant human breast, as shown in this study, and also at the growing tips of the end-buds in normal human infant breast tissues.
Fig. 5 A, B Double immunofluorescence labelling on the human breast at the 15th week of pregnancy for collagen I in red and collagen IV in green and Hoechst stained nuclei in blue. Extensively dilated acini (A) and less developed acini (B) within the same section. Bar: 20 µm.

Fig. 6 A-D Immunoperoxidase labelling for collagen IV on the non-pregnant human breast (A) and the human breast at the 12th (B), 15th (C), 18th (D) weeks of pregnancy. Bar: 50 µm.

[3] and in the lactating rodent mammary gland at both the protein and mRNA levels (J Oliver and CH Streuli, personal communication, [22]). The immunohistochemical examination of the luminal epithelial cells failed to show any change in the expression patterns of other integrin subunits α1, α3, α6, β1, and β4 during the examined duration of pregnancy. This agrees with the findings in the pregnant mammary gland in rodents (J Oliver and CH Streuli, personal communication). Therefore, the overall patterns of integrin subunits existing in the quiescent breast prior to pregnancy seem to support adequately the expansion of acini, polarisation of luminal epithelial cells, and induction of milk protein synthesis, although there may be functional changes such as ligand bound status that the reagents used in this and other studies [9] do not detect.

The present study investigated the human mammary functional differentiation in vivo for which the currently available literature is notably restricted. The breast tissues utilised here were essentially normal by careful histological examination, and the morphological development observed in the pregnant breast samples was consistent with previous reports using formalin-fixed paraffin embedded tissues [8]. The multiple immunofluorescence labelling technique allowed cell by cell correlation of various molecular markers on this limited resource, and it has provided an in vivo evidence of the ability of differentiated luminal epithelial cells to remain in the cell cycle. The simultaneous presence of Ki67 and the milk proteins within a single cell does not exclude the possibility that the proliferative state might still shut down the expression of milk proteins at the RNA level, if only temporarily. As well as their importance in understanding human breast biology, the findings reported here are relevant to understanding breast cancer. Many breast tumour cells express cytokeratin 19—a marker of full phenotypic differentiation of luminal epithelial cells [40]. A few also express the milk proteins [7, 36], and a number of human breast cancer cell lines have been shown to synthesis the milk proteins in vitro [34]. The ability of breast cancer cells to express signs of differentiation without losing proliferative potential is, therefore, not an abnormality unique to carcinogenic
transformation but may reflect the inherent nature of mammary luminal epithelial cells from which the majority of breast cancer cells derive [40].

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