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Development of a fully "humanized" xenograft model of breast cancer

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To my parents and lovely wife

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Abstract

Until now there has been a distinct lack of a truly representative breast cancer model. The development of a complex heterologous multi-compartment xenograft model incorporating the relevant stromal elements will provide a realistic alternative to currently available chimeric xenograft models. The recent ability to immortalize primary human mammary endothelial cells and fibroblasts by the insertion of the hTERT and a temperature sensitive mutant variant of SV40 LT has made this possible. We have commenced the development of an organotypic, 3-compartment xenograft model of human breast cancer. The immortalized mammary stromal cells provide a viable and much needed tumour microenvironment of human origin for tumour proliferation.

Results have confirmed the crucial importance of stromal cell support as well as tumour-stromal interactions in tumourigenesis. MCF-7 cells when xenografted in numbers insufficient to produce tumours alone, consistently produced tumours when combined with stromal cells. Also, heterologous xenografts produced not only faster growing but larger tumours than tumour cell lines alone. Immunohistochemical analysis using human cell specific markers demonstrated that initial tumour growth was supported by the stromal cells before the recruitment of host vasculature. The xenografts were composed predominantly of proliferating tumour cells, host ECM and vasculature. Surprisingly the LT antigen expressing immortalized stromal cells did not survive, proliferate or form organized structures in this environment for any reasonable time period.

To conclude, these findings parallel observations made in 3-D organotypic cultures which indicates that progression of the epithelial cell tumour

is not cell autonomous. Rather, that tumourigenesis is promoted, and probably sometimes induced, by the anomalies in the surrounding stroma and microenvironment. With further refinement and adjustments this model can be utilized as a credible pre-clinical model for the development and testing of new therapeutic strategies, such as those that target breast cancer stroma itself. This is very timely as stroma is now emerging as the dominant factor in modulating epithelial morphogenesis and mitogenesis.

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Introduction

1.1 General introduction

Breast cancer is the most commonly diagnosed form of cancer and the second leading cause of cancer deaths in western women. Despite an underlying rise in incidence, 5-year survival has improved steadily over the last 25 years due to earlier detection and improved treatment. The only way to sustain or even accelerate this progress is to develop and test new therapeutic and preventative strategies. However, there is still no effective cure for metastatic breast cancer. The development of breast cancer models will hopefully facilitate the development and testing of novel therapeutic approaches that target metastatic breast cancer both in the form of new drugs, vaccines or even gene therapy.

In the early 20th century, during the search for the ultimate model of human biology, tissue culture and organ modelling was developed when an unlikely collaboration between aviator and engineer Charles Lindbergh and surgeon Alexis Carrel resulted in the manufacture of a glass perfusion pump that was able to prolong the life of animal organs in culture (Carrel and Lindbergh 1935, 1938). Carrel's revolutionary work resulted in the Nobel Prize for his microvascular surgery techniques (which were critical in his attempts at maintaining organs *ex vivo*) and his recognition as the "father of tissue culture science". The fundamental principles of Carrel's studies have resulted in the tissue culture models of today. However, problems in maintaining human tissues and mature organs *in vitro* (Hayflick and Moorhead 1961, Trowell 1959) together with difficulties in interpreting results led to a step back to more

simplistic first principle models involving cultivation of single cell types. Conversely, these models were too simplistic and therefore poorly representative. In an attempt to overcome these problems there has been renewed interest in the original ideas of Carrel, which involve the development of more complex and physiologically relevant systems, by using animal models. Unfortunately, there have been no significant improvements in the technology of animal modelling since the discovery of the first xenograft model. In order to rectify this situation and improve the authenticity of xenograft models there is a pressing need for the development of realistic pre-clinical models representative of breast cancer in humans. For this to occur we need to develop multi-compartment heterologous organotypic xenograft models, either by inserting intact whole tissue xenografts and or more human components.

Xenograft of human breast carcinoma cells in experimental animals represents the closest model we have to breast cancer in humans. Presently there are numerous human xenograft models available for use in breast cancer research (Kim et al. 2003). However, currently available human xenograft models are very basic and unsophisticated, simply involving the growth of human breast cancer cell lines in immunodeficient mice. Xenografted human breast cancer cell lines growing in immunodeficient mice; are not very representative, markedly differing from human breast cancer in that they contain fewer stromal cells and what stroma that does exist is of course murine in origin, resulting in a chimeric tumour. The biology of rodent/human tumours can differ significantly from that of humans resulting in unpredictable growth, differentiation and metastatic patterns (Cardiff 2001).

Breast cancers in humans are not simply comprised of epithelial tumour cells but are a heterogeneous mix of stromal cells (endothelial cells, fibroblasts,

macrophages and lymphocytes), extracellular matrix and its protein. However a distinct lack of a truly representative heterologous xenograft model of breast cancer exists. The great hurdle impeding the development of more complex xenograft models, at the present time, is a lack of readily available stromal cells which are relevant and accessible in an easily manipulatable form. With the recent ability to immortalize normal human mammary stromal cells, a convenient and reliable source of stable stromal cells has been found. However the characteristics and behaviour of these immortal cells *in vitro* and *in vivo* remains to be determined. Therefore, until they are, other sources of human stromal cells need to be explored. Furthermore the most effective method of incorporating stromal cells as well as their interaction with the host animal needs to be investigated. The development of a complex multicellular organotypic heterologous xenograft model incorporating the relevant stromal elements will provide a realistic alternative to currently available chimeric xenograft models. Such a model containing the relevant and essential constituents of a tumour microenvironment, human stroma and extracellular matrix, will provide a more authentic breast cancer model for the testing of new and emerging therapeutic regimes. In order to develop the most accurate model the biology of the human breast as well as the pathology of breast cancer must be considered.

1.2 The breast

1.2.1 Anatomy

The breast, also known as the mammary gland, is present in both men and women, and its structure is essentially similar in both sexes until puberty. It lies on the thoracic wall, attached to the pectoralis muscles by fascia over the 2nd and 6th ribs. The mature female breast is composed of essentially four structures: lobules or glands; milk ducts; fat and connective tissue (see Figure 1.1) (Osborne 2000, Lamarque 1984). Each fully developed adult breast consists of on average of 15-20 lobes. The lobes are arranged roughly in a wheel spoke pattern emanating from the nipple/areolar area. The distribution of the lobes is not even however; there is a preponderance of glandular tissue in the upper outer portion of the breast. This is responsible for the tenderness in this region that many women experience prior to their menstrual cycle. It is also the most common site for breast cancers. Each lobe is 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, lymphatics and blood vessels lined by endothelial cells (Osborne 2000, Lamarque 1984).

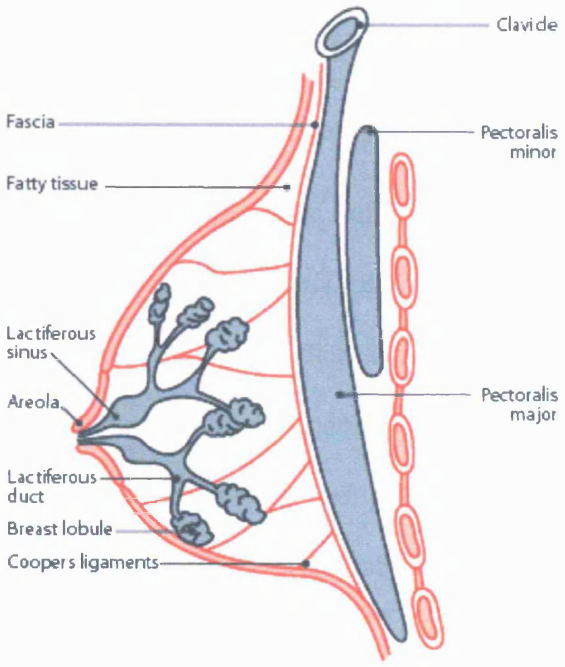


Figure 1.1. Gross anatomy of the mature female breast. Diagram from Breast Cancer Source website.

1.2.2 Physiology

At birth, the degree of morphological development in the human breast is highly variable (Anbazhagan et al. 1995). Maternal hormones may cause transient secretory activity in the infant breast during the first few post-natal days, with additional secretory developments occurring for a further two months subsequently demonstrated by areas of localised hyperplasia.

At puberty the female breast increases in size, mainly due to the deposition of interlobular fat, but further ductal and alveolar development is also believed 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 MC 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. This is accompanied by dramatic morphological changes, characterised by glandular-alveolar growth, expansion of acini and polarisation of luminal epithelial cells (Battersby and Anderson 1988, Vorherr 1974).

At parturition, an abrupt drop in the level of oestrogen and progesterone in blood 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 the release of colostrum, the secretory luminal epithelial cells become fully active, producing large volumes of milk. Muscular cells (myoepithelial cells) surrounding the alveoli contract to express the milk during lactation. 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 involute and dense connective tissue replaces the intralobular loose connective tissue.

1.2.3 Biology

The normal human breast gland comprises a branching ductal-lobular system lined by an inner layer of luminal epithelial cells which are in direct contact with an outer layer of myoepithelial cells separated from the interstitial stroma by an intact basement membrane.

The luminal epithelial cells are polarized glandular cells with specialized apical and basolateral membrane domains expressing sialomucin and adhesion molecules, respectively (Ronnov-Jessen et al. 1996). 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. The myoepithelial cells contribute significantly to the formation of basement membrane, and their myogenic differentiation is responsible for the contractile phenotype mediated by oxytocin responsible for the ejection of milk (Murrell 1995). However it was found that cellular proliferation in the human breast was virtually confined to luminal cells while the myoepithelial cells did not divide when fully mature (Joshi et al. 1986). Also that age and parity of women affected the type of parenchymal structures present in the breast with nulliparous and older age groups observed to have more undifferentiated structures (less ductules per lobular unit) (Russo et al. 1992).

The two cell populations can easily be distinguished by their different phenotypic expression profiles of cytokeratin (CK) 18, CK19, mucin 1 (MUC1), epithelial membrane antigen (EMA) and oestrogen receptor (ERα) in luminal epithelial cells and expression of CK4, CK14, CK17, 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 (Suzuki et al. 2000). Once phenotypically differentiated, under normal physiology, the expression of these markers is stably maintained, and the phenotypically differentiated cells are destined to perform their differentiated function. Recently, comprehensive expression profiles of these two cell types have been produced using gene expression microarray analysis (Jones et al. 2004). New prognostic markers for cell survival were identified when the luminal- and myoepithelial-specific molecules were evaluated on breast tumour tissue microarrays.

As breast cancer arises mainly in the luminal epithelial compartment on the basis that most typical breast cancers express luminal markers (e.g cytokeratin 18, 19, EMA etc.), this has been the focus of the majority of studies with less attention paid to the role of the surrounding cells in breast function. A number of studies have postulated that intact myoepithelial cells are an important determinant of normal breast differentiation, and that loss of their functions could contribute to induction and/or progression of epithelial cancer (Zou et al. 1994, Radice et al. 1997, Sternlicht et al. 1997, Péchoux et al. 1999, Slade et al. 1999). Indeed a number of myoepithelial-specific proteins have been shown to inhibit epithelial tumour formation and, thus, these cells are postulated to have tumour suppressive activities (Gomm et al. 1997, Sternlicht et al. 1997, Shao et al. 1998, Sun et al. 1999).

1.3 Breast Cancer

1.3.1 Epidemiology

With 1 million new cases in the world each year, breast cancer is the commonest malignancy in women and comprises 18% of all female cancers. Breast cancer has now become the most common cancer in England and Wales. One in nine women will develop breast cancer at some point in their lifetime with four in five of them being over 50 years old. In 2000 there were almost 36,000 new cases diagnosed, comprising 30% of all cancers in women and a rate of 114 per 100,000 (Office of National Statistics UK, Cancer Research UK). Breast cancer incidence is increasing particularly among women aged 50-64 (with four out of five new cases being diagnosed in women over the age of 50) with the peak in the distribution of new cases in the 50 to 54 age group. This peak may reflect the breast screening programme because many of these women will have been screened for the first time.

Around 11,500 women died from breast cancer in England and Wales in 2002, a rate of 30 per 100,000 women making it the most common cause of cancer death in women. The age standardised incidence and mortality in the U.K. is presently the highest in the world. Men can also get breast cancer but it is very rare, with around 300 cases being diagnosed each year.

Although a specific cause for breast cancer has not been identified, there are risk factors that increase the likelihood that a woman will develop a breast cancer (Baum 2002). Most of the known risk factors for breast cancer relate to a woman's reproductive history such as early first period, late first pregnancy, low parity and late menopause. Oral contraceptive use, hormone replacement therapy (HRT), obesity and increased alcohol consumption also increase the risk. A

genetic predisposition has now been discovered involving the germline mutations of *BRCA1* and *BRCA2* genes. The incidence of the mutant *BRCA1* gene on chromosome 17q may be 1 in 800 women. Although the *BRCA2* gene on chromosome 13q is less frequently mutated it is also associated with early onset breast carcinomas. The presence of these genes explains some of the familial cases, and may be the aetiology for about 5-10% of breast cancers overall (Eeles 2000, Cannon-Albright and Skolnick 1996). The overall risk of mutation carriers developing (female) breast cancer is estimated to be 80-85% by the age of 80 years (Ford et al. 1995, Ford et al. 1998). Aside from the genetic predisposition, the common factor in many of these risk factors is the increased endogenous oestrogen exposure over a long time.

Although more women in the UK are being diagnosed with breast cancer than ever before, fewer are now dying from the disease, according to new figures. Statistics released by Cancer Research UK reveal that three out of four women are surviving for five years or more. Due to earlier detection and improved treatment (notably the widespread use of tamoxifen), breast cancer death rates in the UK is falling, with around 13,000 women dying from the disease in 2001, a 21% decrease over the last decade. This trend is set to continue at a rate of around 2% decrease each year.

1.3.2 Diagnosis

Breast cancer is most commonly diagnosed by the patient following the discovery of a palpable breast mass and or other signs (e.g. nipple discharge, inversion of the nipple, skin tethering, lymphadeopathy etc.). However, a breast cancer may have been present for 5 to 10 years before reaching a size (about 1

cm) that is detectable by palpation. The other common method of diagnosis, in the older age group, is mammography (in the UK participation of the National Breast Screening Programme). The National Health Service (NHS) offers mammograms (breast X-rays) to women between the ages of 50-69, 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 considerably less than original studies indicated (Quinn and Allen 1995, Baum 2000, Gotzsche and Olsen 2000). Most breast cancers are found the upper outer quadrant (50%) probably because this contains a significantly larger amount of tissue.

Once referred to a specialised breast clinic a triple assessment is performed involving: 1) Clinical evaluation which includes history and physical examination of the breast lump and regional lymph nodes, 2) Imaging (ultrasound <35 years old or mammography >35 years old) and 3) Cytology (fine needle aspiration) or histology (core biopsy or Tru-Cut biopsy). From this assessment a diagnosis of malignancy can be made with a relatively high level of confidence (~90%) (Drew et al. 1999).

1.3.3 Management of early breast cancer

Primary treatment

Treatment of breast cancer can take a variety of forms, depending upon the grade and stage of the cancer as well as the overall health and wishes of the patient. Therapy needs to be appropriate for each individual woman.

The first line of treatment for early breast cancer is surgery. The aims of breast cancer surgery are to achieve cure if excised before metastatic spread has

occurred and to prevent the unpleasant sequelae of local recurrence. Surgical options for the breast are a) Breast conserving surgery (BCS) + radiotherapy with BCS regarded as either wide local excision, quadrantectomy or segmentectomy, b) Simple mastectomy, c) Radical mastectomy which now obsolete and d) Mastectomy + reconstruction (immediate or delayed) (Fisher et al. 2002).

Tumours suitable for breast conservation are small single tumours in a large breast, peripherally located tumours and tumours with no local advancement or extensive nodal involvement. For tumours that are suitable for breast conservation there is no difference in local recurrence or overall survival when BCS + radiotherapy is compared to mastectomy (Bundred and Downey 1996, Hortobagyi 1998).

30-40% of patients with early breast cancer have nodal involvement determined pathologically, sometimes with the aid of epithelial specific immunohistochemistry (e.g. pan-cytokeratin). The aims of axillary surgery are to eradicate local disease and determine prognosis to guide adjuvant therapy. Clinical evaluation of the axilla is unreliable (30% false positive, 30% false negative) with no reliable imaging techniques available; therefore, surgical evaluation is important and considered for all patients with invasive cancer. As a recently introduced alternative sentinel node biopsy is a technique which aims to accurately stage the axilla without the morbidity of axillary clearance. The technique is used to identify the first nodes that tumour drains to following injection of peritumoural, subdermal or subareolar sites with radioisotope labelled colloid, blue dye or usually both. It allows more detailed examination of nodes removed. However the prognostic significance of micrometastatic deposits identified in sentinel nodes is unclear.

Secondary treatment

Surgical procedures may be combined with radiation therapy and or chemotherapy, depending upon the type of cancer present and hormone receptor status. 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, Small and Lurie 2001). Adjuvant therapy consists of drug treatments given after the initial surgery to reduce the risk of recurrence and micrometastasis (Fisher 1999). Over the past three decades conventional adjuvant chemotherapy regimens have also been employed pre-operatively (neo-adjuvant or primary chemotherapy) (Smith et al. 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) is the standard treatment (Pinedo and Giaccone 1997). However, recent results from the ATAC trial (Dec. 2004) demonstrated that aromatase inhibitors produced superior results when compared to Tamoxifen. 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).

1.3.4 Pathology

Classification of Breast Cancer

Breast cancers can be classified histologically based upon the types and patterns of cells that compose them. Carcinomas can be invasive (extending into the surrounding stroma) or non-invasive (confined just to the ducts or lobules) and epithelial or mixed tumours.

The WHO Classification of breast cancer is as follows:

- Epithelial
    - Non-invasive
        - Ductal carcinoma in situ (DCIS)
        - Lobular carcinoma in situ (LCIS)
    - Invasive
        - Ductal
        - Lobular
        - Mucinous or colloid
        - Papillary
        - Medullary
- Mixed Connective tissue and Epithelial
- Miscellaneous

The great majority of invasive breast cancers are of the 'ductal' type (~80%) with 5-10% lobular and the remainder accounted for by specific types (e.g. mucinous, papillary, medullary, tubular).

Hormone receptor and immunoperoxidase techniques

The hormone receptor status of the breast cancer cells can give very useful information for treatment and prognosis. The neoplastic cells can express a variety of receptors. The presence of these receptors can provide a means for controlling cell growth through hormonal agents.

In general, cancers in which the cells express ER in their nuclei will have a better prognosis. This is because such positive neoplastic cells are better differentiated, and they can respond to hormonal manipulation. The drug tamoxifen is often utilized for this purpose. Almost three-fourths of breast cancers expressing ER will respond to this therapy, whereas less than 5% not expressing ER will respond (Barnes et al. 1996). The significance of progesterone receptor positivity (PR) in a breast carcinoma is less well understood (Pichon et al. 1996).

There are other markers that can be identified in breast carcinomas. One important marker for breast cancer is erbB2 (HER2), was identified by staining around the cytoplasmic membrane of the cells. There is a correlation between HER2 (erbB2) positivity and high nuclear grade and aneuploidy, and a specific drug (Herceptin, trastuzumab) is available as a therapeutic option with HER2 positive carcinomas.

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 et al. 2001). The most widely applied system in the UK is the Scarff-Bloom-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 lumina. 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 vesicules 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. These scores are added together to indicate the Grade 1 (score 3-5), Grade 2 (score 6-7) and Grade 3 (score 8-9).

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 distant metastasis. Stage II breast cancers are 2-5cm or with lymph node involvement or both, but without signs of distant 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).

The Nottingham Prognostic Index (NPI)

Out of these prognostic indicators, lymph node status is considered the most important pathological factor (Galea et al. 1992, Sloane et al. 2001). However, it is a time-dependent factor, not taking account of the innate aggressiveness of tumours. Therefore, the three most significant prognostic indicators; tumour size, lymph node status and grade have been integrated (NPI) to provide a more accurate prognosis {0.2 x size in cm + 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%.

1.3.5 Management of 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. Effective treatment relieves symptoms and increases the quality and quantity of life.

Hormone therapy

Historically hormone ablation was carried out simply by oophrectomy (Beatson 1896) and or adrenalectomy, more rarely pituitary ablation. In recent years the pharmacological manipulation of endogenous hormones has predominated. Tamoxifen (Fisher 1999), most widely used hormone therapy, 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). There are other selective oestrogen receptor modulators (SERMs) available such as Toremifene (Fareston), 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 megestrol acetate (Megace) and medroxyprogesterone acetate (Farlutal, Provera); aromatase inhibitors that block the production of oestrogen in adipose tissues such as anastrozole, letrozole and exerestase; 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

Chemotherapy is now the first line of treatment for distant metastases affecting the liver or lungs. 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).

Need for new therapeutic regimes

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 are 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 new drug design

The current best example of intelligent drug design is based on an oncogene human epidermal growth factor receptor-2 ERBB2 / HER2 (ErbB2) that is amplified in approximately 20% 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 this specific breast cancer marker. Herceptin achieves significant improvement in survival of metastatic ErbB2 positive breast cancer patients when combined with chemotherapeutic agents (Piccart 2001, Kumar et al. 2000). Another receptor specific therapy in development is IRESSA (ZD1389)/epidermal growth factor receptor-tyrosine kinase inhibitor (Wakeling et al. 2002, Ranson et al. 2002). More recently, dual ErbB-2/EGFR tyrosine kinase inhibitors (572016) are in the process of Phase III trials (Rusnak et al. 2001). Whilst the success of the intelligent drug design remains statistically significant yet modest (Piccart 2001, Kumar et al. 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.

With the massive explosion in therapeutic strategies becoming available more authentic breast cancer models are urgently required for preclinical trials. Only when an authentic and validated model becomes available can we reliably consider testing on human patients. Development of better *in vitro* and *in vivo* models of human breast cancer is of crucial importance in the fight against breast cancer mortality. Prime among the factors that need to be considered in this context are the tumour microenvironment *in vivo* and the cells that comprise the tumour associated stroma. The objective of this study is to explore different methods of recreating the tumour microenvironment in an experimental model system.

1.4. Tumour microenvironment

1.4.1 Stroma

The normal human mammary gland, which has a highly structured and segregated architecture, contains two distinct tissues: epithelium and mesenchyme (embryonic) and stroma (postnatal). The mammary ducts are

formed by a double layer of cells: luminal epithelial cells surrounded by a layer of myoepithelial cells, enclosed by the basement membrane. The basement membrane separates the epithelial cells from the interstitial stroma. Stroma is composed of extracellular matrix (ECM) and mesenchymal cells, which provides a structural scaffold and conduit for blood and lymphatic vessels, nerves, and leukocytes.

Breast carcinomas are often characterized by a stromal reaction that consists of modifications in the composition of both the cellular elements (infiltration of fibroblastic cells, endothelial cells and inflammatory cells) and the ECM. This reactive stroma actually constitutes a major part of the neoplasm. For a long time, only the neoplastic cells were the focus of interest in cancer research and the stroma was rather considered a reactive component without major significance. However, it has become clear that the stromal cells and their products (matrix components, growth factors, proteases, etc.) regulate the phenotype of cancer cells. Tumours, therefore, represent a complex ecosystem where multiple host cells-ECM and tumour cells-ECM, as well as tumour cells-host cells interactions lead to reciprocal influences resulting in tumour promotion, invasion and metastasis (Mareel and Leroy 2003).

Although the majority of breast malignancies are epithelial cancers they are a complex and relatively unorganized heterogeneous mix of epithelium, stromal cells (endothelial cells, fibroblasts), inflammatory cells (e.g. macrophages, lymphocytes and mast cells), ECM and its proteins (Ghosh et al. 1980). In breast tumours the epithelial carcinoma cells intermingle with the stromal elements in the stroma with tumour angiogenesis producing poorly developed and therefore ill defined blood vessels. In contrast, blood vessels are well defined and centrally located in the normal mammary gland with epithelial

cells prevented from coming into contact with fibroblasts and other stromal cells as they are separated from them by the basement membrane (Ghosh et al. 1980). The stroma provides the vascular supply, specific soluble and ECM molecules that are required for tumour growth and progression (Hanahan and Folkman 1996). Growth and dissemination of a breast cancer involves interaction between the tumour cells and stromal elements. Two important stromal cells that are consistently found in and compose the vast majority of the stromal cells present in breast cancer are fibroblasts and endothelial cells.

Fibroblasts

The principal cells of breast stromal tissue are called fibroblasts. Fibroblasts are cells of mesenchymal origin with many vital functions during development and in adult organisms. Fibroblasts, as they are traditionally defined, comprise a diverse class of distinct functional and differentiated cell types separable based on their gene expression profiles (Chang et al. 2002). However the label fibroblast does not accurately cover their diversity. For example the label 'fibroblast' has also been used loosely to define multipotent cells of mesodermal origin that that can give rise to other cells of mesodermal origin such as fat cells, bone cells, cartilage cells and smooth muscle cells. In addition it is used as an umbrella term to describe other cells such as fibrocytes and myofibroblasts. For example, the mixture of cells identified in the stroma of infiltrating breast carcinomas have been described as resting fibroblasts, active fibroblasts, early myofibroblasts, and mature myofibroblasts (Martinez-Hernandez et al. 1977, Schurch et al. 1981). The fibroblasts that comprise the tumour stroma have also been termed myofibroblasts, peritumoral fibroblasts, reactive stroma and carcinoma-associated fibroblasts .Despite the evidence that the cells we call fibroblasts from different stromal sites may comprise a host of distinct

differentiated cell types, neither the diversity of these cells nor the extent or nature of local specificity in their differentiation has been systematically examined.

Fibroblasts are usually identified by their spindle-shaped morphology containing an abundant and branched cytoplasm surrounding an elliptical and speckled nucleus which has one or two nucleoli; their ability to adhere to plastic culture vessels; and the absence of markers for other cell lineages. Active fibroblasts have abundant rough endoplasmic reticulum and a well developed Golgi apparatus characteristic of synthetic cells (Ghosh et al. 1980). Inactive fibroblasts, called fibrocytes, are smaller, spindle shaped and have reduced rough endoplasmic reticulum, a smaller, elongated nucleus, and are embedded in ground substance.

Fibroblasts present in breast stroma, as distinct from multipotent fibroblasts that can differentiate into other cell types (cartilage, smooth muscle etc.), are considered the least specialized of this family of connective tissue cells, making the structural fibres and ground substance of ECM. As the predominant cell in the stroma, the fibroblast is responsible for the elaboration of most of the components of ECM. They make different collagens, reticular and elastic fibres, glycosaminoglycans and glycoproteins found in the extracellular matrix (Tlsty and Hein 2001). Collagen is the most abundant protein in the human body and 90% of that is fibrillar Type I collagen. They are also responsible for the release of proteolytic enzymes, proteolytic inhibitors and growth factors. Each organ has specialized requirements, and hence fibroblasts from different organs demonstrate organ-specific variations of the classes of basic molecules listed above. Furthermore, in response to different physiologic signals, be they normal or pathologic the fibroblasts of the stroma change accordingly (e.g.

myofibroblasts and carcinoma associated fibroblasts). Several growth factors can induce the activity of fibroblasts. Platelet derived growth factor, PDGF, and connective tissue growth factor, CTGF, stimulate chemotactic movement of fibroblasts (Takehara 2000). Fibroblast growth factor (b-FGF) is involved in the stimulation of fibroblasts during angiogenesis and transforming growth factor beta (TGF-ß) stimulates fibroblasts to produce matrix proteins (Takehara 2000).

In the normal human mammary gland two distinct stromal areas can be identified morphologically (Atherton et al. 1994). Firstly a loose relatively cellular stroma (the intralobular stroma) within which the acini of the lobules are embedded and secondly the densely collagenous and relatively acellular interlobular stroma which surrounds the lobules and their associated intralobular stroma. The fibroblast sub-populations found in each of these compartments are distinct phenotypically and functionally. The presence of the cell-surface enzyme dipeptidyl peptidase IV (DPP-IV) was demonstrated on the cell membranes of mammary interlobular fibroblasts, whilst intralobular fibroblasts were DPP-IV-negative (Atherton et al. 1992). Fibroblasts isolated from the interlobular and intralobular stroma produced similar amounts of type I and IV collagen but the interlobular fibroblasts produced 3- to 5-fold more type XIV collagen/undulin than intralobular fibroblasts (Atherton et al. 1998). Also phenotypes of fibroblasts in the normal mammary gland differs from that present in tumourigenic breast stroma. For example, neutral endopeptidase also known as CALLA or CD10, an ectoenzyme from a large family of membrane-associated metalloproteases, is not expressed by normal adult breast fibroblasts but is found in the stroma associated with over 60% of breast cancers. The maintenance of two types of structurally distinct stromas and its components may be important

during developmental processes in the mammary gland and during tumourigenesis.

Myofibroblasts, a sub-population of fibroblasts, are modulated fibroblasts which have acquired the capacity to neoexpress α-smooth muscle actin, the actin isoform typical of vascular smooth muscle cells, and to synthesize important amounts of collagen and other ECM components (Desmouliere et al. 2004). Myofibroblasts, normally inconspicuous in healthy tissue, are active during tissue development, remodelling, inflammation and repair (Gabbiani 1996) and are intimately involved in wound healing. They have more contractile proteins (actin and myosin) than fibroblasts and can therefore effectively act to pull tissues together after injury. They secrete collagen and their involvement late in wound healing can cause excessive scarring. Indeed, the uncontrolled activity of myofibroblasts is thought to be involved in the development of fibrosis of the liver, kidney, heart and lung (Powell et al. 1999). Myofibroblasts are not only capable of remodelling ECM but also interact with epithelial cells and other stromal elements and thus may control important functions such as tumour invasion and angiogenesis (Desmouliere et al. 2004).

It is important to note that although myofibroblasts are not a component of normal breast stroma they are known to be a prominent feature in the stroma of infiltrating breast carcinoma (Ghosh et al. 1980) and scirrotic breast cancers (Seemayer et al. 1979). Invasive or infiltrating ductal carcinomas, which represent the most common type of breast cancer, are characterized by a pronounced degree of desmoplasia and are often referred to as scirrhous carcinoma. Desmoplasia is a common host response to epithelial tumours and is classically described as fibroblast proliferation in conjunction with ECM remodelling. The infiltrating process of cancer cells is analogous to wound

production and healing with continuous granulation tissue and scar formation resulting in the characteristic desmoplastic reaction seen in certain breast carcinomas. The degree of myofibroblastic proliferation is related to the growth pattern of the tumour. The stromal myofibroblast reaction common to many invasive and metastatic carcinomas characterized by the production of a dense collagen with a contractile state of such tissue may constitute an attempt by the host stroma to contain the neoplasm and impede vascular invasion.

Cancer associated fibroblasts (CAF)

Mammary stromal fibroblasts can differentially regulate breast cancer cell proliferation. Both the fibroblasts' tissue source, normal or tumour associated, as well as the target tumour cell's phenotype will determine the extent of the proliferative response. This source and target dependency has been reported in several studies. (van Roozendaal et al. 1992, van Roozendaal et al. 1996, Brouty-Boye and Raux 1993, Dong-Le Bourhis et al. 1997, Gache et al. 1998). Different studies have reported different results regarding the varying stimulation or inhibition of breast cancer cell lines *in vivo* by normal or CAF. Fibroblasts are thought to affect mammary cells by secreting several classes of compounds including: (1) matrix proteins, (2) soluble chemokines, cytokines and growth factors, and (3) proteases. Such matrix proteins together with growth factors could promote the proliferation of endothelial cells, tumour cells or both (Noel et al. 1993, van Roozendaal et al. 1992).

Studies of fibroblasts in the vicinity of the malignant lesion also support a role for stromal cells in tumourigenesis (Adam et al. 1994, Dong-Le Bourhis et al. 1997, Skobe and Fusenig 1998). The fibroblast is a major cell type of the stromal compartment, and as such is intimately involved in orchestrating the stromal half of the dialogue in tissue homeostasis. Modification of fibroblasts in

the stroma immediately adjacent to transformed epithelial cells has been documented in several tumour systems (Basset et al. 1990, Chiquet-Ehrismann et al. 1986, Singer et al. 1995, Wright et al. 1994, Yee et al. 1991). The different phenotypic and genotypic expression patterns of these different CAFs are the subject of many studies. For example, Ronnov-Jessen et al. (Ronnov-Jessen et al. 1996, Ronnov-Jessen et al. 1995) demonstrated that the histology and growth characteristics of mammary CAFs were different from those of fibroblasts associated with normal breast epithelial cells. They described the presence of 'activated' or abnormal myofibroblasts associated with invasive breast carcinoma cells. Other phenotypic changes ascribed to CAFs include abnormal migratory behaviour *in vitro* (Schor et al. 1988) and altered expression of growth factors such as platelet-derived growth factor, insulin-like growth factors I and II, transforming growth factor-ß1, hepatocyte growth factor/epithelial scatter factor, and keratinocyte growth factor (Yee et al. 1991, Ellis et al. 1994, Frazier and Grotendorst 1997, Nakamura et al. 1997, Ponten et al. 1994, Yan et al. 1993). Although these phenotypic changes have been documented in CAFs, their consequences or contributions to tumour growth and development *in vivo* have not yet been investigated.

Endothelial cells

The endothelial cell is a flattened cell type that forms a sheet (the endothelium) lining all blood vessels. They, like fibroblasts, represent a structurally and functionally heterogeneous population of cells. Endothelial cells may be derived from any part of the vascular tree, from large and small veins and arteries, from capillaries, or from specialized vascular areas such as the umbilical vein of newborns, blood vessels in the brain or from vascularized solid tumours. In addition, endothelial cells can also arise from precursor cells called angioblasts

or haemangioblasts, the latter giving rise not only to endothelial cells but also to blood cells.

Although they present many common functional and morphological features, they also display remarkable heterogeneity in different organs (Rajotte et al. 1998, Trepel et al. 2000). Their complexity and diversity has long been recognized, yet very little is known about the molecules and regulatory mechanisms that mediate the heterogeneity of different endothelial cell populations. However, endothelial cell heterogeneity has now been described at the level of morphology, function, antigen composition, and signalling networks (Cines et al. 1998, Gerritsen 1987, Page et al. 1992). Each type is uniquely adapted to meet the demands of its local environment. Even in the same organ, the endothelium of large and small vessels, veins, and arteries exhibits significant heterogeneity. An extreme case is the kidney, which contains different types of endothelial cells: fenestrated in the peritubular capillaries, discontinuous in glomerular capillaries, and continuous in other regions (Risau 1995).

Evidence that endothelium in macro- and microvascular compartments arise from distinct embryological origins suggests that a developmentally determined phenotype may contribute to the cell's site-specific function. As an important corollary, the embryological origin and local environment of the endothelial cell may importantly contribute to both its normal function and its response to pathophysiological stimuli. There are two possible mechanisms for the development of organ-specific endothelial cell phenotypes. In one case, endothelial cells would arise from the mesoderm with a predetermined phenotype and migrate to specific vascular beds in an organ, where they subsume site-specific function. In the second possibility, all endothelial cells would arise from the mesoderm and have a similar generic endothelial cell phenotype that would

include universal markers such as CD31, but such cells would be devoid of vascular bed-specific endothelial cell markers. For this latter hypothesis to be correct, multipotent endothelial cells would undergo a terminal differentiation, under the influence of inductive signals from the involved organ.

Besides forming a monolayer in every single blood vessel in the circulation endothelial cells are actively involved in several regulatory processes in the body (Cines et al. 1998, Edgington 1995, Rodgers 1988). Examples include: a) the endothelium forms a semi-permeable physical interface between blood and surrounding tissue and acts as a functional barrier for the transport of blood-borne peptides, proteins, and other soluble molecules to underlying tissue to regulate nutrient and blood component traffic (e.g. blood brain barrier); b) via the regulated expression of pro- and anti-coagulative activities, endothelium actively participates in the haemostatic balance in the body; c) under the influence of pro-inflammatory cytokines, endothelial cells up-regulate a variety of cellular adhesion molecules to tether and activate leukocytes (leucocyte trafficking) and facilitate leukocyte adhesion and transmigration from the blood into the tissue; d) endothelial cells play an active role in wound healing and tumour angiogenesis.

In the mammary gland several different types of endothelial cells are present. These are macro- and microvascular, lymphatic, arterial and venous. Although endothelial cells of lymphatic capillaries have many properties in common with the endothelium of blood vessels, they also have distinct structural characteristics reflecting their specific functions (Daroczy 1988, Skobe and Detmar 2000). Lymphatic capillaries lack mural cells and are characterized by an incomplete or absent basement membrane. Lymphatic endothelium typically contains numerous invaginations and cytoplasmic vesicles as well as

characteristic overlapping intercellular junctions. Whereas the junctions in blood vessels connect adjacent endothelial cells over entire cell boundaries, the junctions in lymphatics are generally more sparse. Finally, one of the most striking characteristics of the lymphatic capillary is its integration within the interstitium; lymphatic endothelial cells are connected to the ECM by fine strands of elastic fibres, i.e., anchoring filaments (Leak 1976, Gerli et al. 1990).

Cancer associated endothelium

Like most embryonic tissues, tumours have the ability to build up their own blood vessel networks. However, the architecture of tumour vessels is fundamentally different from that found in healthy tissues. Tumour vessels are usually irregular, heterogeneous, leaky, and poorly associated with mural cells. Endothelial cells in tumour vessels are also disorganized and express imbalanced surface molecules. These unusual features may provide some molecular and structural basis for selective inhibition or even destruction of tumour vessels by angiogenesis inhibitors.

It has long been suggested that an organized lymphatic system, as such, is not present in tumours (Gullino 1975) with studies in human and animal tumours involving injection of tracers into lymphatics revealing that tumours do not have an intrinsic lymphatic vascular supply (Zeidman et al. 1955, Tanigawa et al. 1981). However, it has also been agreed that this observation reflects the collapse of lymphatics within the tumour due to mechanical stress generated by proliferating cancer cells, and that this in turn contributes to increased pressure within the tumour interstitium (Leu et al. 2000). Although the lack of intratumoural lymphatics appears to be a consistent feature, dilated and engorged lymphatics in peritumoural stroma, with occasional penetration into the tumour periphery, are features that are observed with equal frequency (Leu et al. 2000,

Hartveit 1990). It has been suggested that these lymphatics are likely to be existing lymphatics that have enlarged in response to VEGF-C, rather than new lymphatic vessels (Leu et al. 2000).

The presence of tumour cells in peri- or juxtatumoural lymphatics is not an uncommon feature for many primary tumours. In a detailed study on breast carcinoma, Hartveit (1990) has described the presence of an open lymphatic labyrinth in close association with the primary tumour and suggests that tumour cells lying free in the periductal lymphatic spaces will be washed with the tide of tissue fluid into the labyrinth through the sinuses and on into the lymphatic drainage channels

Numerous investigations have focused on the tissue origin of tumour endothelial cells (Lin et al. 2000, Asahara et al. 1997) and the individual genes that promote the growth and survival of tumour vessels. Tumour cells are proposed to mediate tumour angiogenesis by secreting soluble factors that enhance endothelial cell proliferation, migration and tube formation as well as by direct cellular interactions with endothelial cells. The interactions between tumour cells and endothelial cells which are complex, might be analogous to those observed during embryonic development and have been hypothesized to be important in the process of tumour angiogenesis (Darland and D'Amore 1999).

Circulating endothelial progenitors

Traditionally it was believed that tumours recruit neighbouring blood vessels and vascular endothelial cells to support their own blood supply. Recent evidence has indicated, however, that tumours are also capable of mobilizing bone marrow-derived endothelial precursor cells, inducing them to migrate to the tumour and become incorporated into the developing vasculature. Tumour-derived angiogenic factors promote the recruitment of these cells, which include

circulating endothelial progenitor cells and haematopoietic stem and progenitor cells.

Circulating endothelial progenitor cells (CEP) are highly proliferative cells that are derived from bone marrow, foetal liver, umbilical-cord blood and cytokine-mobilized peripheral blood and have several characteristics that distinguish them from circulating endothelial cells. The latter are mature terminally differentiated cells that have sloughed off from the vascular wall and enter circulation as a result of shredding or traumatic vascular injury. Mature endothelial cells have a low proliferative potential and are less likely than CEPs to contribute to tumour neo-angiogenesis (Rafii et al. 2002).

Both CEPs and mature endothelial cells express similar endothelial-specific markers, including vascular endothelial growth factor receptor-2 (VEGFR-2), Tie-1, Tie-2, and vascular endothelial (VE)-cadherin (Rafii et al. 2002, Peichev et al. 2000). Therefore, these markers do not provide a method in which to differentiate between the two populations. Furthermore, identification of the differences between CEPs and circulating mature endothelial cells is complicated by the fact that haematopoietic stem and CEPs express markers similar to those of mature endothelial cells such as VEGFR-1 (Flt-1), platelet endothelial cell adhesion molecule (PECAM), Tie-1, Tie-2, and von Willebrand's factor (vWF), and they also have the capacity to incorporate acetylated low-density lipoprotein (Ac-LDL). However haematopoietic stem and CEPs express unique surface markers, such as CD34 and the newly discovered early haematopoietic stem cell marker CD133. Expression of CD133 diminish with maturation and differentiation (Miraglia et al. 1997, Yin et al. 1997).

CD133 is a novel 120-kd glycosylated polypeptide that contains 5-transmembrane domains with an extracellular N-terminus and a cytoplasmic C-

residue (Miraglia et al. 1997, Yin et al. 1997). The function of CD133, which does not share homology with any previously described haematopoietic cell surface antigen, is not known. However, isolation of a subpopulation of $CD34^+$ cells using monoclonal antibody to human CD133 has resulted in the identification of functional $CD34^+$ population of haematopoietic stem cells. Human-derived $CD133^+$ cells were shown to repopulate sheep bone marrow (Yin et al. 1997) and, therefore, were considered pluripotent haematopoietic stem cells. Expression of CD133 is rapidly down regulated as haematopoietic progenitors and stem cells differentiate into more mature post mitotic cells. In fact, virtually all mature haematopoietic cells, including mature myeloid, megakaryocytes, erythroid, and lymphoid cells and terminally differentiated haematopoietic cells, fail to express CD133 (Miraglia et al. 1997, Yin et al. 1997). Therefore, subsets of $CD34^+$ cells that express CD133 represented a unique phenotypic and functional marker of an immature population of haematopoietic stem and progenitor cells. Taken together, these results suggest that circulating $CD34^+$ cells expressing CD133 comprise a functional population of CEPs cells that may play a role in postnatal angiogenesis or vasculogenesis.

Several studies have reported that CEPs when inoculated into immundeficient mice, incorporate into the vasculature of xenotransplanted tumours (Lyden et al. 2001, Reyes et al. 2002, Moore 2002, Asahara et al. 1999, Gehling et al. 2000).

Inflammatory cells

One of the responses to breast cancer is an immune response with an influx of inflammatory cells. These include macrophages, mast cells, lymphocytes (T and B cells), and antibodies, a reflection of immunologic surveillance against neoplasia. However inflammatory cells provide a very small contribution to the

total population of stromal cells present in breast cancer stroma. The function of all these different immunocompetent cells has not been clarified as yet. Even so the focus of most studies has limited themselves to the lymphocytic infiltration of breast cancer.

Ben-Hur et al. (2002) investigated the presence of lymphoid elements in the tumourigenesis of human breast cancer. They examined the lymphocytic and macrophage content of several different types of breast cancers (fibrocystic disease, fibroadenoma, carcinoma in situ, infiltrating ductal and lobular carcinoma with high lymphoid infiltration, and infiltrating ductal and lobular carcinoma with lymphoid depletion). The two benign conditions showed little lymphoid and macrophage infiltration whereas the other malignant conditions, with the exception of the lymphoid depleted tumours, showed an extensive lymphoid infiltration which were predominantly T-cells. Results demonstrated that the host's reaction to the disease was reflected in high correlations between the densities of the lymphoid cellular elements as tumourigenesis evolved (increased lymphocyte numbers as tumours progressed). It also showed that the majority of the stromal immunocompetent cells were T- killer cells which acted as a reservoir as it were that eventually crossed into the breast parenchyma and joined T helpers and B lymphocytes in the anti-tumour immune response. In later stages of cancer the response was exhausted as shown by the decrease in lymphocytes and macrophages in the late stage tumours (Ben-Hur et al. 2002).

The different patterns of inflammation and or inflammation associated with different histological types of carcinoma have been ignored somewhat, probably due to the fact that the significance of inflammation in breast cancer is controversial. Lee et al. (1996) looked at the pattern of inflammation in 123 invasive mammary carcinomas (including 46 lobular), and characterised the

inflammatory cells by immunohistochemistry. They found different patterns of inflammation in ductal and lobular carcinoma. Diffuse inflammation was seen more in ductal carcinoma, particularly of high grade, and was predominantly composed of macrophages and T cells. Perilobular inflammation was seen most frequently in lobular and high-grade ductal carcinomas, particularly at the tumour edge. Perivascular inflammation was also largely at the tumour edge, but was not more common in any tumour type. In contrast to the diffuse inflammation, the perivascular and perilobular inflammation was composed of T and B cells (Lee et al. 1996).

The effect of mast cell presence in breast cancer stroma is also unclear. Aaltomaa et al. (1993) counted the number of stromal mast cells in 187 breast carcinomas specimens. The numbers present were studied in relation to the clinical, histological and quantitative prognostic factors and survival characteristics. Results showed that a high mast cell count (over 10 g per mm^2 of tumour stroma) was related to a favourable prognosis in their survival analysis (Aaltomaa et al. 1993). Fisher et al. (1985) also counted the number of mast cells within and at the periphery of invasive breast cancers from 424 patients and related it to prognosis. No differences in 10 year disease-free survival were detected in patients without mast cells and those exhibiting varying numbers of such cells. Their information indicates that identifiable mast cells do not represent a prognostic pathologic discriminant in patients with breast cancer. However, this does not unequivocally exclude a role of mast cell secretory products, since only intact and not degranulated or disrupted forms of these cells can be counted (Fisher et al. 1985). These two studies contrast as to whether the presence of mast cells is favourable or unrelated to breast cancer prognosis.

In summary, the effect of the presence of immunocompetent cells in the stroma of breast is not clear. However they do interact with adjacent stromal cells (fibroblasts and endothelial cells) and epithelial cells and so further investigation is merited. In order to construct a more realistic breast cancer model, immunocompetent cells should be incorporated, however this will only be useful once their role in tumourigenesis is clarified.

1.4.2 Extracellular matrix

The extracellular matrix (ECM), often referred to as connective tissue, is a complex meshwork of glycoproteins and proteoglycans that determines tissue architecture and also modulates breast tissue homeostasis *in vivo*. It is produced in collaboration between stromal fibroblasts and epithelial cells to provide structural scaffolding for cells, as well as contextual information. It is composed of 3 major classes of biomolecules: 1) structural proteins: collagen and elastin, 2) specialized proteins: e.g. fibrillin, fibronectin, and laminin and 3) proteoglycans: these are composed of a protein core to which is attached long chains of repeating disaccharide units termed glycosaminoglycans forming extremely complex high molecular weight components of the ECM.

The breast stroma and its cells communicate with the epithelial cells through the ECM with an important function of stromal cells being the deposition and remodelling of the ECM (Noel and Foidart 1998). Constituents of the ECM produce a large variety of specific signals which directly influences cell growth, migration, morphology, proliferation, differentiation, and biosynthetic activities (LaFlamme and Auer 1996, Weaver et al. 1997). It has now clear that the ECM is made up of specific structural modules that encode information

interpreted by cells through interactions with specific plasma membrane receptors, mostly of the integrin family (LaFlamme and Auer 1996). Integrins, a family of adhesion receptors used by cells to interact with their ECM, function as signalling receptors integrating information from the ECM and other environmental cues including growth factors and hormones. Signals triggered by integrins direct cell adhesion and regulate other aspects of cell behaviour including cell proliferation and differentiation as well as determine cell survival. In addition, the ECM plays an essential role in normal epithelial cell survival, and loss of contact with the ECM and its components results in apoptosis termed anoikis (Frisch and Francis 1994). For example several studies have revealed the importance of the basement membrane in the morphogenesis and differentiation of mammary epithelial cells (Roskelley and Bissell 1995, Weaver et al. 1997).

Although the precise mechanisms of matrix effects remain to be elucidated, there is considerable evidence that the ECM influences cell properties and behaviour by modification of cytoskeletal organization and activation of second messenger and protein kinase pathways (LaFlamme and Auer 1996). In addition, the ECM is a site of sequestration of various factors that are likely to affect cell activity such as growth factors, mobility factors, natural proteases and their inhibitors.

In addition to functioning as a structural scaffold the ECM produces matrix macromolecules for cell adhesion and migration to exert profound effects on cell behaviour by a variety of mechanisms. Common matrix constituents elicit signal transduction cascades as a result of their ligation by integrin receptors, and that these matrix induced signalling networks share many common pathways with their cytokine induced counterparts (Juliano 1996). Cytokines and matrix macromolecules have a bi-directional co-modulatory effect on each other's

synthesis. Cytokines affect the expression of matrix molecules, matrix receptors and matrix degrading enzymes, whereas matrix macromolecules modulate the synthesis of cytokines and their respective cell surface receptors (Schor 1994). In addition, the effects of cytokines on cell behaviour may be mediated by the matrix macromolecules whose synthesis they regulate. In other words the observed cellular response to a cytokine may be a secondary consequence of the bioactivity of the matrix macromolecules whose expression is under primary cytokine control. Also, matrix macromolecules bind cytokines and present them to cell surface receptors in a functionally optimal state (Streuli 1999). Finally, matrix macromolecules modulate cellular response to cytokines (i.e. the nature of the macromolecular matrix to which the cells are adherent determines cellular response to cytokines) (Schor 1994, Lee and Streuli 1999).

Breast carcinomas are characterized by a stromal reaction that consists of modifications in the composition of both the cellular elements (infiltration of fibroblastic cells, endothelial cells, inflammatory cells) and the ECM. Disruption of communication between the epithelium and stroma can lead to alterations in the ECM which can both induce and promote breast cancer. Alterations in cell-ECM interactions are a consistent feature of mammary tumours *in vivo* and in culture (Petersen et al. 1992, Bernfield et al. 1993, Zutter et al. 1993, Sympson et al. 1994, Bergstraesser and Weitzman 1993, Howlett et al. 1995). The stromal cells surrounding tumours have increased or altered expression of many ECM proteins which change with cancer progression (Ronnov-Jessen et al. 1996, Rabinovitz and Mercurio 1996, Ziober et al. 1996). Tumour associated fibroblast stroma has been shown to produce ECM molecules such as fibronectin and tenascin that influence cell adhesion and proliferation. Tenascin, known to reduce epithelial cell adhesiveness, is absent in normal breast, its expression

being restricted to embryonic and malignant tissues (Kadar et al. 2002). Thus we can see that the ECM is involved in both normal and pathological processes.

Desmoplasia is a good example of ECM remodelling during tumourigenesis. The desmoplastic stromal reaction is characterized by both quantitative and qualitative modifications in the composition of connective tissue matrix (Dvorak 1986, Gregoire and Lieubeau 1995, Wernert 1997). An excessive accumulation of ECM components including different types of collagen (types I, III, V), fibronectin, elastin and proteoglycans is often observed. In addition, non basement membrane type IV collagen was shown to be increased in elastotic breast tumour tissues (Verhoeven et al. 1990). Interestingly bone sialoprotein, a bone matrix protein involved in hydroxyapatite crystal formation is ectopically expressed in human breast cancers and results in the formation of microcalcifications which can be detected in early lesions (Bellahcene et al. 1996).

1.4.3. Tumour-stromal interactions

To achieve a better understanding of cancer we must study the function of the complex tumour microenvironment and not just focus on the genes involved. It is clear now that the heterotypic interactions between cancer cells and their recruited normal neighbours as well as the associated ECM have a crucial role in tumour progression and so it is no longer sufficient to just understand intracellular signalling (Shekhar et al. 2001).

As described above breast carcinomas are composed of not only tumour epithelial cells but also of associated endothelial cells, fibroblasts, macrophages, and lymphocytes (Ghosh et al. 1980, Gregoire and Lieubeau 1995). Several lines

of evidence indicate that breast stromal cells play a central role, via the ECM (remodelling, growth factors, proteases etc.), in tumour invasion and dissemination (Camps et al. 1990, Picard et al. 1986, Grey et al. 1989). Perturbations in crosstalk between the mammary epithelium and stroma together with alterations in the ECM can, therefore, lead to the induction and promotion of breast cancer.

Stroma is well known to support and respond to tumourigenesis, directly influencing the tumour process (Noel and Foidart 1998). This was demonstrated by culturing primary breast tumours cells, in combination with stromal elements (fibroblasts), in 3-D collagen type 1 matrices. The addition of stromal fibroblasts caused the tumour to spread and become invasive, with greater concentrations of stromal elements having a proportional effect on tumour growth (Ronnov-Jessen et al. 1995). The exact mechanism of tumour-stromal interactions is not clear at present but it has now been established that fibroblasts by modulating the ECM, and/or by their direct influence on tumour cells are crucial.

As the predominant cell in the stroma, the fibroblast is responsible for the elaboration of most of the components of the ECM in addition to several other important factors (proteases, matrix proteins, chemokines, cytokines and growth factors). Proteases that remodel the ECM play an important role in the progression of neoplasia (Werb 1997). Excessive protease activity can lead to major changes within the microenvironment of tumour tissue to promote cell migration, and it thereby contributes to metastasis. Moreover, subtle changes in the levels and activities of proteases can expose cryptic sites in ECM molecules that alter integrin usage, and release matrix-bound growth factors, which both potentiate proliferation and survival of tumour cells, and induce angiogenesis (Streuli 1999, Vu et al. 1998, Stetler-Stevenson 1999). Thus, several of the

hallmarks of tumour progression occur as a result of alteration in protease activity within the extracellular environment of a progressing tumour (Hanahan and Weinberg 2000). Direct evidence that inappropriate expression of both matrix metalloproteinases (MMPs) and serine proteases, the two main classes of ECM-degrading proteases, is involved in tumour progression comes from mis-expression studies in genetically altered mice (Wilson et al. 1997, Johnsen et al. 1998, Sternlicht et al. 1999).

The regulation of ECM-degrading proteases is normally kept under tight control. Tissue inhibitors of MMPs suppress the activity of MMPs, whereas serpins are a class of serine protease inhibitor. The expression of these protease inhibitors is closely regulated in developmental morphogenetic processes. For example, tissue inhibitors of MMPs control ECM remodelling during mammary gland development, suppressing excess MMP activity and therefore preventing matrix remodelling from occurring prematurely in post-lactational involution (Talhouk et al. 1992, Fata et al. 2001). However, there can be severe consequences if the expression of matrix proteinase suppressing enzymes is misregulated. Just as over-expression of MMPs and serine proteases can contribute to carcinogenesis, so can down-regulation of their inhibitors. Levels of one such inhibitor of serine proteases, namely maspin, are frequently reduced or even absent in invasive cancer (Zou et al. 1994).

Information from ultrastructural, immunohistochemical and biochemical analyses have demonstrated that the mammary stroma is altered in critical aspects during the neoplastic process. Several studies have documented an acquired expression of α-smooth-muscle actin, vimentin, smooth muscle myosin, calponin, tenascin and desmin in tumour stroma (van den Hooff 1988, Ronnov-Jesson et al. 1996). Myofibroblastic differentiation of fibroblasts with the

activation of a partial smooth muscle differentiation programme and the excessive elaboration of collagen types III and V in the area of a neoplastic lesion occurs in the desmoplastic reaction. The above described proteins are also often expressed as a response to wound healing or inflammation, as myofibroblasts orchestrate the repair response (Sappino et al. 1990). In addition, the distribution of laminin, a molecule critical for the architectural integrity of undisturbed tissue, is reduced and altered in ECM found associated with malignant cells (van der Hooff 1988). More recent studies documented alterations in dipeptidyl peptidase IV, MMPs, inhibitors of metalloproteinases, growth factors and collagens (Basset et al. 1990, Atherton et al. 1992, Garin-Chesa et al. 1990, Nakamura et al. 1997a, Rasmussen and Cullen 1998). These numerous alterations in the mammary stroma drive the tumourigenic process.

Growth and progression of breast cancers are accompanied by increased neovascularization (angiogenesis) (Ausprunk and Folkman, 1977, Nicosia et al. 1982, Hanahan 1988, Hanahan and Folkman 1996). Most of the available data is concerned with fibroblast-epithelial cell interactions in breasts cancer. However endothelial cells in the stroma also play a major role in tumourigenesis in particular angiogenesis (Rak et al. 1996). Accumulating evidence points to the local environment particularly interaction with adjacent cells and ECM, as being a major factor in endothelial cell differentiation (Kubitza et al. 1999). Heterogeneity of mature endothelial cell phenotypes is proof different organs produce different endothelial phenotypes. The study of how changes in stroma can affect tumour formation represents a new direction in tumour therapeutics. A variety of factors, including hypoxia and genetic changes in the tumour epithelial cells, contribute to increased production of angiogenic factors (Boudreau and Myers 2003). Furthermore, cells within the activated tumour stroma also

contribute to the increase in production of vascular endothelial growth factor and other angiogenic factors, including basic fibroblast growth factor and platelet-derived growth factor (Kern and Lippman 1996, Kushlinskii and Gershtein 2002). The contribution of the microenvironment to tumour-induced angiogenesis is underscored by findings that breast tumours implanted into different tissue sites show marked differences in the extent and nature of the angiogenic response (Boudreau and Myers 2003). These findings have important implications for designing anti-angiogenic therapies.

The fact that while normal stroma can be protective in delaying or preventing tumour formation (Shekhar et al. 2001, Hayashi and Cunha 1991, Cooper and Pinkus 1977), abnormal stroma can promote tumourigenesis, underlines the crucial importance/role of stroma in tumourigenesis. Shekhar et al. (2001) showed that, while normal reduction mammoplasty fibroblasts could inhibit or retard the morphological transformation of both normal MCF10A and preneoplastic EIII8 breast epithelial cells, respectively, tumour-derived breast fibroblasts could override or enhance genetic constraints imposed by MCF10A or EIII8 cells, respectively, causing them to undergo ductal-alveolar morphogenesis. However the stromal (cell) effect may be organ type specific. A study by Moinfar et al. (2000), using a 3-D cell culture model with Matrigel, showed that only breast fibroblasts, but not soft tissue sarcoma-derived malignant mesenchymal cells, normal lung, or embryonal 3T3 fibroblasts, could confer morphogenesis inducing effects on primary mammary epithelial cells. Therefore, although the cultures were performed in Matrigel, a reconstituted basement membrane matrix containing a variety of ECM molecules, induction of breast epithelial growth and morphogenesis appeared to be mediated by the ECM that was laid down by organ-specific mesenchyme.

A recent report has shown that stromal alteration(s) can precede the malignant conversion of tumour cells (Moinfar et al. 2000). Following examination of multiple human breast tumours they found a high frequency (up to 75%) of alleic loss (loss of heterozygosity) in mesechymal mammary stromal cells (fibroblasts) close to the cancer cells which were not present in normal breast tissue. Moreover, the genetic alterations found in the tumour associated fibroblasts preceded the genotypic changes found in the epithelial cells. In an alternative approach, a study by Olumi et al. (1999) reported, using both an *in vitro* co-culture and an *in vivo* tissue recombinant system, that prostate carcinoma-associated fibroblasts but not their normal equivalents were able to stimulate tumourigenesis in non-tumourigenic prostatic epithelial cells. Oncogenic signals from tumour derived fibroblast can change non-malignant epithelial cell populations to malignant ones. Furthermore only transient stimulation is required to transform cells into tumourigenic lesions. This evidence from a similar cancer system clearly shows that the surrounding stroma has a functional role in tumour development. Similarly in a mouse mammary model, only irradiated mammary stroma of the fat pad and not its normal equivalent was shown to give rise to tumours when non-tumourigenic mammary epithelial cells grafted into the mammary fat pad (Barcellos-Hoff and Ravani 2000).

Oncogenic mechanisms of stromal cells

Genetic change in the form of 'loss of heterozygosity' (LOH) may be the trigger in the development of breast cancer. A study by Kurose et al. (2002) analyzed 50 breast tissue samples taken from epithelial and stromal tumours looking for LOH in two known tumour suppressor genes, TP53 and PTEN. Altered forms of both

genes have been associated with the development of breast cancer. Half of the tumour samples contained mutated TP53 genes: 11 samples had mutations in the epithelium alone, nine had mutations in the stroma alone and five had mutations in both tissue layers. Nearly one-third of the breast cancer samples contained PTEN mutations. However these alterations were found in either the stroma or the epithelium; only one breast tissue sample contained PTEN mutations in both tissue compartments. In addition to finding variations in the frequency and distribution of genetic mutations, the researchers also discovered that, with the exception of two samples, PTEN and TP53 mutations did not occur together in the same tissue compartment. Yet when genetic mutations were found in both tissue compartments, at least three of the samples had different types of mutations between epithelium and the respective stroma. This effectively ruled out the possibility of cross contamination between the tissues. Their exclusive nature and the presence of discordant mutations even within the same gene suggest that genetic alterations occur independently in the stroma and the epithelium. Put simply, a mutation could first occur in the stroma, rather than in the epithelium. These mechanisms are not limited to breast cancer with similar genetic changes occurring in other solid tumours.

The knowledge that stromal cells have the ability to stimulate oncogenesis has been taken a step further by recent data suggesting that stroma may develop oncogenic properties independently even in the absence of a tumour. Four possible mechanisms are; carcinogen induced mutations, use of enzymes (matrix metalloproteinases), recruitment of inflammatory markers and viral alterations of stromal signals.

I. Carcinogen induced oncogenesis

Carcinogens in addition to promoting tumourigenesis by directly damaging epithelia, can also promote tumourigenesis by altering the surrounding stroma. Barcellos Hoff and Ravani (2000) showed that irradiated murine mammary stroma promoted tumour progression of transformed normal murine epithelial cells. Also these mammary epithelial cells developed tumours faster and more often and reached a greater size than the same cells transplanted with un-irradiated stroma. This effect has also been clearly documented with skin and bladder tissue where carcinogen treated stroma combined with untreated epithelial cells resulted in enhanced tumour formation (Billingham et al. 1951, Hodges et al. 1977, Uchida et al. 1990).

II. Recruitment of matrix metalloproteinases (MMP)

MMPs have many functions and activities. They can degrade ECM as well as being involved in the promotion of inflammatory responses, normal tissue remodelling, wound healing and angiogenesis. These enzymes, however, also have an important function in malignancy. The sustained presence of these proteinases in the tumour microenvironment, produced both by cancer and activated cells, leads to the degradation of normal ECM. Breakdown of ECM stimulates both proliferative and apoptotic mechanisms, which can lead to the selection of apoptosis-resistant carcinoma cells and an enhanced invasive potential (Sethi et al. 1999, Mitsiades et al. 2001). In some tumours direct association of MMPs with specific ECM receptors provide spatial control of MMP activity and directional signals to the invading tumour cells. Alterations in epithelial tissues together with disrupted intercellular signals, found in wounding for example, can promote inflammation. This process when unregulated or persistent can lead to upregulation of enzymes such as MMPs by stromal

fibroblasts that can disrupt the ECM causing invading immune cells to overproduce factors that promote abnormal proliferation. Consequently this can cause epithelial cells present in the microenvironment, which already possess tumourigenic potential, to be activated or promote genomic instability within nearby epithelial cells leading to tumourigenesis.

Emerging new data underlines the fact that matrix remodelling enzymes like MMPs can affect mammary stroma, so that it directly influences the carcinogenic potential of neighbouring epithelial cells. Several studies have indicated that over activity of these MMPs can not only promote carcinogen induced mammary tumourigenesis but also spontaneous mammary tumour formation. Conversely reduced MMP activity levels can even suppress tumourigenesis. Data has suggested that transgenic expression of the metalloproteinase MMP-3/stromelysin-1 in murine mammary epithelia is sufficient for tumour formation. Prior to tumour formation stromelysin-1 expression leads to the development of reactive stroma implying alterations in the stroma have an important role in tumour formation. Also tumours formed after a given time period lose the transgenic expression of MMP-3 to gain specific genomic alterations (Sympson et al. 1995, Rudolph-Owen and Matrisian 1998, Thomasset et al. 1998, Sternlicht et al. 1999). Also a new gene coding for the enzyme stromelysin-3 (ST-3), has been identified, that is expressed specifically in stromal cells surrounding invasive breast carcinomas. ST-3 is a new member of the family of metalloproteinase enzymes which degrades the ECM. ST-3 is postulated to play an important role in the progression of epithelial malignancies (Schor and Schor 2001). Two very important facts to consider have arisen from these studies; a) in the absence of a previously formed

tumour, altered MMP expression is causative of the neoplastic phenotype, and b) aberrant expression of a mutated MMP can generate genetically altered cells.

III. The recruitment of inflammatory cells

Tumour development, similar to inflammation, involves the recruitment of inflammatory cells. Research using a transgenic mouse model that expresses the human papilloma virus-16 in basal keratinocytes to study tumour development demonstrated inflammatory stromal cell infiltration during tumourigenesis. The infiltrating stromal cells were mast cells, observed to activate matrix metalloproteinase-9 by the release of serine proteins. These findings were significant because tumour models with mast cell deficient mice coincided with a lack of pre-malignant angiogenesis, an important marker for tumourigenesis. This suggested that neoplastic progression involves the exploitation of an inflammatory response to tissue disruption or aberrations (Coussens et al. 1999, Coussens et al. 2000)

IV. Stromal signals transformed by viruses

The study of viral associated cancers has raised the possibility that viral affected non malignant stromal cells may stimulate tumourigenesis in normal cells which eventually produce tumours. For example, multiple myeloma research has shown that viral sequences for the cytokine interleukin-6 (IL-6), coded by the human herpes virus, were present in the stromal dendritic cells. As dendritic cells are well known to influence the growth control of haemopoietic cells in a paracrine pathway, it was suggested that abnormal regulation of IL-6 expression in stromal cells may promote tumourigenesis in haemopoietic cells leading to multiple myeloma. Also a similar theory has been proposed regarding HIV and Karposi's sarcoma (McGrath et al. 1995). Here IL-6 produced by virus infected stromal cells, macrophages, is thought to trigger the initial sequence for

tumourigenesis in the neighbouring cells. In both cases virus and viral sequences were absent from the malignant cells. This evidence together with the presence of viral expression in tumour associated stromal cells; macrophages and dendritic cells provides good evidence of the correlation between viral induced cytokine expression in stromal cells with the appearance of transformed cells (Coussens et al. 1999).

With the increasingly recognised fact that tissue microenvironment is very important in the regulation of normal cell function, we need to develop models to test how structural and biochemical cues provided by the tissue microenvironment play critical roles in the suppression of tumourigenic phenotypes (Bissell et al. 1982). These extracellular cues themselves influence intracellular gene expression that in turn results in fundamental alterations in the composition of the microenvironment (Bissell et al. 1982). Inappropriate alterations of cell-microenvironment interactions can result in abnormal cellular behaviour, as seen in tumour progression (Ronnov-Jessen et al. 1996).

Targeting stroma as potential therapies

Vascular endothelial cells are already the focus of intense therapeutically relevant research, but tumour-associated fibroblasts, macrophages, neutrophils, lymphendothelial cells, etc. provide additional largely unexplored territory in the ongoing search for efficient countermeasures against invasive cancer.

Better understanding of breast cancer biology has lead to the realisation that tumour stromal interactions are also of crucial importance in cancer biology. Understanding the effect these interactions have on cell growth and morphogenesis as well as the complex signalling pathways between the two offers the possibility of revealing new potential therapeutic targets, such as those that target breast cancer stroma itself. Stroma appears to be the dominant factor

in modulating and controlling epithelial morphogenesis and mitogenesis, and therefore direct targeting of stroma may result in a more efficient method of tumour regression and treatment (Camps et al. 1990, Picard et al. 1986, Grey et al. 1989). For example it is now recognized that tumour fibroblasts, which behave differently to mature tissue fibroblasts, offer a potential target for anti-tumour therapy (Mersmann et al. 2001). Moreover, the study by Moinfar et al. (2000) reported that stromal alterations (fibroblasts) precede the malignant conversion of mammary epithelial cells.

Also the discovery of a cell surface antigen called fibroblast activation protein (FAP), present on reactive tumour stromal fibroblasts found in epithelial cancers and in granulation tissue during wound healing, presents a potentially attractive stromal target. Reactive stromal fibroblasts found in injured/healing tissue and epithelial tumours characteristically induce the formation of FAP a type II integral membrane protein that belongs to the serine protease gene family (Garin-Chesa et al. 1990, Niedermeyer et al. 1998, Scanlan et al. 1994, Park et al. 1999). It is absent from most normal adult human tissues, with resting fibrocytes in normal tissue generally lacking detectable FAP expression (Garin-Chesa et al. 1990, Rettig et al. 1988). FAP-positive stromal fibroblasts are seen in the stroma of over 90% of malignant breast, ovarian, colorectal, lung, skin and pancreatic tumours. A proportion of bone and soft tissue sarcoma tumour cells are also FAP positive (Rettig et al. 1988). Some reports have shown that FAP expression may be an independent prognostic factor in certain tumours such as breast cancer (Ariga et al. 2001).

The targeting of antigens selectively expressed on tumour stroma or tumour capillary endothelial cells with monoclonal antibodies has emerged as a novel targeting approach for immunotherapy (Burrows and Thorpe 1994, Old

1996, Scott and Cebon 1997). The FAP antigen, mentioned above, is an attractive antigenic target because it has broad applicability to several common and as yet poorly treatable cancer types, and it also displays a restricted expression pattern in normal tissue (Garin-Chesa et al. 1990). Welt et al. (1994) using the F19 monoclonal antibody to FAP and recently Tahtis et al. (2003) using a different ^{131}I-labeled humanized anti-FAP monoclonal antibodies (BIBH-7) to target human FAP-expressing tumour xenografts, established in a human skin/SCID mouse chimeric model, demonstrated specific binding to the stromal components of the tumour.

However as animal models and *in vivo* studies are costly and complex with problems of unpredictable characteristics and ethical approval, physiological 3-D model systems using human cells to create an authentic model is an obvious choice. With the recent advances in tissue engineering 3-D cultures are now more morphologically and functionally differentiated (lobulo-alveolar structures that express and secrete milk). In addition 3-D models can be produced in larger quantities as a result of greater control over culture composition, new bioreactors and greater choice in the method of inducing 3-D growth. The great hurdle impeding model development is a lack of a readily available supply of organ-specific stromal cells such as fibroblasts and endothelial cells. However, recent advances in genetic engineering have allowed the generation of cells with immortal growth to be produced.

1.5 Immortalization

1.5.1 Immortalization of normal human cells

Normal human somatic cells undergo a limited number of divisions before entering an irreversible growth-arrest state defined as senescence (Hayflick et al. 1961). However genetic alterations can allow some cells to escape senescence. These cells pass through a 'crisis' phase, which is usually accompanied by extensive cell death, after which they become immortalized and continue to proliferate indefinitely. They may then progress and acquire the characteristics of cells that are fully cancerous or 'transformed' through additional mutations in tumour-suppressor genes or oncogenes (genes that cause cancer when mutated). Unlike normal cells, transformed cells can survive without growth factors, and they do not need to be anchored to a solid support. The acquisition of unlimited proliferative potential defined as true immortality is probably a critical step during tumourigenesis (Newbold et al. 1982).

In normal human somatic cells the progressive shortening of telomeres with each cell division has been demonstrated as the single key factor determining the length of the cell cycle (Allsopp et al. 1992). Telomeres are short repetitive DNA sequences that define the ends of chromosomes and are synthesized by the enzyme telomerase (Blackburn and Gall 1978, Harley et al. 1990). With each round of cell division, the DNA at each telomere gets progressively shorter (~50 base pairs per division) (de Lange 1994), leading, in turn, to shortening of the whole telomeric structure. Adult somatic cells lack functional telomerase, a multi-component enzyme comprising a template RNA plus an essential catalytic subunit of human telomerase (hTERT), telomeres are not regenerated (Zhu et al. 1999, Meyerson et al. 1997, Lingner et al. 1997,

Harrington et al. 1997, Kilian et al. 1997, Nakamura et al. 1997b, Counter et al. 1998). Several observations suggested that this process of telomeric erosion could impose the limits on cellular lifespan. Normal human tissues have low levels of telomerase. In contrast, many tumour cells and immortalized cell lines have up-regulated telomerase activity, suggesting a mechanism by which cells may escape normal ageing controls. To overcome telomere shortening and bypass senescence and crisis, it has been demonstrated that artificial expression of hTERT can immortalize normal human cells (fibroblasts and epithelial cells) (Bodnar et al. 1998, Vaziri and Benchimol 1998, Yang et al. 1999, Ouellette et al. 2000). However, O'Hare et al. (2001) reported that hTERT alone could not immortalize primary human mammary fibroblasts or endothelial cells. On the other hand a combination of hTERT and the oncogene SV40 Large T antigen when transduced by amphotropic retroviruses into primary human mammary endothelial cells and fibroblasts consistently generated stable immortal human cells in a reproducible and convenient manner (O'Hare et al. 2001) and thereby providing potential components of a multi-compartment tumour-stromal model system. The value of these cells very much depends on whether they retain normal cell function and do not exhibit transformed characteristics.

Transformation

Tumourigenesis is a multi-step process involving the accumulation of genetic alterations and mutations, of genes controlling cell proliferation and survival, which allow tumour cells to overcome cellular hurdles on its way. Normal human cells have a number of intrinsic mechanisms, involving molecular 'gatekeepers', to protect them from going out of control and forming tumours. For example, mutations of the p53 tumour suppressor gene have been reported to be present in over half of all breast tumours (Ozbun and Butel 1995). Breast

cancers also frequently carry mutations that deregulate the retinoblastoma protein (pRB) pathway including loss of expression of pRB or $p16^{INK4a}$ (Varley et al. 1989, Brenner et al. 1996) or amplification or over-expression of cyclin D1 (Gillett et al. 1994). Breast carcinoma cells also commonly acquire alterations in the Ras-signaling pathway (Clark and Der 1995), which may occur by several mechanisms, most notably amplification or over-expression of the ERBB2 gene (Slamon et al. 1989). C-*myc* is also frequently amplified or over-expressed (Escot et al. 1986).

It has been shown that hTERT immortalized cells (normal human mammary epithelial cells, mammary fibroblasts and hepatocytes) (Hahn et al. 1999, Elenbass et al. 2001) can be fully transformed into a malignant phenotype by adding two well known oncogenes. The first is an activated ras gene (H-rasV12), which although is rarely mutated in breast cancers (~5% of cases) (Clark and Der 1995) is mutated in many human tumours. The second is a viral oncoprotein, the simian virus 40 large-T antigen. This protein is involved in transforming normal host cells into tumour cells by inhibiting two central growth-control factors; the tumour-suppressor proteins p53 and pRB (Varley et al. 1989, Brenner et al. 1996, Ozbun and Butel 1995, Hahn et al. 1999). When Hahn et al. (1999) introduced the ras oncogene alone, the human cells became senescent (as previously reported for mouse cells). This senescence could not be overcome by combining ras with introduction of hTERT. But cells expressing large-T and hTERT became immortalized, and when these proteins were combined with ras expression increased growth of the cells was observed (Hahn et al. 1999). Hahn et al (1999) demonstrated that these different cells formed colonies in soft agar, a simple, *in vitro* test for transformation and were tumourigenic in nude mice. The results clearly showed that only the combination

of all three genetic elements was sufficient to cause efficient tumour growth. Therefore in tumourigenic human cells, mutations/alterations causing telomerase to become active can cooperate with other mutations in oncogenes to induce transformation.

The findings by Hahn et al. (1999) indicated that at least 3 distinct pathways were involved in genetically manipulating normal human cells so that they are transformed into a fully tumourigenic cell. These are: 1) expression of the telomerase enzyme (hTERT) to overcome telomere shortening and maintain length, 2) expression of the large-T protein inactivating both the pRB and p53 pathways and 3) the expression of the *ras* oncogene to induce transformation to a cancerous state.

1.6 *In vitro* tumour models

Since human *in vivo* models are not ethically available, the development of more relevant models has relied upon *in vitro* systems. These have been based on both cell lines derived from breast tumours and normal human mammary epithelial cells. Problems of authenticity with *in vitro* systems are caused by acquisition of any number of additional events, all of which contribute to transformation.

1.6.1 Two and three dimensional tissue culture models

At present *in vitro* tissue culture of breast cancer cell lines is the most widely used experimental model system of breast cancer (Suzuki et al. 2001). Although simple and convenient to use, this model is far from satisfactory in that it represents a very artificial environment. Important physiological processes that are lacking *in vitro* include three dimensional (3-D) structure, tumour-stromal

interaction and angiogenesis (presence or lack of circulating activating/inhibiting factors) with its influence on tumour development and tumour growth, and metastatic spread to other tissues.

Two dimensional culture models

Until the last 10 years, 2-D monolayer cultures together with organ cultures have been the most popular *in vitro* models for breast cancer research. Monolayer culture models are easy and convenient to set up with good viability of cells in culture but they lack some major elements such as the 3-D microenvironment of intact tissue in particular stroma; this being important as stroma of the mammary gland accounts for greater than 80% of the resting breast volume (Drife 1996). Due to their lack of structural architecture they are also faced with transport limitations resulting in the quality of the monolayer being strongly influenced by the physicochemical properties of the support. Moreover, growth of normal epithelial cells as monolayers on artificial supports leads to partial loss of the original epithelial cell characteristics resulting in observations that normal epithelial cells when grown in 2-D monolayers are highly plastic and express many characteristics displayed by tumour cells *in vivo* (Bissell 1981, Petersen et al. 1992). In addition, not all normal epithelial cell types are able to adhere and grow well on artificial substrata. In contrast, the microenvironment of tumours remains intact in organ cultures. This is of vital importance as cell-cell, cell-matrix interactions and interstitial fluid affect differentiated cell function within the 3-D environment. However organ culture is not without its own problems; with difficulty in obtaining specimens and poor viability of the tissues in culture being major obstacles encountered.

Three dimensional tissue culture models

It has not been long that 3-D culture models, in the form of multicellular spheroids, have replaced 2-D models as more representative and complex *in vitro* culture models. The advantages 3-D cultures have over 2-D monolayers are their well defined geometry, which makes it possible to directly relate structure to function and in turn enable theoretical analyses. Their close resemblance with micrometastases and initial avascular regions of metastatic tumours augments their authenticity. In addition, 3-D cultures are composed of cells with different phenotypes such as proliferating, non-proliferating and necrotic cells very similar to the situation within intact human tumours. Phenotypes present, therefore, more closely resemble those present *in vivo*. Cellular heterogeneity within these tumour models caused by mass transport limitations, resembling the multiple phenotypes found in solid epithelial tumours, is far more realistic than the cellular homogeneity found in monolayer cultures. The majority of 3-D cultures used were in the form of multicellular tumour spheroids.

3-D multicellular tumour spheroids

Multicellular tumour spheroids are very small spheroids consisting of cells (epithelial or a mixture of epithelial and stromal cells) organized in a 3-D spheroidal arrangement. Many cells types, present in 3-D multicellular spheroids, were found to assume a more or less normal cellular architecture and exhibit gene expression profiles that were reflective of an authentic differentiated phenotype (Stoker et al. 1990, Kleinman et al. 1987). 3-D spheroids also contain extensive ECM that differs in the relative amount and assembly from the corresponding monolayer cultures. The complex 3-D network of cell-cell and cell-matrix interactions is not only relevant in the penetration and action of drugs but it also affects the distribution and function of physiologically occurring

factors. These biological effectors such as hormones and growth factors fundamentally determine the regulatory mechanisms of cell growth, differentiation and death. For example, human mammary epithelial cells, isolated from reduction mammoplasty, grown in culture on reconstituted basement membrane form polarized acinus type structures capable of gland specific function such as milk production (Stoker at al. 1990). These results were also reproduced using primary mouse epithelial cells isolated from pregnant mice. However the same cells grown in a different substrate, Type 1 Collagen, show altered integrins, abnormal cellular polarity and disorganization emphasising the importance of matching cell type with appropriate substrata (Howlett et al. 1995). As mentioned above 3-D spheroids have been shown to recapitulate the drug sensitivity patterns of tumour cells *in vivo*. MDA-MB-231 breast tumour cells exhibited a much lower IC50 to cis-platinum when plated in monolayer cultures than as suspended spheroids. In a study by Ohmori et al. (1998) up-regulation of TGF-βs in breast tumour cells, has been shown to be protective against cytotoxic agents such as cis-platinum. Treatment of MDA-MB-231 spheroids, but not MDA-MB-231 monolayers, with cis-platinum induced up-regulation of TGF-β1 mRNA and protein which is highly predictive of the patterns of drug response of tumour cells *in vivo*.

With its many advantages over conventional monolayer cultures, 3-D cultures more closely resemble the *in vivo* situation with regard to cell shape and its environment. This is important as it is recognised that cell shape and environment can determine the behaviour and gene expression of the cell. For example, K-*ras* mutations frequently found in colorectal tumours, have been noted to occur more consistently during transition from early to intermediate

adenoma stage which coincides at what time tumours acquire 3-D growth patterns (Rak et al. 1995).

Heterologous 3-D co-culture models

Currently heterotypic co-cultures of luminal and myoepithelial cells; tumour and fibroblasts; and tumour and endothelial cells are available. This is important as human breast cancers are now well recognised as a heterogeneous mix of stromal and epithelial cells and also because it has been demonstrated that the presence of stromal cells promotes cell differentiation and structure formation. For example it has been reported that human mammary epithelial cells when injected alone, without stromal cells (fibroblasts), into mammary fat pads do not support complex differentiated structures (e.g. ductal structure formation (Sheffield 1988)).

Three mandatory requirements are necessary to develop human tissue; 1. co-localization of different cell types with cell-cell interactions and the exchange of growth factors and other biological effectors; 2. manufacture of an extracellular matrix (ECM) to provide a 3-D scaffold for mechanical stability and to regulate cell function; 3. synthesis of interstitial fluid containing the necessary nutrients and biological effectors required for tissue differentiation and maturation. Heterologous 3-D co-culture models incorporating stroma satisfy these three broad requirements and so produce credible and reliable preclinical tumour models.

As 3-D cultures support co-cultivation of multiple cell types, interaction between epithelial and stromal cells which regulate normal and neoplastic development can be studied. For example, the value of experimental culture models using normal epithelial cells often depends on the degree of epithelial cell polarization as well as other critical features observed in natural tissues,

including the formation of tight junctions, desmosomes and membrane interdigitations. As the control of epithelial cell polarity is one of the important functions of the stroma, inclusion of stroma and its elements is vital for maintaining authenticity. Indeed loss of epithelial cell polarity has been proven to lead to increased cell proliferation and tumourigenesis.

Breast tumour epithelial cells found in human breast cancer are apolar, exhibit disorganized cell-cell junctions, contain distorted cell nuclei, and fail to organize into ductal structures. Cell shape and tissue architecture is partly dependent on various adhesion molecules, and so any alterations in these molecules have been implicated in cancer progression (Alford and Taylor-Papadimitrou 1996). Heterologous 3-D models, therefore, can recreate or maintain these vital cell-cell adhesion interactions preserving the tissue architecture. For example, tumour cell adherence and migration in spheroid co-cultures reflect the distinct metastatic potential of (different) breast tumour cells. Given the crucial importance of the tumour-stromal interaction in cancer biology, a more realistic environment is created better able to elicit an *in vivo* like response to cancer therapies.

With the microenvironment of normal tissue and tumours being of crucial importance in the control of growth and development it is vital to clarify the origin of the ECM present within *in vitro* and *in vivo* model systems. The ECM produced and present in the majority of 3-D *in vitro* tumour model systems is predominantly of tumour cell origin. In heterologous (tumour-stromal) model systems incorporating stromal cells such as fibroblasts, ECM is also produced by these stromal elements. However the situation present *in vivo* with animal models is very different. In xenograft and transgenic models it is the host cells that produce the ECM; this being significant as it is of rodent non-epithelial tumour

tissue origin. This raises serious implications and issues regarding tumour-stromal cell interactions as well as cell signalling.

1.6.2 3-D cell culture techniques and models

The concept of the 3-D *in vitro* co-culture system was invented due to the many advantages it gave to cancer research. These models can reproducibly reflect the distinct invasive behaviour of breast tumour cells, mimic the cell-stromal interactions of breast carcinomas and allow for systematic investigation into the multiple unknown regulatory feedback mechanisms between breast tumour and stromal cells in a well defined 3-D environment. Multicellular tumour spheroids have a well organized spherical symmetry of morphological and physiological features including complex cell-cell and cell-matrix interactions resembling avascular tumour sites and or micrometastatic regions *in vivo* and thus are a very useful model in tumour biology.

The most popular methods used to manufacture 3-D spheroids are spontaneous aggregation, liquid overlay cultures, spinner flasks and more recently the NASA developed Rotary Cell Culture System (Kim et al. 2004). As an alternative, 3-D prefabricated biodegradable scaffold based cultures, and pre-engineered collagen scaffold have been developed. The scaffold based culture system is gaining in popularity as the 3-D matrix is used to promote multilayer growth of cells derived from breast cancers. The design of the Rotary Cell Culture System is a vast improvement on the spinner flask in that larger more morphologically and functionally differentiated breast cancer spheroids can be produced. The individual merits of each method are discussed in more detail in Chapter 5

1.7 *In vivo* model systems

1.7.1 Animal model systems

Many of our medical advances over the last century have been made using animals. Our extended lifespan, treatments for heart disease, cancer, diabetes, vaccinations, and many other things are all a testament to the benefits of animal models in research. Animal tumour models of the past have led us to the drugs of the present. Not only are they used in the discovery and screening of drugs, they are used for therapeutic development such as predicting drug efficacy, safety and learning about the mechanism of action. They also have major role in improving our understanding of tumour biology such as cell proliferation, progression and apoptosis. Furthermore, as patients bearing tumours of a given type do not necessarily provide a perfectively predictive model for others with the same tumour type, *in vivo* models may provide a more consistent and reliable indicator.

As with any disease prevention is the key, more so with breast cancer as it frequently occurs in women without established major risk factors. Great advances in other areas of breast cancer research have led us to some very exciting ideas as well as therapeutic strategies with enormous potential. Therefore we clearly need suitable models for initial preclinical testing of these new preventative and therapeutic strategies. Development and testing of preventative strategies is very important in understanding the controls of normal mammary gland development, differentiation and involution, and genetic and epigenetic changes/developments that are associated with precancerous proliferative breast disease. Further research into developing better *in vitro* and *in vivo* models of human breast cancer is urgently required. Animal models used in

preclinical studies should possess genetic and other biomarker abnormalities similar to their human counterparts. These include animal xenograft models, human precancerous cell lines, and *in vivo* human precancerous models for long term study.

Animal models are a vast improvement on *in vitro* cell culture systems which are limited in that they represent tissues taken out of the body. Cells in culture do not behave normally when removed from the blood, immune system, nervous system and neighbouring tissues that help make them what they are. In addition, even cells for culture must come from an animal initially. Sometimes, however, an animal model is too complex and a cell culture system or computer model allows a more effective study of a single aspect of the disease. Moreover, an animal model can't be created until patients are studied and the disease is described and defined.

With the massive explosion in therapeutic strategies becoming available an authentic breast cancer model is urgently required for preclinical trials. Only when an authentic and validated model becomes available can we reliably consider testing on human patients. Development of better *in vivo* models of human breast cancer is of crucial importance in the fight against breast cancer mortality.

The mutant nude mouse

The introduction of the immunodeficient (*Foxn1*) mice in the 1960s opened a brand new era of cancer research enabling the development of xenograft models of human breast and other cancers. The absence of a functional immune response has made it possible to transplant and grow foreign tissues with very little interference and complications. Prior to the discovery of the immunodeficient mouse, syngeneic transplantable mouse tumour systems or autochtonous rat

tumours were employed as the main or only tool in the development of anti-tumour agents. Sometimes complex procedures were used to render wild type mice immunodeficient, involving a combination of surgery (thymectomy), radiation and/or drug treatments; however the specialised techniques used meant that this approach was not widely used.

Today the nude (*Foxn1*) and severe combined immunodeficiency (SCID) mice, which have naturally occurring single gene mutations which affect their immune system, are the most commonly used research models in xenograft experiments. Nude mice have a naturally occurring autosomal recessive mutation in chromosome 11 which causes failure of hair growth and other defects, including thymic epithelial dysgenesis, which renders them T cell deficient (Kindred 1971). The thymus remains rudimentary and produces reduced numbers of mature T cells. This means nude homozygotes (animals with identical mutant genes at corresponding chromosome loci) do not reject allografts and often do not reject xenografts (tissue from another species). The discovery that human neoplasms (tumours) could be grown in nude mice was immediately recognized as an important research tool. Thus, the spontaneous mutation of *Foxn1* among laboratory mice was a serendipitous development that led to the nude mouse becoming the first animal model of a severe immunodeficiency. The first successful transplantation of a human malignant tumour into nude mice was reported in 1968 (Pantelouris 1968). In the decades since, the nude mouse has been widely utilized by researchers studying factors regulating transplantable human tumour growth and cancer metastasis. Although it lacks T cells, the nude mouse has a normal complement of bone-marrow-dependent B cells. It thus presented a unique tool for the study of the role of the thymus on lymphocyte differentiation, investigations of B cell functions (including interactions with

other immune cells) and other immune cells, including the natural killer (NK) cells. Nude mice have been used extensively in studies of the tumourigenesis of *in vitro* cultured cells. They are also widely utilized in evaluating anticancer agents prior to human clinical trials.

1.7.2 Xenograft models of breast cancer

The breast cancer xenograft model exploits the fact some human breast cancer cell lines formed tumours in immunodeficient mice. Unfortunately human breast cancer is one of the more difficult tumours to transplant directly into experimental animals. Primary human breast cancer has proven to be among the least likely of human tumours to grow progressively in immunodeficient mice, with relatively low rates of tumour take, and few forming spontaneous metastases (Price 1996). The reported success (take rate) for invasive human breast cancer (primary metastatic cells) is 7%-20% (Mehta et al. 1993). The differences are accounted for by site of implantation (orthotopic being better) the age and strain of mice used, and whether or not hormonal supplementation is used (nude mice have low oestrogen levels compared with humans). In comparison the success rates of pancreatic/colonic, renal cell, lung and urological carcinomas have been reported as 40-60%, 40%, 38-51% and 56% respectively (Cui et al. 2001, Angevin et al. 1999, Mourad and Vallieres 1995, Mattern et al. 1985, Okada and Yoshida 1984). Serially transplantable breast xenografts are much rarer still (Mehta et al. 1993). Paradoxically, better success has been reported with pre-invasive disease samples e.g. ductal carcinoma in situ (Gandhi et al. 2000, Chan et al. 2002).

Directly established mammary tumour xenograft lines with the capacity to metastasize were not developed until the early 1990s (Hurst et al. 1993), although prior and subsequent to this a number of established *in vitro* lines have been adapted to xenograft cultures. Some such lines are able to locally invade or metastasize, sometimes as the result of further genetic engineering to a more aggressive phenotype. Of the generally available human breast cancer cell lines, only MDA-MB-435 reliably forms spontaneous metastases after implantation into the mammary fat pad of immunodeficient mice (Price 1990).

Advantages of xenograft models

No single disease model can accurately reflect all features of the disease being tested. One must therefore select the model type which most closely mimics that aspect of the disease required by the hypothesis. Currently several different classes of animal based breast cancer models exist, these include rat models (chemically induced tumours), human tumour xenografts, syngeneic, transgenic (gain), and knockout mice. The chief advantage the xenograft models convey over other model systems is that it represents a model system of human origin allowing the use of fresh surgical patient material and or human tumour engraftments. Human breast cancer cells that grow progressively in nude mice generally retain their karyotypic, morphologic and histologic appearances as well as their production of specific enzymes and antigens, thus providing more opportunities to study the tumour cells *in vivo* rather than only *in vitro* (Fogh et al. 1977).

Human tumour xenograft and tissue transplant models are thus powerful tools for analyzing the complexities of organ function, especially when results can be reciprocally tested and scrutinized in simpler 3-D *in vitro* culture models. As with any model the similarity with the original is of paramount importance.

Therefore incorporating more human components such as the relevant stromal cells will only improve the authenticity and credibility of xenograft models.

Orthotopic vs heterotopic xenotransplanation

Over the last decade opinion has differed with respect to the optimum site of tissue transplantation within the animal and its preparation. For example, does the outgrowth of human mammary epithelium from immunodeficient subcapsular kidney transplantations truly represent normal mammary events or does the mammary fat pad provide a more relevant environment for outgrowth? Indeed, it has long been recognised that mouse mammary epithelial cells display differential developmental responses depending upon their site of delivery (Miller et al. 1981, Neville et al. 1998). In addition, it is now widely accepted that tumours grown subcutaneously are less successful in terms of take rates and metastases than those grown in the anatomically correct site (i.e. mammary fat pad) (Price 1990). Furthermore orthotopic implantation of breast tumour cells encourages the development of both lung and lymphatic metastases (Killion et al. 1998, Fidler 1999). For example, this is seen with the MDA-MB-231 model of human breast cancer metastatic to the bone (Yoneda et al. 1994), human colorectal cancer lines metastatic to liver, melanoma lines metastatic to nodes, prostate lines metastatic to nodes and bone, and pancreatic lines metastatic to liver and many other syngeneic and xenogeneic models.

1.7.3 Alternative models of breast cancer

It is important to note that other animal models of breast cancer exist. These reflect various aspects of breast cancer biology, but despite their non-human origin, have been widely and successfully used. They fall into three groups: the

chemically induced (rat) models, syngeneic, and models generated by genetic manipulations (transgenic gain and knockout mice).

Naturally each of the models has its specific uses, disadvantages and advantages. The majority of non-xenograft mouse models involve both *in vitro* and *in vivo* models which have utilized rodent cell systems, often in conjunction, with mutant rodent oncogenes. The results obtained from these models, therefore have questionable relevance to the human disease of breast cancer. For example, manipulation of a single gene can lead to high tumour incidence in transgenic and knockout mice; however this is very different to the mechanism of tumour formation in humans as multiple genetic and epigenetic changes are evident in human tumours and even in some benign breast lesions (Millikan et al. 1995, Dietrich et al. 1995, Simpson et al. 2005). These models, therefore, may more adequately reflect inherited beast cancers, since the altered genetic events are present from foetal life. On the top of these problems, unfortunately the expressed transgene is generally expressed throughout the mammary epithelium, which probably does not occur in the majority of sporadic human breast cancers (Clarke 2000). There may be additional problems of interpretation when the transgene is not controlled by a tissue-specific promoter but is expressed in every cell in the animal.

Chemically induced models

Chemically induced and oncogenic virus derived mouse mammary tumours (e.g. mouse mammary tumour virus) were not ideal models for human cancer in that they were highly immunogeneic, failed to metastasise, and were not hormonally responsive as human cancers. Researchers began to recognize the importance of using strictly syngeneic inbred strains and the fact that spontaneous tumours may

be closer to their human counterparts than those induced with strongly oncogenic chemicals and viruses. As such these models are now rarely used.

Syngeneic mice

Syngeneic mice are strains of mice produced by repeated inbreeding (over 20 generations), in order to eliminate heterozygocity, so that each pair of autosomes within an individual is identical (Shi et al. 2001). They are virtually genetically identical (over 98% genetically identical) in addition to being immunologically compatible so as to allow tissue transplantation. Therefore they allow the study of the immune response in the absence of other variables caused by genetic differences (i.e. MHC haplotype, immunoglobulins (Ig) idiotype). Ig from one animal can be injected into another animal of the same inbred strain with out eliciting an immune response. Furthermore specific cells (e.g. tumour cells) can be transferred without rejection.

Syngeneic mouse tumour models have a somewhat limited role in cancer research because the biology of the rodent and their tumours can differ significantly from that of human cancer. To begin with, the difference in developmental programmes of mouse and humans will manifest in many ways with size being an obvious example. As a consequence the cellular targets for oncogenic transformation are present in vastly reduced numbers in mouse tissues than in their human counterparts. This is coupled with the fact that mice have a much shorter lifespan than humans, and so tumours that occur in mice must have an accelerated programme of progression. In addition a smaller number of genetic changes are required for rodent cell transformation *in vitro*, so it follows that this situation will be mimicked *in vivo*. These fundamental differences between humans and mice will undoubtedly have an influence on tumour formation. It therefore comes to no surprise to learn that the tumours formed

differ as well. For example, most human breast cancers are hormone dependent, whilst the vast majority of mouse tumours are hormone independent with much lower levels of oestrogen/progesterone receptors (Nandi et al. 1995). Another point of note is that although similar morphological patterns of lesions appear in both species, the morphology of most mouse tumours does not resemble the common human breast cancers (Cardiff 2001). Finally to round things off the metastatic pattern between the two species is also very different. Breast cancer in humans involves either regional spread, by lymphatic channels to local lymph glands, or haematogenous systemic spread. In contrast mouse mammary cancers metastasize almost exclusively to the lung (Cheung et al. 1997). A major technical problem with using syngeneic models involves studies where monoclonal antibodies against human antigens failed to cross react with the rodent equivalent.

Although human tumour xenografts are now the preferred model, applications requiring an intact immune system still present uses for syngeneic mouse models. In addition, as metastasis is more readily observed in syngeneic models they are still useful for such specific studies.

Transgenic gain and knockout mice.

The development of techniques for introducing foreign DNA into the germ line of mice by microinjection and those of engineering embryonic stem cells prior to injection into mouse blastocytes has yielded two powerful experimental tools: the ability to over-express or suppress genes in specific tissues of a mouse (transgenic gain mice), and the power to ablate specific gene expression in the whole mouse or in a tissue specific (knock out mice). Since the establishment of these technologies, their use has allowed the exploration of the role of a variety

of genes during development, as well as the importance of these genes for certain physiological and biochemical processes and for the development of disease.

Transgenic mice result from genetically altered embryos: introduction of a foreign or synthetic gene (called a transgene) or combination of genes by microinjection into developing oocytes. This results in a chimeric mouse with a gain of a gene with the genetic alteration affecting the germ plasm, and subsequently can be transmitted to their progeny (heterozygous progeny).The transgene inserts randomly into the genome (non-homologous recombination). Through selective breeding, it then is possible to maintain a strain of mice consisting of individuals with particular traits of interest. A specific trait, such as a predisposition to develop a particular type of tumour, can be introduced into a mouse strain by injecting an oncogene into the embryo. Transgenic mice can therefore permit the study of cancer in specific tissues including their initial development.

The knock out mouse is generated in a very similar manner to the transgenic gain mice except in this case a normal gene is replaced with a mutant gene resulting in the loss of a gene or function (thus allowing the investigation of that specific gene function).

To determine the value of genetically engineered mice (GEM) we must look closer at the biology and genetics. It is well recognised that cancer involves the accumulation of genetic abnormalities within a cell with sporadic cancers arising when the initiating mutation affects a single cell in an otherwise normal microenvironment. However oncogene bearing transgenic mice and tumour suppressor gene knockouts do not mimic sporadic tumour development because the initiating mutation is present throughout the body or a particular tissue. As a consequence the functions of the microenvironment in either stimulating or

inhibiting tumour growth can be impaired. Following on from this other side effects such as the induction of mechanisms that balance the effects of the mutation and tumourigenesis outside the tissue of interest can occur. This situation more closely resembles rare familial cancer syndromes in humans. Another major limitation of transgenic and knockout mice is their dependency on promoters that are ultimately sensitive to hormonal stimulation.

Currently most genetically engineered mice transgenes are placed behind promoters that have some target tissue specificity, with MMTV-LTR (mouse mammary tumour virus long terminal repeat) and WAP (whey acidic promoter) being the most commonly used promoters. These promoters have hormone regulated enhancer elements that are stimulated by various hormones and so are not the natural promoters for the activated oncogenes associated with human breast cancer. Therefore these and other promoters can result in tumours with strange biologies very different to the human situation. As a consequence it is difficult to assess the interaction between oncogene coupled signalling pathways and the action of endocrine hormones. For example tumourigenesis can be enhanced in pregnancy with both promoters, whereas in humans pregnancy tends to be protective (Cardiff 2001).

1.8 Aims of the thesis

The aim of this thesis was to develop a more authentic xenograft model of breast cancer by incorporating the relevant and essential constituents of a tumour microenvironment: human stromal cells and extracellular matrix. Ideally this would be an organotypic, 3-compartment xenograft model of human breast cancer in mice comprising solely of human cells. The development of a complex

multi-compartment heterologous xenograft model incorporating the relevant stromal elements will provide a realistic alternative to currently available chimeric xenograft models. If successful this model can be utilized as an accurate and credible preclinical model for the development and testing of new therapeutic strategies, such as those that target breast cancer stroma itself.

The great hurdle impeding multi-compartment xenograft model development has been the lack of readily available human stromal cells. The required cells have to be robust enough and in sufficient supply to be incorporated into these model systems. With the recent availability of a series of immortalized human mammary stromal cell lines (O'Hare et al. 2001) it is has now become possible to commence development of a multi-compartment breast tumour model. There are two broad components to this thesis: *in vitro* and *in vivo*.

In vitro

The first objective was to characterize each of the cell lines by comparing their growth properties against each other and also their equivalent normal cells in order to select the cell line or cell lines most suitable for incorporation into the 3-compartment xenograft models (Chapter 3). Next, the vascularization of the xenografts was investigated by determining the ability of the human endothelial cells to interact and integrate with host (mouse) endothelial cells, *in vitro*, to form chimeric vasculature (Chapter 4). Prior to *in vivo* xenografting, the ability of the immortalized stromal cell lines, with the aid of new 3-D tissue culture techniques, to interact and form viable complex heterologous tumours *in vitro* was investigated (Chapter 5). If successful, the goal was to xenograft these pre-formed heterologous *in vitro* tumours directly into immunodeficient mice in order to streamline model development.

In vivo

The first objective was to investigate whether the available cell lines survived *in vivo* and for what period of time. The next objective was to examine the extent to which surviving cells formed organised structures (e.g. blood vessels) and whether they proliferated. Once the basic parameters of survival, growth and differentiation had been established, the appropriate stromal cell lines were co-grafted with different breast epithelial tumour cell lines (e.g. VEGF over-expressing mammary epithelial tumour cell lines) and with primary breast tumour cells (Chapter 6). The last part of the thesis describes experiments in which an alternative source of stromal cells, in the form of circulating endothelial progenitor cells, was investigated as regards to their suitability for use in this model system (Chapter 7).

Chapter 2

Materials and methods

2.1 Routine cell culture

2.1.1 General tissue culture methods

All cell culture procedures were carried out using aseptic technique in a laminar flow cell culture hood with the operator wearing protective clothing and nitrile gloves. Surface decontamination was achieved with 70% (v/v) ethanol, and any cell contaminated or potential pathogen containing liquids drained into 10% (v/v) 'Chloros' solution (Sodium Hypochlorite, Haychlor Hayes Chemicals) (See appendix I for details of all sources). All vessels, flasks and pipettes used were left to soak in Chloros solution for >12 hours prior to being discarded in appropriate yellow bags or containers for incineration.

Cells were grown in $25cm^2$, $75cm^2$, or $175cm^2$ Nunc® tissue culture flasks (Nalge Nunc International) in an atmosphere of 5% (v/v) carbon dioxide (CO_2)/air in 37°C or 33.5°C humidified incubators. Cells were monitored daily and passaged at 80-90% confluency by trypsinisation. The culture medium was drained and the cells washed once with phosphate buffered saline (PBS, Biowhittaker) and then twice using PBS containing 0.02% (w/v) ethylene diaminetetra-acetic acid (EDTA) (disodium salt) (PBS/EDTA), to chelate calcium ions. This PBS was then drained and ~1 ml of thawed bovine trypsin (1mg/ml, Sigma) in 0.02% (w/v) EDTA was added to the flask, and the flask incubated at 37°C for approximately 1-2 minutes to disrupt cell-cell and cell-flask attachment. Intermittent removal of the flask from the incubator and rocking of its contents to assess cell detachment from the flask surface was

carried out to prevent over trypsinisation of the cells. Detached cells were washed from the flask with air buffered L15 medium (Biowhittaker) containing 10% (v/v) of foetal calf serum (FCS, First Link), pelleted by centrifugation for 3 minutes at approximately 300×*g* (1500 revolutions per minute (rpm)) and then re-plated as required. All cell lines were routinely passaged at a split ratio of 1:6.

2.1.2 Freezing, thawing and storage of cells

Cells to be used in xenograft experiments were previously grown up in bulk and stored in dedicated liquid nitrogen (N_2) tissue culture banks in replicate ampoules. Cells to be frozen were trypsinised from their flasks as previously described, centrifuged into a pellet and then resuspended at a cell density of approximately 5 x10^6 cells/ml in freezing medium which contained 10% (v/v) dimethyl sulphoxide (DMSO, Sigma) as a cryoprotectant in L15 medium containing 10% (v/v) FCS. The cell suspension was then aliquoted into 1ml cryotubes (Nalge Nunc International) and frozen in a liquid N_2 vapour phase freezing head using a two stage freezing protocol. After freezing, the tubes were stored under liquid N_2 in dedicated tissue storage facilities. Cells were thawed by placing the frozen cryotubes in a 37°C water bath immediately after removal from the liquid N_2. Once the contents were fully thawed the cell suspension was transferred to 5-10 ml of growth medium. The cells were pelleted by centrifugation for 3 minutes at approximately 300×*g* (1500 rpm), resuspended in 5ml of growth medium and plated out in a 75cm^2 flask containing the appropriate medium. After 24 hours the medium was changed to remove dead cells, debris and any remaining traces of DMSO.

2.1.3 Culture conditions for different cell lines used

The cells used in this study are summarised in Table 2.1. The individual culture conditions are described below.

	Temperature sensitive 33.5 °C	**Non-temperature sensitive 37 °C**
Human mammary endothelial cells	HMME 2 HMME 7	HMME HMME 7wt (wild type)
Human mammary fibroblasts	HMF 3A HMF 3B* HMF 3C HMF 3D	HMF HMF 3wt (wild type) HMF 3wt *ras*
Human breast tumour cell lines		MCF-7 MCF-7 V12 T47D ZR-75-1

Table 2.1. Table showing all the cells and cell lines used together with their temperature sensitivities. (* derived with the temperature sensitive LT construct but lacking temperature sensitivity).

Human breast cancer cell lines

Four established oestrogen sensitive breast tumour cell lines were used; these were MCF-7 V12 (VEGF over-expressing variant of the MCF-7 cell line from Professor Adrian Harris, Institute of Molecular Medicine, Oxford), MCF-7, T-47D and ZR-75-1 (originally obtained from the American Type Culture Collection, ATCC).

Banked cell lines of MCF-7, ZR-75-1, T-47-D, and MCF-7 V12 from frozen stocks were thawed as described in section 2.1.2. The contents of a frozen vial were transferred by pipette into a 75 cm^2 culture vessel containing a 1:1 mix of Dulbecco's Modified Eagle's medium (DMEM, Biowhittaker)/Ham's F-12

medium (Biowhittaker) containing 10% (v/v) FCS, 2mM L-glutamine (Gibco), 100U/ml penicillin (Gibco) and 100μg/ml streptomycin (Gibco). The flasks were then placed on a horizontal incubator shelf in a humidified atmosphere of 5% (v/v) CO_2 at 37°C. The cells were allowed to settle and attach to the surface of the culture flask. Feeding of the cells in culture was carried out at regular intervals depending on the culture conditions and the apparent pH as denoted by the change in colour of the phenol red. When the cell population had reached 80-90% confluency across the tissue culture flask surface the cells were either used for experiments or passaged further. All experiments were carried out within 5-10 passages of original thawed stocks.

Human mammary stromal cells and immortalized cell lines

Unaltered human mammary fibroblasts and endothelial cells (non-transformed) and immortalized human mammary stromal cell lines (temperature sensitive and non-temperature sensitive, derived by O'Hare et al. (2001) used are shown in Table 2.1. All stromal cell lines used with the exception of the unaltered cells (HMF and HMME) were immortalized by the insertion of the catalytic subunit of human telomerase (hTERT) and a temperature sensitive mutant (U19tsA58) variant of the Simian virus 40 large T antigen (SV40 LT). The HMF variants A-D differed in the timing and order of the immortalizing gene transfection. The HMME variants were derived in a similar manner from two separate donors. Wild type (non-temperature sensitive) large T (LT) variants of each cell type (HMF 3wt, HMME 7wt) were also created by insertion of a wild type LT antigen in cells previously engineered to express hTERT. HMF 3wt *ras* was derived by transfection of the wild type HMF 3wt cell line with the activated (V12) *ras* oncogene.

Human mammary fibroblasts (HMF). HMF 3A, HMF 3B, HMF 3C, HMF 3D, HMF 3wt, HMF 3wt *ras*, HMF were obtained from Prof. Mike O'Hare, UCL.

HMF 3A, HMF 3B, HMF 3C, HMF 3D are the four conditionally immortalized human mammary fibroblasts lines. The condition being that they are temperature sensitive with the permissive growth temperature being 33.5 °C. HMF 3wt, and HMF 3wt *ras* are wild type LT variants of HMF 3D and unlike the temperature sensitive (ts) lines grow well at 37 °C. HMFs are the unaltered sub-cultures of primary human mammary fibroblasts.

The HMF cells and HMF cell lines were cultured in growth medium consisting of DMEM, 2 mM glutamine, 10% (v/v) heat-inactivated FCS, 50 units/ml penicillin, and 50 μg/ml streptomycin at either 37 °C or 33.5 °C in a humidified incubator in an atmosphere of 5% (v/v) CO_2 + 95 % air. Flasks were examined daily during the first week.

Human mammary endothelial cells (HMME). HMME 7, HMME 2, HMME 7wt, HMME were obtained from Prof. Mike O'Hare, UCL.

HMME 7 and HMME 2 are the two conditionally immortalized human mammary endothelial cell lines which grow only at 33.5°C. HMME 7wt is a wild type LT variant, with HMME being the sub-cultured unaltered endothelial cells.

The HMME cells were cultured in a specially optimized microvascular endothelial medium EGM-2™ - Endothelial Growth Medium-2 (EGM-2 Bullit kit, Biowhittaker) consisting of basal growth medium developed for normal human microvascular endothelial cells in a low serum environment with a final serum concentration of 2% (v/v). The complete medium (500ml) contained various supplements and growth factors. These were heparin 0.5ml, hydrocortisone (HC) 0.2ml, human fibroblast growth factor (hFGF) 2ml, vascular endothelial growth factor (VEGF) 0.5ml, insulin like growth factor R^3-

IGF-1 0.5ml, ascorbic acid (aa) 0.5ml, human epidermal growth factor (hEGF) 0.5ml, gentamicin and amphotericin (GA-1000) 0.5ml, and foetal bovine serum (FBS) 10ml (precise concentrations of the additives are not supplied by the manufacturer). All cultures were kept in a humidified incubator in 5% (v/v) CO_2 at their respective optimum temperature of 37°C or 33.5°C.

2.1.4 Culture conditions for freshly isolated cells

Mouse microvascular endothelial cells

Murine microvascular endothelial cells (isolation method described in section 2.3.1) were serially sub-cultured at 37°C in a humidified atmosphere with 5% (v/v) CO_2 in DMEM supplemented with 2mM glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 1mM sodium pyruvate (Gibco), 20mM HEPES (Gibco), 1% non-essential amino acids (Gibco), 50mM 2-mercaptoethanol (Gibco), freshly added 20% (v/v) heat inactivated FCS, 150µg/ml EC growth supplement (Sigma) and 12U/ml heparin (Sigma). They were cultured in either fibronectin (1mg/ml) or 2% (w/v) gelatin (type B from bovine skin, Sigma) coated tissue culture flasks (see section 2.2.1 for coating method). At confluence the endothelial cells were detached from the culture flasks using a solution of 0.125% (w/v) bovine trypsin (Sigma) in 0.02% (w/v) EDTA and then passaged. In some experiments, enzyme free cell dissociation solution was used. Cells were washed twice with PBS containing 0.02% (w/v) EDTA and mechanically disrupted by gentle tapping of the tissue culture flasks.

Circulating human endothelial progenitor cells (CEPs)

Immediately following isolation (for method see Chapter 2.3.2) approximately $1x10^5$ $CD34^+CD133^+$ enriched circulating endothelial progenitor cells were

plated on fibronectin coated 25cm^2 tissue culture flasks (see Chapter 2.2.1 for coating method). Growth factors required for CEPs differentiation and expansion have been identified as VEGF, bFGF, IGF, EGF as noted by Kalka et al. (2000) and so were incorporated into the growth medium.

Cells were grown in 60% (v/v) low-glucose DMEM and 40% (v/v) MCDB-201 (all from Sigma), supplemented with 1x insulin-transferrin-selenium, 1x linoleic acid–BSA, 10^{-8} M dexamethasone, 10^{-4} M ascorbic acid 2-phosphate (all from Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin, 10ng/ml VEGF, bFGF-2 1ng/ml, 2ng/ml IGF-1, and 10% (v/v) FBS (Sigma) as described by Reyes et al. (2002). Cultures were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2 with medium changes every 4–5 days.

2.2 Specialized culture methods

2.2.1 Fibronectin and gelatin coating of tissue culture flasks

Fibronectin

Fibronectin coating helps cells maintain native differentiated characteristics and functions as well aid attachment to the tissue culture plastic. 2ml of human fibronectin solution (1mg/ml, BD Laboratories) was added to 25cm^2 tissue culture flasks. The flasks were then transferred to an incubator and the coating solution was allowed to sit for a minimum of two hours or overnight. The excess coating solution was then drained from the flasks. The flasks were then allowed to dry flat in the hood for at least one hour. The coated flasks were stored at room temperature for use within 1 month. A similar procedure was followed for preparing all culture flasks and culture dishes for growing and culturing freshly

isolated murine microvascular endothelial cells and circulating endothelial progenitor cells.

Gelatin

Sufficient amount of 2% (w/v) gelatin (Sigma) was added to culture vessel so that it completely covered the bottom. Approximately 3 ml per 25cm^2 flask or 7-8 ml per 25cm^2 flask was used. The gelatin solution was left to sit in contact with the plastic for at least 20 minutes at room temperature. The excess gelatin solution was then aspirated off and the remainder was allowed to evaporate by leaving the container sitting open in the hood until no traces of the liquid remained. The cap was then replaced once the surface had dried. Gelatinized flasks were stored at room temperature or at 4°C for up to 2 weeks maintaining sterility until use.

2.2.2 Preparation of ECM-related substrates for differentiation assays and liquid overlay cultures

Matrigel

BD Matrigel™ Matrix is a solublized basement membrane preparation extracted from EHS mouse sarcoma, a tumour rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin. At room temperature, BD Matrigel™ Matrix polymerizes to produce biologically active matrix material resembling the mammalian cellular basement membrane. Two varieties of matrigel were used: Matrigel and growth factor reduced Matrigel (GFR) (Table 2.2).

Matrigel was stored at -80°C, after aliquoting into vials. Vials of Matrigel were thawed by placing on ice or at 4°C for 24 hours. The thawed matrigel was

mixed to homogeneity using pre-chilled pipettes. A required amount of Matrigel (300µl/well for 96-well plates and 1ml/well for 48-well plates (both from Nalge Nunc International) was placed in the multiwell plates to obtain an even spread. The plates then were placed in an incubator for 30 minutes at 37°C to gel the matrix. Media was added and incubated for 24 hours to elute any residues and toxic contaminants.

Amounts of Growth Factors (GF) Present in BD Matrigel™ Matrix vs. GFR BD Matrigel™ Matrix			
Growth Factor	Range of GF Concentration in BD Matrigel Matrix	Average GF Concentration in BD Matrigel Matrix	Typical GF Concentration in GFR BD Matrigel Matrix
EGF	0.5-1.3 ng/ml	0.7 ng/ml	< 0.5 ng/ml
bFGF	< 0.1-0.2 pg/ml	n.a.*	n.d.**
NGF	< 0.2 ng/ml	n.a.*	< 0.2 ng/ml
PDGF	5-48 pg/ml	12 pg/ml	< 5 pg/ml
IGF-1	11-24 ng/ml	16 ng/ml	5 ng/ml
TGF-β	1.7-4.7 ng/ml	2.3 ng/ml	1.7 ng/ml
* n.a. - not applicable **n.d. - not determined			

ECM Composition of BD Matrigel™ Matrix vs. GFR Matrigel Matrix		
Basement Membrane Matrigel Component	Percent in BD Matrigel™ Matrix	Percent in GFR BD Matrigel™ Matrix
Laminin	56%	61%
Collagen IV	31%	30%
Entactin	8%	7%

Table 2.2. Table summarizing the constituents of growth factor reduced and standard Matrigel (data supplied by manufacturers product specifications).

Agarose gels

Purified low-melting-point agarose powder (A-4018; Sigma Type VII: low gelling temperature, Sigma-Aldrich) was mixed with PBS in a glass beaker to obtain the desired concentration (1-1.5% (w/v) agarose) and then sterilized by autoclaving. The beaker was then heated in a water bath until all the agarose had completely melted. After cooling the solution to about 40°C, 100 µl of 1.5% agarose was pipetted for 96-well plates, 300µl for 48-well plates and 500µl for 24-well plates.

Fibrin gels

Human fibrinogen (Sigma) was dissolved in PBS to give a solution of 30mg/ml. 1ml of this solution was added to 10 ml of MCDB 131 medium (Biowhittaker). This solution was dialysed using medium sized dialysis tubing (25kD) against MCDB 131 medium for 24 hours while stirring at 4°C to remove any residues and contaminants. Following dialysis 10% (v/v) FCS and 2mM glutamine were added to the mixture. For the manufacture of liquid overlay cultures, 500µl of the fibrinogen solution was added to the wells of a 48-well plate. The fibrinogen was solidified into fibrin by adding 5µl of thrombin (Sigma) (1µl of thrombin for 100µl of fibrinogen solution). Once the fibrinogen had solidified it was then placed at 37°C for 2 hours for the fibrin gel to fully form before cells were plated. For multiple layer cultures this process was repeated on the top of the seeded cells.

Collagen gels

Rat tail collagen, Type 1 (3.82 mg/ml) (Collaborative Biomedical Products) was used to prepare solid gels in microwell plates. The collagen solution was diluted to 50µg/ml using 0.02N acetic acid and then neutralized with 0.1M NaOH. 200µl of the diluted collagen material was added to each microwell of a 96-well plate.

The microwell plates were then incubated at 37°C where the gels polymerized in 60 minutes. The plates were then rinsed well using PBS to remove the acid and air dried. They were then stored at 2-8°C for up to one week under sterile conditions before use.

2.3 Cell separation techniques

2.3.1 Isolation of mouse endothelial cells

The isolation and long term culture of murine endothelial cells has often proven a difficult task. A quick and efficient magnetic bead separation protocol outlined by Dong et al. (1997) was adopted for the isolation of microvascular endothelial cells from murine tissues.

Animals

Female NCr (*nu/nu)* mice (original stocks from National Cancer Institute) aged 4 to 6 weeks bred in on site facilities were used. The ethical guidelines that were followed met the standards required by the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia (Workman et al. 1998).

Preparation of tissue

Tissues (murine lungs and hearts) from 8 mice were dissected out and removed aseptically, rinsed in 30ml of Hanks' balanced salt solution (HBSS, Biowhittaker) containing 100U/ml penicillin and 100μg/ml streptomycin, minced into small pieces (1 x 2 mm squares) in a 60mm tissue culture dish using sterilized razor blades, and then enzymatically digested in 20ml of 1ml/mg collagenase A (Sigma) in L15 medium and 1mg/ml of bovine trypsin at 37°C for 45 minutes with occasional agitation. Following digestion, DMEM with 10% (v/v) FCS was added and centrifuged for 3 minutes at approximately 300×*g* (1500 rpm) to form

a pellet. The pellet was then resuspended in 5ml of DMEM with 10% (v/v) FCS and then the cellular digest was filtered sequentially through sterile 40μm and 31μm nylon meshes (Falcon, Becton Dickinson Labware) to remove clumps of cells, centrifuged at 400×*g* for 10 minutes to pellet cells, and washed twice in DMEM medium with 10% (v/v) FCS. The cell pellet was then resuspended in 4 ml of DMEM with 10% (v/v) FCS prior to Dynabead separation.

Preparation of antibody coated magnetic beads for endothelial cell isolation

Dynabeads (Dynal Biotech) coated with sheep anti-rat IgG (30μl aliquot per 5ml tube) were washed three times with serum free DMEM and then incubated with rat anti-mouse PECAM-1 monoclonal antibody (MEC13.3, BD Pharmingen) overnight at 4°C (10μl/50μl beads in DMEM). Following incubation, beads were washed three times with DMEM containing 10% (v/v) FCS, centrifuged at 300×*g* (1500 rpm) for 5 minutes and re-suspended in 5ml of the same medium.

Separation of cells

1ml of cell suspension (from digested murine tissues) was added to the tube containing beads that had been pre-coated with anti-PECAM-1 as described above. After affinity binding for 30 minutes at 4°C with occasional agitation, the bead-bound cells were recovered using magnetic bead separation (Dynal magnetic particle concentrator MPC-1, Dynal Biotech), washed five times with DMEM containing 10% (v/v) FCS and once with FCS free DMEM, and then digested for 5 to 10 minutes at 37°C in 1ml of 1mg/ml of trypsin/ 0.02% (w/v) EDTA to release the cells. The bead-free cells were centrifuged at 300×*g* for 5 minutes in DMEM containing 10% (v/v) FCS and then re-suspended in 7 ml of growth medium (described in Chapter 2.1.4) for culture. This solution was then plated in a 25cm^2 fibronectin pre-coated tissue culture flask.

2.3.2 Isolation of circulating endothelial progenitor cells

Separation of mononuclear cells from bone marrow aspirates and cytokine mobilized blood (buffy coats)

Bone marrow. After informed consent, bone marrow samples (20-50ml) collected from healthy donors for allogeneic bone marrow transplantation, were placed in sterile tubes containing preservative-free lithium heparin (20U/ml) in preparation for layering on a Ficoll-Hypaque gradient (specific gravity 1·077 g/ml, Sigma). Low-density mononuclear cells (LD-MNCs) were isolated from the bone marrow sample by density gradient centrifugation over 15ml of Ficoll-Hypaque for 25 minutes at 500×*g*. The LD-MNCs at the interface were carefully harvested using a Pasteur pipette. The harvested cells were then re-suspended in 10ml of PBS (containing 1-5% (v/v) FCS) and washed 3 times, at 18-20°C, by centrifuging at 300×*g* for 3 minutes and re-suspending each time. After washing the cells were then centrifuged sequentially at 200×*g*, 300×*g*, and 500×*g* for 10 minutes each to remove platelets. The LD-MNCs were then washed twice in 20ml of HBSS and re-suspended in 10ml of Iscove's modified Dulbecco's medium (IMDM, Biowhittaker) supplemented with 10% (v/v) FBS and 5mmol/l EDTA.

Peripheral blood mononuclear cell isolation. Cytokine mobilized (Granulocyte-macrophage colony-stimulating factor) pheripheral buffy coat residues rich in mononuclear cells were obtained from the National Blood Transfusion Unit, London. 25 ml of the anti-coagulated blood was mixed with 25ml of PBS (containing 1-5% (v/v) FCS) in a 50 ml tube at room temperature. 2 volumes of diluted blood (30ml of diluted blood mixture) were carefully layered over 1 volume of Ficoll Hypaque (15ml). This solution was centrifuged (low

acceleration and deceleration in a non-braked centrifuge) at 800-1000×*g* for 30 minutes in a swinging motor at room temperature. The LD-MNCs at the interface were carefully harvested using a Pasteur pipette. The harvested cells were then re-suspended in 50ml of PBS (containing 1-5% (v/v) FCS) and centrifuged at 290×g for 10 minutes at 18-20°C and the supernatant discarded. This washing process was repeated twice. The LD-MNCs were then washed twice in 20ml of HBSS and filtered through a 50μm nylon mesh to remove clumps before being re-suspended in 10ml of IMDM supplemented with 10% (v/v) FBS and 5mmol/l EDTA. A 500ml bag of buffy coat residue provided a yield of approximately 10^8 cells.

Positive selection of $CD133^+$ cells by magnetic cell sorting

The haemopoietic stem/progenitor cells were isolated by positive selection of $CD133^+$ expressing cells using the AC133 Cell Isolation Kit (Miltenyi Biotec) and the MS Magnet (MiniMACS™, Miltenyi Biotec). Briefly LD-MNCs were added to $CD133^+$ monoclonal antibody conjugated super paramagnetic microbeads (AC133 Cell Isolation Kit) to attach, washed and processed through a MACS magnetic separation column to obtain a pure population of $CD133^+$ (Figure 2.1).

Figure 2.1. Isolation of $CD133^+$ cells using the MACS AC 133 Cell Isolation Kit.

Modified diagram taken from Miltenyi Biotec protocol instructions sheet.

Standard buffer used for washing the LD-MNCs was made from PBS (pH 7.2), supplemented with 0.5% (w/v) bovine serum albumin (BSA, Sigma) and 2 mM EDTA. The buffer was kept cold (4°- 8°C) at all times. 100µl of FcR blocking reagent provided was added to a total cell count of 10^8 LD-MNCs cells suspended in 300µl of buffer solution, to inhibit unspecific or Fc-receptor mediated binding of antibodies to non-target cells. The LD-MNCs were then labelled by adding in 100µl of $CD133^+$ coated microbeads (500µl of beads per10^8 total cells) producing a final volume of 500µl per10^8 total cells. The cells were mixed well and incubated for 30 minutes at 4-8°C. The cells were then washed by adding 10-20 times the labelling volume of buffer solution and centrifuged at 300×*g* for 10 minutes. The supernatant was then pipetted off and the cell pellet was re-suspended in 500µl of buffer. In the meantime a MS column was prepared by placing it in the high-gradient magnetic field of an MS magnet and rinsing with 500µl of buffer. Following two washes the cell suspension was applied and the negative cells were allowed to flow through the column. The column was then washed four times with 500µl of buffer solution followed by another 1ml of buffer. After removing the column from the magnet the positive fraction with the magnetically labelled cells was firmly flushed out using the supplied plunger to obtain purified $CD133^+$ cells. These cells was then plated in a $25cm^2$ tissue culture flask pre-coated with fibronectin containing either the endothelial cell growth medium EGM-2 or the specialized medium described in Chapter 2.1.4.

Cytospin slide preparation for staining of CEPS

Glass slides, filter cards (Shandon #190005 thick white) and funnels (0.5ml capacity, Shandon) were set up in the Shandon Cytospin-2 device. 0.2ml aliquots of diluted cell suspensions were placed into the chamber. Another device was

placed opposite for counter balance. The Cytospin-2 device was set at 100 revolutions per minute for 5 minute at high acceleration. The funnel and filter were then discarded from the glass slide and the cell preparation was allowed to air dry overnight, protecting it from dust. Immunofluorescence staining was performed on the slide.

2.4 Cell labelling techniques

In this study both cytoplasmic and membrane marking dyes were evaluated. The cytoplasmic dyes were the CellTracker™ dyes (Molecular Probes) and the membrane labelling dyes were the fluorescent liphophilic Vybrant Cell-Labeling Solutions (Molecular Probes). Emission spectrum information for both types of dye is shown in Figure 2.2.

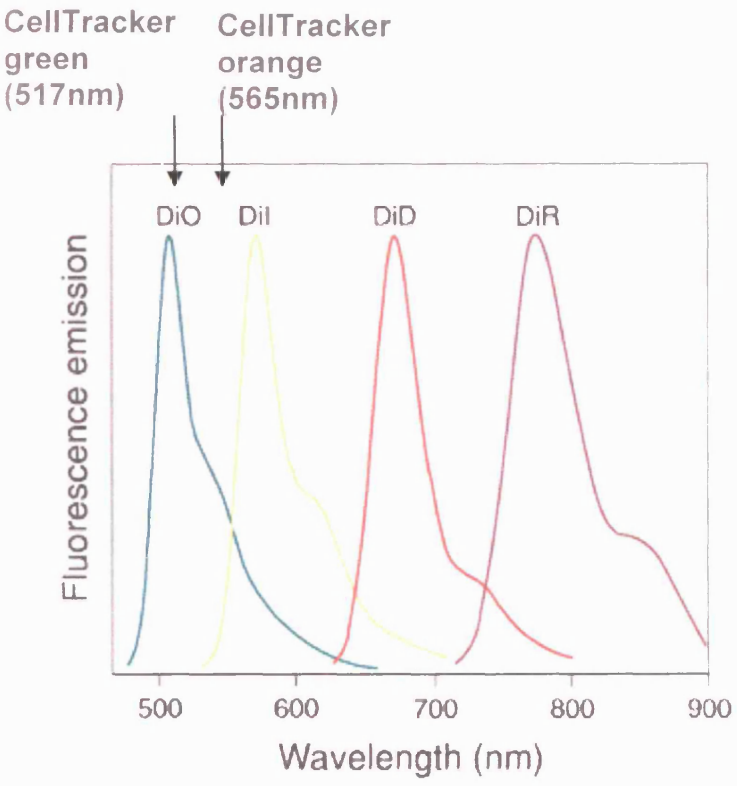


Figure 2.2. Normalized fluorescence emission spectra of DiO (3,3'-dioctadecyloxacarbocyanine perchlorate), **DiI** (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), **DiD** (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate), **DiR dyes (bound to phospholipid bilayer membranes) and the green and orange CellTracker dyes** (taken from instruction protocol, Molecular Probes).

2.4.1 CellTracker™ Dyes

5mg of CellTracker fluorescent probes; either Orange CellTracker™ CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine) or Green CellTracker™ CMFDA (5-chloromethylfluorescein diacetate) were dissolved in 10% (v/v) DMSO to obtain a stock solution with a final concentration of 10mM. The stock solution was diluted to a final working solution of 0.5 to 25μM in

serum free DMEM medium. Cells were grown in tissue culture flasks containing the appropriate medium until the desired confluency was reached. The medium was removed from the flask by decanting and pre-warmed (37°C) probe-containing (0.5 to 25μM) medium was added. The flask was placed horizontally and the cells were incubated for 45 minutes at 37°C or 33.5°C (depending on cell) in 5% (v/v) CO_2. The loading solution was then replaced with fresh pre-warmed medium and incubated for another 30 minutes at 37°C to ensure complete integration of the probe. The cells were then washed again. 10% (v/v) DMSO alone without dye was used as the control.

2.4 2 Fluorescent liphophilic dyes

Vybrant DiI (1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate), DiD (1,1'-dioctadecyl-3,3,3',3'- tetramethylindodicarbocyanine perchlorate), and DiO (3,3'-dioctadecyloxacarbocyanine perchlorate) cell-labelling solutions were supplied in units of 1 ml. The solutions containing 1mM DiI, DiO or DiD were sterile filtered through 0.2μm polycarbonate filters.

The staining solution was prepared by adding 5μl of the supplied labelling dye solution to 1 ml of normal growth medium. Trypsinised cells at a density of 1×10^6/ml were suspended in serum-free DMEM culture medium. 5μl of the cell-labelling solution supplied was added for each ml of cell suspension. The suspension was mixed well by gentle pipetting and then incubated for 20 minutes at 37°C. The optimal incubation time varied depending on cell type. The labelled suspension tubes were then centrifuged at 1500 rpm for 5 minutes at 37°C. The supernatant was then removed and the cells were gently re-suspended

in warm (37°C) medium. This wash procedure was repeated three times. 10 minutes recovery time was allowed before fluorescence observations were made.

2.5 *In vitro* growth and differentiation assays

2.5.1 Flask growth assays

A required number of cells (1×10^5) were plated in 25cm^2 flasks in their respective growth medium and cultured for various time points. Cells were then trypsinised, centrifuged into a pellet and then gently re-suspended in an appropriate volume of medium ensuring a unicellular suspension was achieved. Cell numbers were determined using a haemocytometer. Experiments were either carried out at single time points after plating, or replicate flasks were harvested at intervals (multi-point assays).

Haemocytometer counting

The haemocytometer (improved Neubauer® chamber) and cover slip were cleaned first with distilled water, then by 70% (v/v) ethanol and wiped dry with a lens tissue. A lightly moistened coverslip was placed squarely on top of the haemocytometer before being gently pressed into position. Pressure was applied until it had firmly stuck with 'Newton's rings' visible. Cells were gently redistributed throughout the medium through repeated re-suspension using a pipette. The haemocytometer was then loaded by placing a drop of suspension near the edge of the coverslip. Every count was duplicated by analyzing two ruled counting areas. Using the 20X objective of a microscope, the upper left primary squares of each grid (10 primary squares) was located and a counter was used to count the cells in 10 primary square using the following conventions: Each large square of the grid equals a volume of 10^{-4} cm^3 or 10^{-4} ml (as 1 cm^3 =

1 ml). Multiplying the number of cells in a large square by the dilution factor (if the sample was diluted) and then multiplying by 10,000 will determine the total number of cells per ml. At least 200 cells were counted for each measurement.

2.5.2 Microwell growth assays

24-well plates (Nalge Nunc International) were used for microwell growth assays. The required numbers of cells (1×10^3) were plated in wells of a 24-well plate and cultured in 1-2 ml of medium containing various growth factors and serum concentrations as described in the results (Chapter 3.2.2). Cell numbers were determined by a methylene blue staining assay.

Methylene blue staining

The methylene blue staining assay was used for estimating the number of fixed adherent cells present in a micro-culture (Oliver et al. 1989). The assay is dependent on the binding of methylene blue to a fixed monolayer at pH 8.5, and after washing, the release of the dye by lowering the pH and then measuring dye absorbance with an automated vertical light-path microplate photometer. This assay is rapid, highly reproducible and easy to perform, making it ideal for screening large numbers of samples.

The methylene blue staining assay was conducted with minor changes to a previously described protocol (Oliver et al. 1989). Borate buffer stock solution (2×) was prepared by dissolving 38.1 g of Di-sodium tetraborate (AnalaR® BDH Laboratory supplies) in 5 litres of Millipure water and the pH adjusted to 8.5 by adding ~ 120 - 140 ml of 1M hydrochloloric acid (HCl). Methylene blue dye was prepared by dissolving 1g of methylene blue powder (BDH Laboratory supplies) in 100 ml of 1 × borate buffer and filtered through Whatman filter paper prior to

use to remove undissolved particulate. Saker elutant was prepared by admixing 98% (v/v) ethanol with 0.1M HCL in 1:1 proportions.

Following microwell growth assay experiments the cells were washed twice with PBS and then fixed with 4% (v/v) formaldehyde in PBS and stored at 4°C. These fixed 24-well plates were inverted to remove excess PBS and tapped dry on to absorbent paper towel. 300µl of methylene blue solution was placed in each of the 24 wells using a Gilson pipette. The plate was left at ambient temperature for 1 hour for the cells to take up the methylene blue dye. Excess methylene blue solution was poured off and the plate was then submerged in 5 consecutive plastic containers of borate buffer, to remove all traces of unincorporated methylene blue. The plate was once again tapped dry, and 300ml of Saker elutant was added to each well. The plate was then left on a shaker platform (Luckham model 804) for 1 hour for elution to take place.

The 24-well plate format was chosen so as to accommodate a reasonable starting cell number (1×10^3) and also to allow the wells to be easily observed using a phase contrast microscope. However the available microplate reader did not accommodate 24-well plates. Hence eluted dye samples from the 24-well plates were transferred in replicate to 96-well plates before measurements were made.

For each 24-well plate, a 96-well plate was labelled and prepared with the first blank row filled with 100µl of Saker elutant. The furthest row on the left of each plate was used as the plate reader standard. Its value was subtracted from the reading of the rest of the rows. The eluted dye solution from each well was transferred in triplicate into a 96 well plate at 100µl/well (e.g. the contents of each well of the 24 well plate was transferred into 3 wells of a 96-well plate. If the colour was found to be too intense for the plate reader to measure the eluted

cell solution was diluted by a factor of 4 or 8, and 100µl of the diluted elutant was added to the 96-well plates in triplicate. The 96-well plates were then returned to the platform shaker for a further 10 minutes to ensure adequate mixing of diluted solutions and to remove any bubbles created during the pipetting process. To check that all bubbles had been removed the plates were viewed on a light box (Kenro® lightbox – Swindon). The plates were then loaded onto a microplate reader (Dynatech laboratories MRX) and the relative absorbance of light at 650 nm wavelength measured against the Saker elutant as standard. The numerical output from the plate reader was analysed with a spreadsheet package in Microsoft Excel.

2.5.3 Co-culture growth assays

To investigate the effect of tumour-stromal interactions, in an *in vitro* setting, a series of co-culture experiments were performed using a cell culture insert and multiwell system (cells were deposited into two compartments in each well). The method used was as follows:

The cell culture inserts consisted of 0.4µm pore sized polyethylene terephthalate membranes ($1.6x10^6$ pores $/cm^2$) (BD Falcon™) with cells deposited on and below the membrane in each well. The pore size chosen prevents physical contact by inhibiting migration of cells from the upper to the lower chamber but allows free exchange of nutrients and growth factors (Figures 2.3 and 2.4).

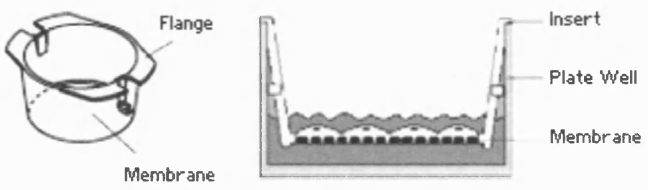


Figure 2.3. Diagram of the co-culture tissue culture dish. Co-culture inserts are constructed of plastic sides with a microporous membrane on the base which is made of polyethylene terephthalate. The insert is suspended inside the corresponding culture dish by means of a lip/flange at the top. (taken from diagram in the instructions protocol, Nalge Nunc International).

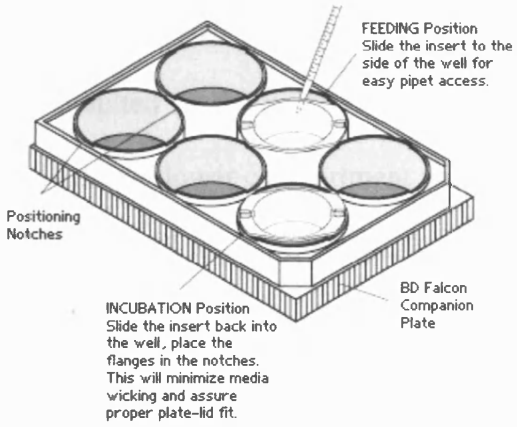


Figure 2.4. Co-culture assays. Separate populations of cells can be grown on the upper surface of the insert and on the base of the culture dish, kept physically separated but allowing the passage of macromolecules through the porous membrane. (taken from diagram in the instructions protocol, Nalge Nunc International).

Prior to seeding, 1ml of pre-warmed culture medium was added to each well of the 24-well plate. The plate was then placed in either a 37°C or 33.5°C incubator with a humidified atmosphere of 5% (v/v) CO_2 for 20 minutes before seeding to equilibrate the pH. Cells were added to the pre-warmed culture media in the 24-well plates and left for minimum of 2 hours to attach.

Cell culture inserts were rehydrated in 2ml of DMEM for 2 hours at room temperature prior to use. Following rehydration, the medium was removed and the required numbers of cells (depending on experiment, usually 1×10^3 cells /insert) were added into each insert chamber in 1ml of its appropriate medium. The seeded inserts were then gently placed in corresponding wells of the 24-well plate. After 24 hours, medium was removed from each compartment and replaced with fresh medium, and thereafter every 2 days. All the experiments were carried out in triplicate. Controls consisted of wells with cells seeded in the lower compartment and cell culture inserts without cells. The end point was determined when the cells, in the lower chamber below the inserts, in whichever experimental condition resulted in the most rapid growth to become 80-90% confluent. All target cells in the lower compartment were then washed and fixed in 4% (v/v) formaldehyde in PBS for 20 minutes after the inserts containing the effector cell had been removed. Cell numbers were measured by the modified methylene blue staining method. All measurements were done in triplicate (described in Chapter 2.5.2)

2.5.4 In vitro endothelial cell differentiation assays

To investigate the differentiation properties of the unaltered and immortalized human mammary endothelial cells, *in vitro* assays were performed to assess the ability of the cells to form primitive microcapillaries on Matrigel.

Aliquoted vials of frozen matrigel and growth factor reduced Matrigel (GFR) were defrosted on ice at 4°C overnight. The required numbers of wells of a 24 well plate were coated with 300µl of Matrigel ensuring all air bubbles were removed (using a heated needle). The Matrigel was left to gel for 30 minutes at

37°C. The total volume was made up to 500µl with media and the plates were incubated for up to 72 hours at 37°C in a humidified atmosphere of 5% (v/v) CO_2 with regular media changes to remove residue and toxic contaminants. Endothelial cells were plated out at densities of $1\text{-}3\times10^3$ cells/well onto either GFR Matrigel or Matrigel in complete EGM-2 endothelial growth media. The wells were visualised under a phase contrast light microscope at hourly time points and images were recorded electronically.

2.6 Manufacture of heterologous 3-D co-culture models and tumour spheroids

To investigate direct cell-cell interactions various 3-D cell culture methods were used. They included microcarrier beads, liquid overlay cultures, a rotary cell culture system and prefabricated 3-D scaffolds.

2.6.1 Microcarrier beads

Cytodex-3 microcarrier beads (Sigma) are dextran beads (133-215µm) coated with denatured porcine-skin collagen bound to the surface. Before use the beads were prepared by swelling and hydrating in 50ml/g of calcium and magnesium free phosphate-buffered saline (CMF-PBS, Biowhittaker) and autoclaved at 120°C. The beads were then sterilized overnight in 70% (v/v) ethanol. Prior to use, the beads were washed three times in CMF-PBS and once in growth culture medium.

5×10^6 cells were added to 5 ml of bead containing solution (1.5×10^5 beads). The beads and cells were transferred to a 125-ml Wheaton Magna Flex Spinner Flask (Wheaton Scientific), containing 75 ml of complete medium, and

incubated under constant stirring 30 revolutions per minute (rpm) in a humidified atmosphere of 5% (v/v) CO_2 at 37°C overnight to attach. The medium was replaced with fresh medium every fourth day by letting the beads settle and changing 50ml of medium. To create a heterologous co-culture spheroid other cell types were added either in free cell suspension or as pre-seeded microcarrier beads.

2.6.2 Liquid overlay cultures

Simple liquid overlay plates and cultures were prepared by inserting 500µl, 300µl, or 100µl of agarose, fibrin, collagen or matrigel into wells of a 24, 48 or 96-well plate (Becton Dickinson Labware) respectively. The preparations of individual substrates are described in Chapter 2.2.2. Substrates were allowed to gel and solidify before the required cells were plated. Cells were counted using a haemocytometer and were added to the substrate-coated wells and allowed to attach for 4 to 5 hours at 37°C. For heterologous spheroid manufacture, cell suspensions (approx. 1×10^4 cells) or pre-seeded microcarrier beads (1×10^4 cells seeded on 5×10^3 beads) were added and mixed with the substrates (500µl) before being allowed to gel and finally applied to a 24-well plate. Then 2ml of growth medium was added to produce the liquid overlay. Following an overnight incubation with the liquid overlay, media were replaced daily. For extended cultures (>2 weeks); a further 2ml of fresh medium was added on the second day. This allowed for replacement of spent medium at the air liquid interface without disturbing the cellular layer on the gel surface.

2.6.3 Prefabricated 3-D scaffolds

Small (approximately 5mm × 3mm × 0.039 cm^3 in size) 3-D biodegradeable pre-engineered scaffolds (BD™ Three Dimensional Open-Cell Polylactic Acid (OPLA®) Scaffolds, BD Biosciences) were seeded with cells using two different methods: still and dynamic.

For the still seeding method a cell suspension containing approximately $5.0x10^4 - 2.5x10^5$ cells in 250µl of growth medium (of each cell type) was added to a scaffold placed in a 30ml universal container. The scaffolds which had adsorbed the medium plus cells were gently transferred to 96-well plates containing 250µl of growth medium. In some experiments the seeded scaffolds were coated in Matrigel or serum. 200µl of liquid Matrigel at 4°C was added to the seeded scaffolds before being transferred to the wells.

For dynamic seeding the tube containing the scaffold was seeded with cells and then incubated with gentle agitation (~50–100 rpm) on an orbital shaker (Stuart Scientific) at 37°C in a gassed incubator for between 2-24 hours depending on the cell type. To maintain the pH during incubation a further 250µl of medium was added after 8 hours. After incubation with agitation was completed, the seeded scaffold was gently placed into a well of a 96-well plate containing 250µl of media for the remainder of the experiment.

Seeded scaffolds were also placed in liquid overlay culture, to further promote growth and differentiation. 5.0×10^4 cells were seeded onto the scaffolds using the dynamic seeding method and then mixed with 300µl of Matrigel before being solidified in wells of a 48-well plate. Liquid overlays were made as described in Chapter 2.6.2. Medium was changed every other day. At

fixed time points scaffolds were removed and fixed in 4% (w/v) formaldehyde for sectioning and immunohistochemistry.

2.6.4 Rotary cell culture system

Rotary cell culture system RCCS-4D (Cellon), 10ml and 50ml high aspect ratio vessel bioreactors were used for the manufacture of heterologous tumour spheroids (Figure 2.5). 5×10^6 cell suspension or $1.5\mathbf{x}10^5$ cells pre-seeded on Cytodex-3 microcarrier beads (depending on study group) were inserted into either a 50ml or 10ml cylindrical culture vessel. This was then filled completely with growth medium making sure all air bubbles were removed. The vessel was then attached to the rotator base and rotated about the horizontal axis. The disposable vessels were rotated at 26 rpm at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 and 95% air. Oxygen supply and carbon dioxide removal was achieved through a gas permeable silicone rubber membrane that forms one surface of the cylindrical vessel. Cells were cultured for fixed time points or alternatively until the desirable spheroid sizes were manufactured. Spheroids meeting the size criteria (1-4 mm) were carefully removed from the culture vessel through access ports using a syringe. Spheroids were either fixed in 10% formaldehyde or snap frozen for histology. They were also used for liquid overlay and *in vivo* studies. The cryopreserved or fixed spheroids were processed for immunohistochemistry as described in Chapter 2.7.

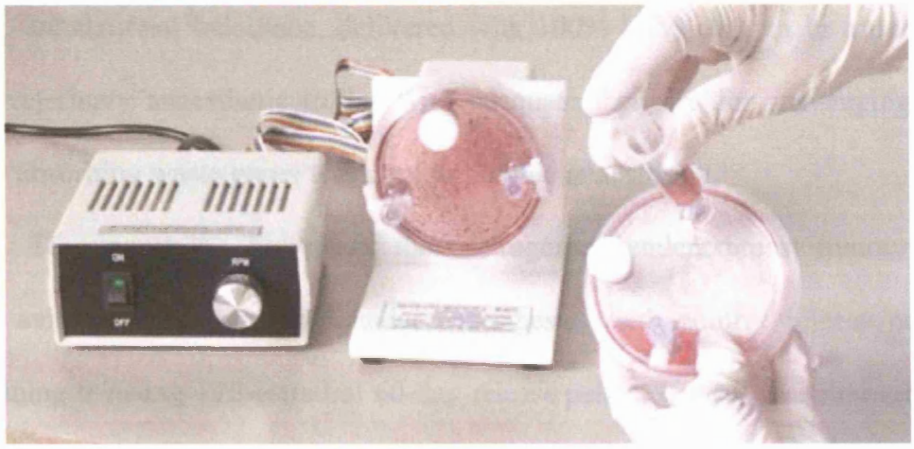


Figure 2.5. Rotary Cell Culture System. Microgravity bioreactor allows the growth of complex three-dimensional tissue models. Picture taken from Synthecon™ showing power source, complete assembly and on the right the harvesting of spheroids from a vessel using a syringe.

2.7 Xenotransplantation

2.7.1 Animals and husbandry

Experimental animals

The mice used in all xenograft experiments were bred on site in the facilities of the Institute of Cancer Research Biological Services Unit from existing populations of *nu/nu* mice. The mice were separated into single sex family groups and only female mice were used for the purposes of the study. The mice were kept in purpose built cages in Maximised laminar flow cages and were allowed free access to food and water.

6 week old female NCr-*nu* athymic nude mice from existing stocks were acclimatized in the laboratory 1 week before experimentation. The animals were housed in microisolator cages, four per cage, in a 12 hour light/dark cycle. The animals received filtered sterilized water and sterile rodent food *ad libitum*. The animals were observed daily and any abnormal clinical signs noted.

Inhalational halothane, delivered with 100% (v/v) oxygen in a purpose built veterinary anaesthetic trolley with adequate facilities for scavenging and safely absorbing waste gases was used to provide anaesthesia.

To support the growth of the oestrogen-dependent breast tumour cell lines, animals were implanted under anaesthesia with control release pellets containing 0.72-mg 17ß-estradiol 60-day release pellets (Innovative Research of America) subcutaneously in the dorsal neck region, under strict asepsis, one week prior to tumour inoculation. This allowed enough time for the mice to recover and for a constant level of oestrogen to be established.

2.7.2 Preparation of cell suspensions and spheroids for inoculation of mice

Cell suspensions

Sub-confluent cells in the exponential growth phase were trypsinised as previously described and collected by centrifugation at 300×*g* for 3 minutes. Normally, unless stated, $5x10^6$ cells were counted on a haemocytometer and resuspended in either a 1:1 mix of Matrigel and serum free medium (SFM) or SFM alone depending on the study group. For threshold cell number xenografts (i.e. cell numbers that will consistently take as a xenograft), MCF-7 cells were inoculated at $5x10^6$ cells /site. For sub-threshold experiments $1x10^5$ cells /site were inoculated. Each animal received 50µl inoculations bilaterally either subcutaneously or into the mammary fat pad. Cells lines were injected in either SFM or together with matrigel in a 1:1 mix of Matrigel and SFM depending on the study group. Anaesthetized mice were injected using a sterile 30-gauge needle under a dissecting microscope into the mammary fat pad or

subcutaneously in the flanks while avoiding leakage from the injection site by pinching the skin at the injection site.

Spheroids

Two methods were used for the manufacture of 3 compartment spheroids to be xenotransplanted into mice; the Rotary cell culture system (RCCS) and liquid overlay cultures. Heterologous 3 compartment spheroids manufactured in the rotary cell culture system were carefully removed from the RCCS vessel at 7 and 14 day time points. Spheroids ranging in size from 1mm to 6mm in diameter were produced and resuspended in either a 1:1 mix of Matrigel and SFM or SFM alone depending on the study group, and inoculated in 50µl/site as described above.

2.7.3 Harvesting and processing of xenografts

Xenograft tissue harvests

The mice were sacrificed by rapid cervical dislocation. Tumours were carefully dissected out whole and removed of skin under aseptic conditions. The tumours were then immediately fixed in either 4% (w/v) formaldehyde for 8-10 hours for preparation of paraffin sections or cryopreserved (snap frozen in liquid nitrogen cooled isopentane) for frozen sectioning. Some xenografts were fixed in 2.5% (v/v) glutaraldehyde for 2-5 hours for preparation of ultra thin sections. For control purposes tissue from other sites were harvested and fixed in a similar manner.

Processing of xenograft tissue for paraffin sections.

A standard dehydration and paraffin wax embedding procedure was performed to process the tumours. The tumours sections were cut on a microtome (Anglia

Scientific AS300 Microtome) set to 3-5μm at room temperature. A standard procedure was used to dewax and rehydrate the tissue sections prior to staining and immunohistochemistry.

Preparation of paraffin blocks. Following fixation the tissue specimens were loaded onto dedicated cassettes and processed through an automated processor (Shandon Citadel 2000 processor) which takes the cassettes through a series of graded alcohol baths to dehydrate the tissues and then into a clearing agent xylene. Hot paraffin was then allowed to permeate the tissues. Tissues processed into paraffin were melted by placing them in a 65°C paraffin bath for 15 minutes. Block molds were warmed on a hot plate. Hot paraffin was then poured into the molds with the melted tissues placed in the molds (Tissue Tek II Wax Embedding Center). Heated forceps were used to prevent the tissues from sticking. When the tissues were in the desired orientation the molds were placed on a cooling plate and the cassette backing was placed on top as a lid. After 30 minutes the hard paraffin block was popped out of the mold. Tissue blocks were stored at room temperature prior to sectioning.

Sectioning paraffin blocks. Formaldehyde fixed paraffin embedded blocks to be sectioned were prepared by placing face down on an ice block for 10 minutes. A water bath of fresh deionized water was also prepared by preheating to 40°C. Following chilling on ice, the block was inserted into the microtome chuck so that the wax block faced the blade and was aligned in the vertical plane. A fresh blade was placed in the microtome and the dial was set to 4μm cut. The block was "faced" by cutting down to the desired tissue plane with the paraffin ribbon discarded. Once the block had began ribboning well then another four sections were cut, picked up with forceps or brush and floated on the surface of the water bath. 3-6 μm thick tissue sections were cut from the paraffin-embedded

blocks.The sections were carefully floated onto the surface of clean Vectabond™ (Vector Laboratories) coated glass slides. If the block was not ribboning well then it was placed back on the ice block to cool and hydrate. Sections were allowed to dry overnight at room temperature or 40°C

Processing of xenograft tissue for frozen sections

Freshly harvested tumours were immediately flash frozen by plunging the tissue specimens into isopentane (BDH Laboratory Supplies) cooled in liquid nitrogen. The frozen specimens were then stored in liquid nitrogen banks prior to processing.

A small mound of Optimal Cutting Temperature compound (OCT, Tissue-Tek) was placed on the cryostat chuck taking care to avoid bubbles. The base of the OCT was then frozen using Cyto-Cool freeze spray before placing another small mound of OCT on top. The tissue specimen was then embedded in the OCT making certain it was completely covered and thus protected from freeze-drying. Another drop of OCT was then added to ensure full coverage. The chuck was then frozen by immersing it and the specimen mounted upon it in liquid nitrogen or pre-cooled isopentane. Once the liquid nitrogen had stopped boiling the specimen was solidly frozen and ready to be sectioned. The position of the block was adjusted to align the block with the knife blade. Before cutting sections, the temperature of the block was allowed to equilibrate with the temperature of the cryostat (-20°C) (Bright Cryostat). The tissue block was cut until the desired tissue was exposed. Sections were cut at the desired thickness (usually 3-5 μm) and then transferred directly to clean Vectabond™ coated glass slides. The slides were air dried at room temperature for 1 hour before being covered in aluminium foil and stored at -40°C. Generally, the technically best-

preserved section out of three adjacent sections processed from each block was selected for subsequent staining.

Processing of xenograft tumours for ultra thin sections

Freshly dissected tumours were rinsed in PBS and then cut into very small pieces (1-2mm^2) in fresh electron microscope grade 2.5% (v/v) glutaraldehyde and fixed for a total of 2.5 hours. The tissue samples were then post fixed in 2% osmium tetroxide in 100mM cacodylate buffer (1 hour on ice).The tissue was then embedded in Epon resin and cut (0.5μm) in an ultramicrotome before being stained with 0.6% toluidene blue in 0.3% (w/v) sodium bicarbonate.

Coating of glass slides

Vectabond™ (Vector Laboratories) treated slides were used to significantly enhance the adherence of frozen sections, paraffin-embedded tissue sections and cell preparations to glass slides and coverslips. This product does not coat slides with a glue or denatured protein, but chemically modifies the glass to form a highly adherent surface.

High quality glass slides (BDH Laboratory Supplies) were thoroughly cleaned in detergent and then rinsed in water before being placed in metal slide racks (25 slots). The slides were then immersed in 400ml of acetone (BDH Laboratory Supplies) for 5 minutes. Vectabond™ Reagent solution was prepared just prior to use by adding 7ml of Vectabond™ Reagent treatment solution (entire contents) to 350ml of acetone and stirring well. Slides were removed from acetone; the rack was tapped several times to drain and immediately placed in the pre-prepared Vectabond™ Reagent solution for 5 minutes. The slides were then removed and excess reagent was eliminated by gently dipping the rack several times over 30 seconds in deionized or distilled water taking care not to create bubbles. The water was changed after every 5 racks. The rack of slides was then

removed for drying. Gentle agitation or tapping of the rack before drying was performed to decrease water droplets and their resulting spots. The slides were air dried thoroughly at room temperature or at 37°C. Slides were used immediately after drying, or stored in a box at room temperature for later use. The 357ml of Vectabond™ Reagent mixture was sufficient to treat at least 500 slides.

2.8 Immunohistochemistry

2.8.1 Haematoxylin and eosin staining

Formaldehyde fixed paraffin embedded sections were deparaffinized in 3 changes of xylene (BDH Laboratory Supplies) for 2 minutes each. They were then rinsed in 2 changes of 100% (v/v) ethanol for 1 minute each followed by 95% (v/v) and 80% (v/v) ethanol for 1 minute each. They were then washed thoroughly in cold running tap water for 1 minute. The sections were then immersed in Harris's Haematoxylin solution (BDH Laboratory Supplies) for 2 minutes followed by rinsing in cold running tap water for 1 minute. This was followed by immersion in Acid alcohol (76.6% (v/v) ethanol, 1/300 (v/v) HCl (230ml 100% (v/v) ethanol, 70 ml H_2O, 1ml of 1M HCl)) for 5-10 seconds before being washed in cold running tap water for 5 minutes The sections were then dehydrated in 80% (v/v) ethanol followed by a 30 second immersion in Eosin Y solution (Sigma) for 15 seconds. Dehydration was completed in 2 changes of 95% (v/v) and 100% (v/v) ethanol, 1 minute each. The sections were finally cleared in 3 changes of xylene for 2 minutes each. Sections remained in the xylene until they were coverslipped.

2.8.2 Section preparation

Formaldehyde fixed paraffin embedded tissue sections

Prior to deparaffinization, the sections were placed in a 55°C oven for ten minutes to melt the paraffin. The slides were deparaffinized in two changes of xylene for 5 minutes each, 100% (v/v) ethanol two changes for 3 minutes each and finally transferred to 95% (v/v) ethanol for 3 minutes. Following this the endogenous peroxidase activity was blocked by incubating sections in 10% (v/v) H_2O_2 solution (BDH Laboratory Supplies) in methanol for 10 minutes. The slides were then rinsed twice in PBS for 5 minutes each time. The sections were now ready for antigen retrieval.

Frozen tissue sections

Frozen sections were removed from the -40°C fridge, allowed to thaw and rinsed twice in PBS to remove the tissue freezing matrix. Care was taken to avoid dehydration of the sections, before being fixed in ice cold 100% (v/v) methanol for 7 minutes. The slides were then washed twice in PBS for 5 minutes each time.

2.8.3 Antigen retrieval techniques

Antigen retrieval mechanisms works by causing either acid- or base-catalysed hydrolysis of methylene cross-links between protein molecules, which are introduced by fixation in formaldehyde, and possibly also by removing calcium ions (citrate and EDTA are common ingredients of retrieval solutions). Most antigens are retrieved by heating in either acidified (pH 1) or alkaline (pH 8-10) water, some by either or neither of these. Less extreme pH levels (e.g. 6) are often effective and less likely to cause section losses.

Enzymatic digestion

For enzymatic digestion, 0.1% (w/v) chymotrypsin (Sigma) and 0.1% (w/v) calcium chloride (Sigma) were dissolved in distilled water and the pH of the solution was adjusted to 7.8 using 0.1N sodium hydroxide. 50ml of the solution was then poured into a Coplin jar and warmed to 37°C in a water bath. Once the temperature of the solution had stabilized at 37°C the slides were transferred into the jar and incubated at 37°C for 10 minutes. The slides were then washed in cold running tap water.

Heat treatment

Two methods were adopted in this study. The first method involved the use of a simple commercially available pressure cooker (John Lewis). Slides were slotted into a metal slide rack and placed in the open pressure cooker containing 3000ml of vigorously boiling citrate buffer (pH of 6.0). The lid of the pressure cooker was the replaced and heated until the pressure indicator signal was turned on (115°C). Once this had occurred the slides were left for 2 minutes of pressure cooking before the pressure was released and the slides were washed in cold running tap water.

The second method involved the use a standard commercially available 700 kW microwave. Slides for staining were placed in a metal slide rack and then place in a microwaveable plastic container containing 700ml of citrate buffer at a pH of 6.0. The container was sealed with a piece of Clingfilm and then pierced with a fork several times. The container was placed in the microwave (Sharp) and heated for 18 minutes at full power (700kW). Following this the container was removed from the microwave and left to cool for 20 minutes at room temperature with the Clingfilm taken off. The slides were then washed in cold running tap water.

2.8.4 Antigen visualization method

Immunoperoxidase staining

Immunoperoxidase staining was performed using a modified version of the 3 step protocol described in the Vectastain Universal Elite kit (Vector Laboratories). If antibody staining required antigen retrieval to unmask the antigenic epitope it was performed as described above, the method used being dependent on the antigen/antibody combination chosen.

Following antigen retrieval the slides were labelled with a solvent resistant pen and the tissue sections were demarcated with a grease pen (Dako). To overcome the problem of non specific staining the sections were blocked for 20 minutes by applying 200µl of a diluted solution of normal horse serum (50µl of normal horse serum, provided in the kit, mixed with 1ml of PBS) to the sections and then tipping off without washing.

Primary antibody was diluted in antibody diluent (PBS with 10% (v/v) FCS) until the desired working concentration was obtained. 200µl of the diluted antibody solution was applied to each tissue sections on the slide. The sections were incubated for 1 hour at room temperature in a humidified chamber taking care not to allow the sections to dry out. The sections were then rinsed 3 times in PBS, 2 minutes each time.

After washing, the Universal biotinylated secondary antibody solution (prepared by adding 100µl of normal horse serum and 100µl of biotinylated antibody, both provided in the kit, to 1ml of PBS) was applied to the tissue sections on the slide (200µl/section) and incubated for 45 minutes at room temperature. Again sections were rinsed 3 times in PBS, 2 minutes each time. In the third step an Avidin-Complex Solution (prepared by adding 100µl of reagent

A and 100µl of reagent B, both provided, to 1ml of PBS) prepared at least 30 minutes prior, was applied to the tissue sections on the slide and incubated for 45 minutes at room temperature. Again sections were rinsed 3 times in PBS, 2 minutes each time.

Finally the diaminobenzidene substrate solution (DAB) was prepared by adding 1 drop of DAB chromogen (50µg) to every 1 ml of DAB buffer. Slides were allowed to incubate for 5 minutes or until the desired colour intensity was reached. The sections were then washed thoroughly in cold running water. The slides were then counterstained in Harris's haematoxylin solution for 60 seconds before being rinsed in water and then dipped quickly twice in acid alcohol. Finally the sections were dehydrated through 4 changes of ethanol (95%, 95%, 100% and 100%) and cleared in 3 changes of xylene. The slides were mounted with a 22×50mm coverslips (BDH Laboratory Supplies) using 10µl of a 1:1 mixture of Hydromount (National Diagnostics) and Citifluor antifadant AF1 (Citifluor Ltd). Excess mounting media was removed with fibre-free paper, without disturbing the coverslip. The primary antibodies were omitted to provide a negative control, and human tonsil sections were used as positive controls.

2.8.5 Indirect immunofluorescence

Incubation with primary antibodies

The primary antibody was diluted to the appropriate desired concentration in antibody diluent (PBS with 10% (v/v) FCS). 200µl of the primary antibody solution was applied to each section in a humid chamber taking care not to allow sections to dry out and incubated for 1 hour at room temperature. Primary antibodies used were either mouse or rat monoclonals. Source and details of all

primary antibodies used are given in Chapter 3. Following incubation the antibody solution was then removed and the sections were washed three times in PBS, 5 minutes each wash.

For the examination of two or three different antigens in the same cell, a double or triple immunofluorescence procedure was used. Sections were incubated simultaneously with the required number of primary antibodies, provided they were monospecific and could be distinguished with secondary antibodies conjugated to different fluorochromes (or with primary antibodies directly conjugated to different fluorochromes).

Incubation with secondary antibodies

The sections were now incubated with secondary antibodies conjugated to a fluorochrome; e.g. anti-mouse IgG FITC or TRITC, depending on the donor species of the primary antibody and the desired fluorochrome. Conjugated secondary antibodies cross-adsorbed against human tissue to minimize non-specific binding and affinity-purified against the appropriate primary antibody isotype were used. The type and source of the secondary antibodies used are given in Table 2.3. The secondary antibody was diluted to the appropriate working concentration in antibody diluent and 200µl of the diluted antibody solution was applied to each section. The sections were incubated for 1 hour at room temperature and then washed again three times in PBS, 5 minutes each wash. Single antibody staining controls consisted of sections stained with only the secondary antibody (no first antibody). Secondary antibodies of different species and antibody subclasses were chosen so that the primary antibody staining could be separately detected.

Detection and visualization of multiple antigens were performed using appropriate mixtures of species, isotype or subclass specific fluorophore

conjugated secondary antibodies. Controls for multiple antigen visualization involved omitting individual primary antibodies as well as sections stained only with the combination of secondary antibodies.

Species	Species specificity	Class	Fluorochrome	Dilution	Source
Sheep	mouse	IgG1	FITC	1:64	Sigma
Sheep	mouse	IgG1	TRITC	1:100	Dako
Goat	rat	IgG2a	FITC	1:500	Serotec

Table 2.3. Table showing the secondary antibodies used.

2.8.6 Direct immunofluorescence

A direct immunofluorescence system was also used (Zenon, Molecular Probes) (Figure 2.6). This consists of a kit whereby a primary antibody can be directly conjugated with an Alexa fluorophore of choice. Zenon™ Human IgG Labeling Kits contains a Zenon human IgG labelling reagent (Component A) and a Zenon blocking reagent (human IgG) (Component B). Subclass specific Zenon reagents for mouse IgG1 and IgG2a were used in the study. Alexa fluors emitting at 488nm (FITC equivalent) and 555 nm (TRITC equivalent) were used.

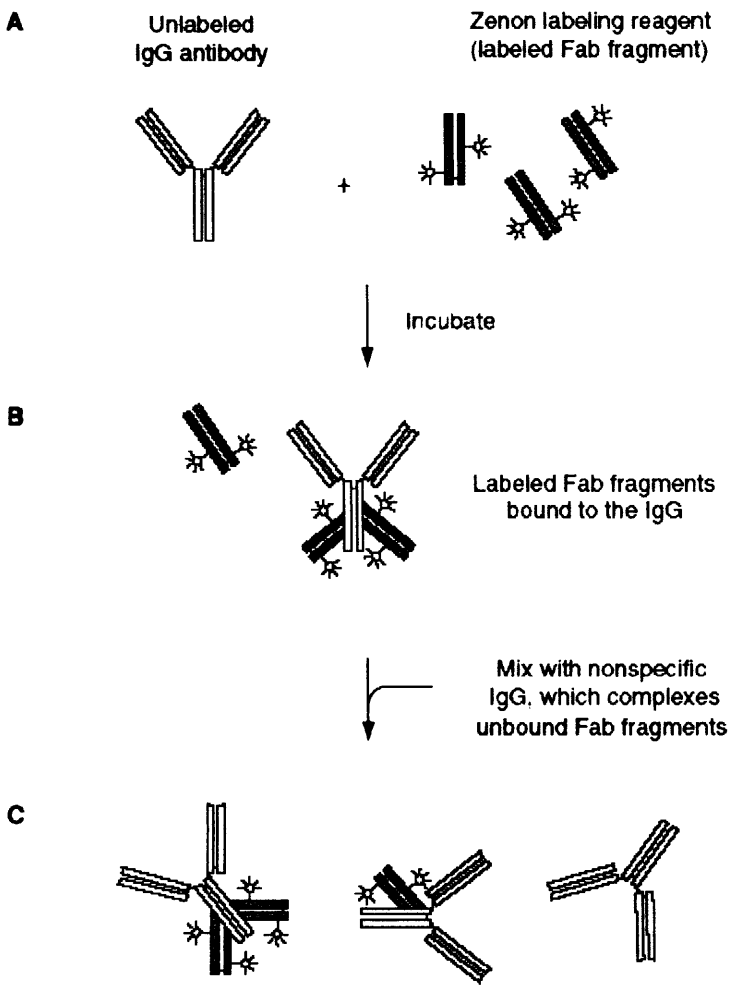


Figure 2.6. The Zenon antibody labelling scheme. An unlabelled IgG is incubated with the Zenon labelling reagent, which contains a fluorophore-labeled Fab fragment (A). The labeled Fab fragment binds to the Fc portion of the IgG antibody (B), and excess Fab fragment is neutralized by the addition of a nonspecific IgG (C). The addition of nonspecific IgG prevents cross-labeling of the Fab fragment in experiments where multiple primary antibodiesof the same type are present. (diagram modified from a picture taken from the product information sheet, Molecular Probes).

1μg of the required primary antibody was diluted in PBS to make an approximate total antibody solution of 5-10μl. 5μl of the Zenon class specific mouse IgG labelling reagent (Fab, Component A) was added to the antibody solution. This mixture was incubated for 5 minutes at room temperature. Then 5μl of the Zenon

blocking reagent was added (Fcr, Component B) to the reaction mixture. Again the solution was incubated for 5 minutes at room temperature. The reaction mixture was then diluted to the appropriate working concentration in antibody diluent equivalent to an antibody concentration of 0.5-0.1μg/ml and applied to sections within 30 minutes of manufacture.

Frozen tissue sections to be labelled with the Zenon reagents were fixed in 4% (w/v) formaldehyde solution in PBS for 15 minutes at room temperature prior to antibody labelling. The sections were then washed two times with PBS for 5 minutes each. The tissue sections were then permeabilized by incubating in PBS containing 0.1% (v/v) Triton® X-100 (Sigma) for 5 minutes at room temperature. The pre-prepared Zenon labelling complex was diluted to the desired working concentration in antibody diluent, and applied in sufficient volume (200μl) to immerse the cell sample. The slides were incubated for 40–60 minutes at room temperature in the dark protecting from light during the incubation. When labelling was complete, the sections were washed twice with PBS for 5 minutes each. When staining with more than one Zenon labelling complex, all complexes were combined into a single staining solution.

Following antibody labelling, a second fixation of the section was performed in 4% (w/v) formaldehyde solution in PBS for 15 minutes at room temperature. When the fixation was complete, the sections were washed twice with PBS for 5 minutes each. This second fixation step was carried out because the Zenon Fab fragment is not covalently coupled to the primary antibody and so can dissociate with time and even transfer to another primary antibody in multicolour applications.

Following this the sections, if required, were counterstained with a DNA stain Hoechst 33258 (5μg/ml) and washed with PBS after any additional staining

procedures. Controls consisted of sections that were stained with the Zenon reagents only (A + B) (no primary antibody added). The sections were mounted with coverslips using 10µl of a 1:1 mixture of Hydromount (National Diagnostics) and Citifluor antifadant AF1 (Citifluor Ltd). Excess mounting media was removed with fibre-free paper, without disturbing the coverslip.

2.8.7 Fluorescence staining of live cells on coverslips

Live cells for labelling were plated onto sterile coverslips (13mm, BDH Laboratory Supplies) placed in 24-well plates at a density of 4×10^4 cells in 2ml of their respective growth medium. When the cells reached approximately 80% confluency, they were washed twice in 2ml of PBS/well, fixed with cold methanol (-20°C) for 7 minutes, washed three times with 2ml of PBS and stored in PBS at 4°C.

Prior to antibody labelling the PBS was drained from the 24-well plates. The cells were first blocked with 500µl /well of 0.5% (w/v) BSA in PBS for 30 minutes at 37°C to minimize non-specific adsorption of the antibodies to the coverslips. The blocking buffer was removed by holding each coverslip on its edge with forceps and draining it onto a sheet of fibre-free paper. Fixed cells were stained by both indirect and direct immunofluorescence as described above.

The coverslips were mounted by inverting then onto a slide containing 10µl of mounting media consisting of a 1:1 mixture of Hydromount and Citifluor antifadant AF1. The edges of each coverslip were sealed with regular transparent nail polish and allowed to dry for 3 minutes to provide semi-permanent preparations. Image data sets depicting immunofluorescence labelling were collected and processed as described in Chapter 2.8.7.

2.8.8 Visualisation and image processing

Light microscopy

Brightfield. Image data files of H&E and immunoperoxidase labelled specimens were collected with a high resolution Leaf camera (ISS) mounted over an Axiophot microscope (Zeiss) fitted with a x10 NA 0.3 ,a x20 NA 0.5 or x40 NA 0.75 objective as described elsewhere (Entwistle 1998, Entwistle 2003). 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 plug-in with Image-Pro Plus (Media Cybernetics). 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 Adobe Photoshop (Adobe Systems Inc). The resulting data set was then subjected to contrast stretching and saved as the 24 bit RGB TIFF files.

Phase contrast. A Nikon camera connected to an inverted Zeiss light microscope fitted with x10 NA 0.3 and x20 NA 0.5 phase contrast objectives was used to take greyscale pictures of cells in culture and endothelial differentiation assays using Technical Pan Film (Kodak).

Fluorescence microscopy

Image data files depicting immunofluorescent 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 a x10 NA 0.3, 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 by proprietary software (Photonic Science) controlled by Image Pro Plus v 4.01. The fluorophores were visualised with various filter combinations, or modes, as follows: Hoechst 33258 using a using a blue bandpass emission filter and ultraviolet illumination, fluorescein isothiocyanate (FITC), using a green bandpass emission filter and blue illumination and tetramethylrhodamine isothiocyanate (TRITC) using a red / near infrared bandpass emission filter and green illumination. The emission from each fluorophore into the other modes, crosstalk, was insignificant, <0.1% (A Entwistle, personal communication). Images depicting multiple immunofluorescences 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 triple immunofluorescent labelling with additional Hoechst labelling were made by assigning the emissions from: Hoechst (exclusively nuclear) to blue, red to TRITC and green to FITC. 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. The protocol used in this study conforms to and meets the requirements of a criterion set in a recently published review on the correct use of a light microscope (Dell and Vale 2004).

Time lapse microscopy

In some experiments sequential phase contrast, brightfield and immunofluorescence images were recorded using an automated time lapse system (Olympus CK2 microscope) controlled by Image Pro plus 4.0 microscopy program. These images were collated to produce short sequential movies visualized on Real Media Player.

Chapter 3

Characterisation of immortalized human mammary stromal cell lines

3.1 Introduction

Tumour-stromal interaction studies have to date relied on the continuous collection and explanation of biopsies, a relatively time consuming process. In addition, normal breast biopsies are limited in supply and long term experiments, or experiments requiring larger quantities of cells, have also been hampered by the difficulty of expanding the different cell populations, especially stromal cells. To overcome the problems of using normal primary tissue, methods to extend the lifespan of the explanted cells have been developed. Ectopic expression of the catalytic subunit of human telomerase (hTERT), as well as Simian virus (SV40) large T antigen oncogene, has been shown to extend life of normal human somatic cells, including fibroblasts and endothelial cells to confer immortality (Hahn et al. 1999, Elenbass et al. 2001). The immortalized human mammary stromal cell lines used in this study were cells previously derived by O'Hare et al. (2001) using the above method.

Derivation and properties of the immortalized stromal cell lines

The transduction and establishment of conditionally immortalized human mammary stromal cell lines by O'Hare et al. (2001), from primary cells, are explained in Figures 3.1 and 3.2. Primary mammary microvascular endothelial cells (1° HMME) were immunomagnetically isolated, from normal reduction mammoplasties (two donors), by using the QBEND-40 monoclonal antibody against thrombomodulin, a marker of endothelial cells. The primary mammary

interlobular fibroblasts (1° HMF) were established from 'fines' (40μm filter passing single cells and small cell clumps) from an enzymatically dissociated normal reduction mammoplasty (one donor). Fibroblasts were separated from other cell populations in the digested mix by selective growth in a medium (DMEM /FCS) which did not support the growth of mammary endothelial or epithelial cells. The conditionally immortalized cell lines were generated by transduction of a temperature sensitive (tsLT) mutant (U19tsA58) of SV40 LT antigen, whereas the wild type variants were generated by transduction of a wild type LT antigen (U19). The conditionally immortalized cell lines varied in the timing and order of the oncogene transductions. The variations in order and timing of transduction, however, did influence the genomic stability with the cells differing slightly in properties (Table 3.1). None of the cell lines displayed anchorage independent growth, a characteristic of transformed cells at early passage. However HMF 3B, unlike the other early passage cells, grew at 37°C. HMF 3C became spontaneously transformed at about 60 population doublings (PD) as demonstrated by its capacity after this time to grow in soft agar. HMF 3A remained stable maintaining temperature sensitivity with no evidence of overt transformation. HMF 3D lost this temperature sensitive status at late passage (>60PD) but did not show anchorage independent growth. HMF 3wt and HMME 7wt have been passaged in excess of 100 PD without evidence of transformation as have the temperature sensitive endothelial cell lines (HMME 2 and HMME 7).

Recent data (PS Jat, personal communication) have shown that the HMF B line but not HMF D has lost the temperature sensitive A58 (tsA58) mutation, probably as the result of a reverse transcriptase caused back mutation, thus explaining its loss of temperature sensitivity.

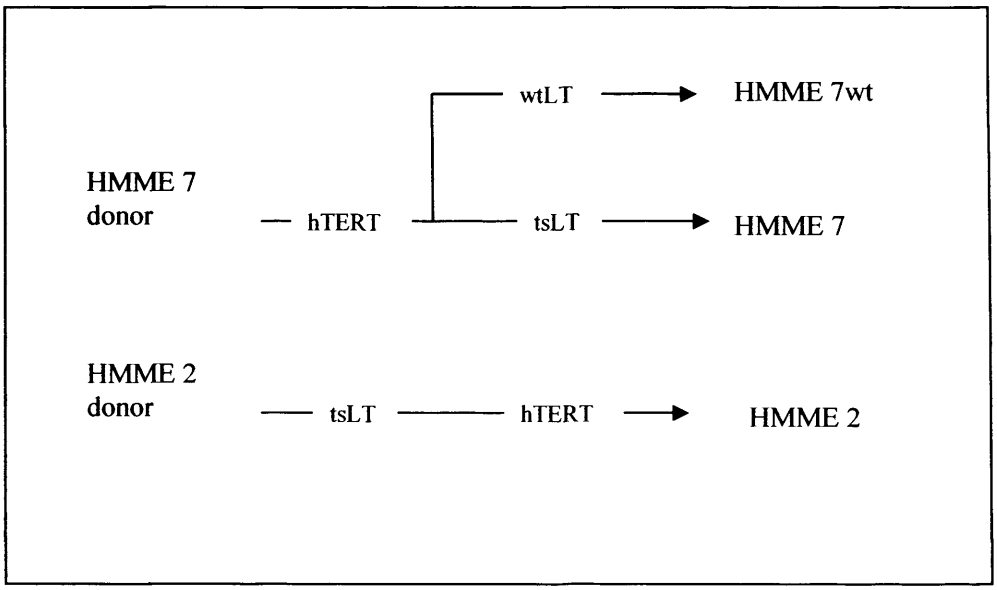


Figure 3.1. Derivation of the immortalized human mammary endothelial cell lines (HMME). The immortalized endothelial cells were derived from two donors. The cell lines differed in the order and timing of the oncogene transductions. HMME 7wt is a wild type LT variant of HMME 7 which grows well at 37.5°C.

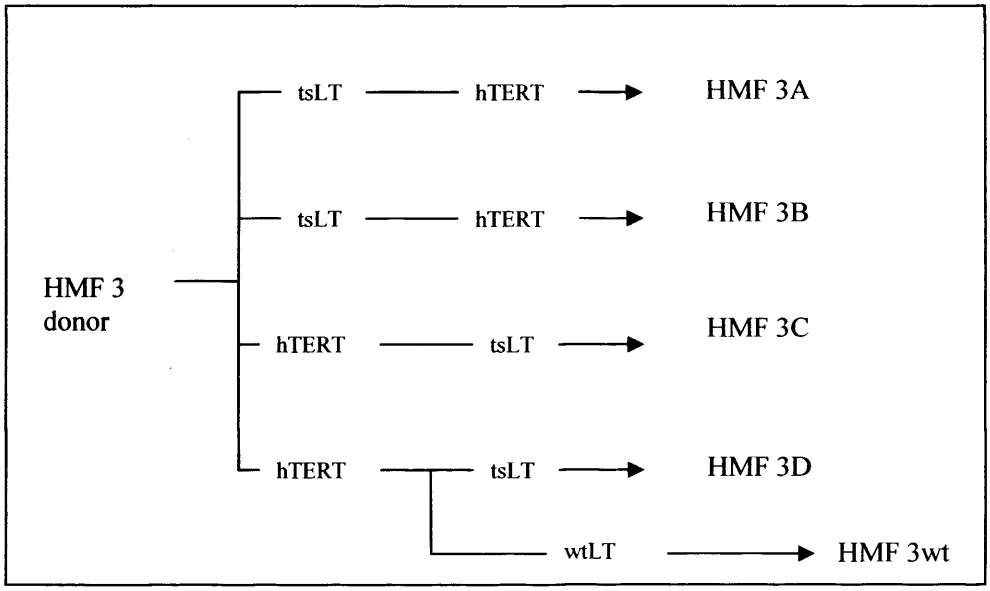


Figure 3.2. Derivation of the immortalized human mammary fibroblast cell lines (HMF). The immortalized fibroblasts were derived from normal breast tissue removed from one donor-HMF 3. The cell lines differed in the order and timing of the oncogene transductions. HMF 3wt is a wild type LT variant of HMF-3 which grows well at 37.5°C.

Immortalized Stromal Cell Line	Temperature sensitivity	Anchorage Independent Growth
HMF 3A	**YES**	**NO**
HMF 3B	**NO**	**NO**
HMF 3C	**YES**	**NO (<60PD) → YES**
HMF 3D	**YES (<60PD) → NO**	**NO**
HMF 3wt	**NO**	**NO**
HMF 3wt *ras*	**NO**	**YES**
HMME 2	**YES**	**NO**
HMME 7	**YES**	**NO**
HMME 7wt	**NO**	**NO**

Table 3.1. Table summarizing the temperature sensitivity and transformation status of the immortalized stromal cell lines.

The properties of immortalized cell lines, such as their genotypic and karyotypic stability, may vary depending upon the method of immortalization (e.g. hTERT vs SV40 LT vs HPV viral oncogene) and/or on the order of genetic events (Fauth et al. 2004). Furthermore the oncogenes inserted to generate these immortal cell lines have other properties and abilities which can have a significant effect on the genome of the cell and its phenotype. For example the inactivation of retinoblastoma (pRB) and p53 tumour suppressor genes (TP53) produced by SV40 LT antigen insertion could alter their properties. The abrogation of Rb/p53 cell cycle check point controlling genes release check points so that cells continue to cycle. In addition p53 also senses mutation damage and may trigger apoptosis of damaged cells. Thus its abrogation by T antigen might result in the

accumulation of genomic damage in such cells. The T antigen has recently been reported to have the ability to cause chromosomal aberrations, aneuploidy and transformation by its interaction with Bub1 and Bub3, both components of the spindle checkpoint complex (Cotsiki et al. 2004). On the face of it, hTERT, which stabilises telomeres and prevents cell senescence by telomere erosion, should be a milder method of immortalizing human adult somatic cells which lacks this activity. However it has been shown, repeatedly, that adult human mammary fibroblasts and endothelial cells do not successfully immortalize with hTERT alone. Consequently a combination of hTERT and SV40 LT antigen was used to generate immortalized lines for these cell types.

Characterisation of the immortalized stromal cell lines

The aim of the experiments described in this chapter was to determine whether these cells had retained basic characteristics of normal cellular growth or whether the immortalization procedure had transformed the cells in full or in part. Cells had already been tested for anchorage independent growth which was negative in all early passage cells. However anchorage independent growth represents a relatively extreme aberration and there remained the possibility that the lines were altered in less obvious ways. Tests were carried out to investigate some of the other characteristics of transformation. Some generic characteristics of transformed cells are listed in Table 3.2 (Chang 1986, Hahn et al. 1999).

Criteria of transformation *in vitro*
Increased growth rate
Growth factor insensitivity
Higher confluent density (loss of contact inhibition of growth)
Cellular overlapping (loss of contact inhibition of movement)
Loss of serum dependence
Acquisition of anchorage independent growth (high colony formation in soft agar)
Ability to produce tumours in immunodeficient mice

Table 3.2. Summary of the characteristics representative of transformation. The characteristics are listed with the most extreme phenotypes last.

Significant alterations in their growth properties would imply that their cellular phenotypes had changed to a degree no longer representative of normal mammary fibroblasts and endothelial cells. Therefore the experiments outlined below should provide an indication of whether these stromal cell lines have essentially normal human characteristics or whether they had become tumourigenic as result of partial or full (tumourigenic) transformation. In addition, the results reported in this chapter will help answer the question of whether these cell lines are suitable for use as normal fibroblasts and endothelial cells in the proposed 3 compartment xenograft model and if one or other line is a better model of normality in this context.

Furthermore, monolayer co-culture growth assays together with 3-D *in vitro* differentiation assays were performed to investigate the reciprocal effects of tumour-stromal interactions between the immortalized cell lines and breast tumour epithelial cells. These studies were carried out to confirm the well

recognised reports that tumour-stromal interactions are stimulatory on both growth and differentiation and to establish the extent to which these cell line models reproduce these effects. The ability of these cell lines to survive and form tumours *in vivo* is described later (Chapter 6).

3.2 Growth kinetics

The questions of growth rate, growth factor dependency and serum requirements were addressed in the current study by performing a series of monolayer growth experiments in a variety of growth medium supplements.

3.2.1 Simple growth assays

A series of monolayer growth assays were performed to examine the growth properties of the immortalized endothelial cell lines; HMME 7, HMME 2, HMME 7wt and fibroblasts; HMF 3A, HMF 3B, HMF 3C, HMF 3D, HMF 3wt, HMF 3wt *ras*. HMF and HMME are unaltered subcultures of primary fibroblasts and endothelial cells respectively. The HMF 3wt *ras* was derived by transducing the HMF 3wt with an activated *ras* oncogene as described in Chapter 2.1.3. The main purpose of these experiments was to look for alterations in growth rates, in comparison to unaltered stromal cells, as these are often taken to be one of the criteria representative of transformation (Chang 1986).

Flask assays were used for multipoint growth measurements, as described in Chapter 2.5.1. 1×0^3 cells of each of the cells and cell lines were plated in $25cm^2$ flasks, in triplicate, in their respective growth medium and conditions. Cells were harvested at intervals of 2, 4, 6, 8, 10 and 12 days and counted using a haemocytometer. A minimum of 200 cells were counted for each determination.

The results were tabulated and graphically represented in Figures 3.3 and 3.4. All cell lines were tested were early passage cells (less than passage 30) equivalent to <100 population doublings. Unaltered fibroblasts and endothelial cells were tested at passage 3-6 (<20 PD).

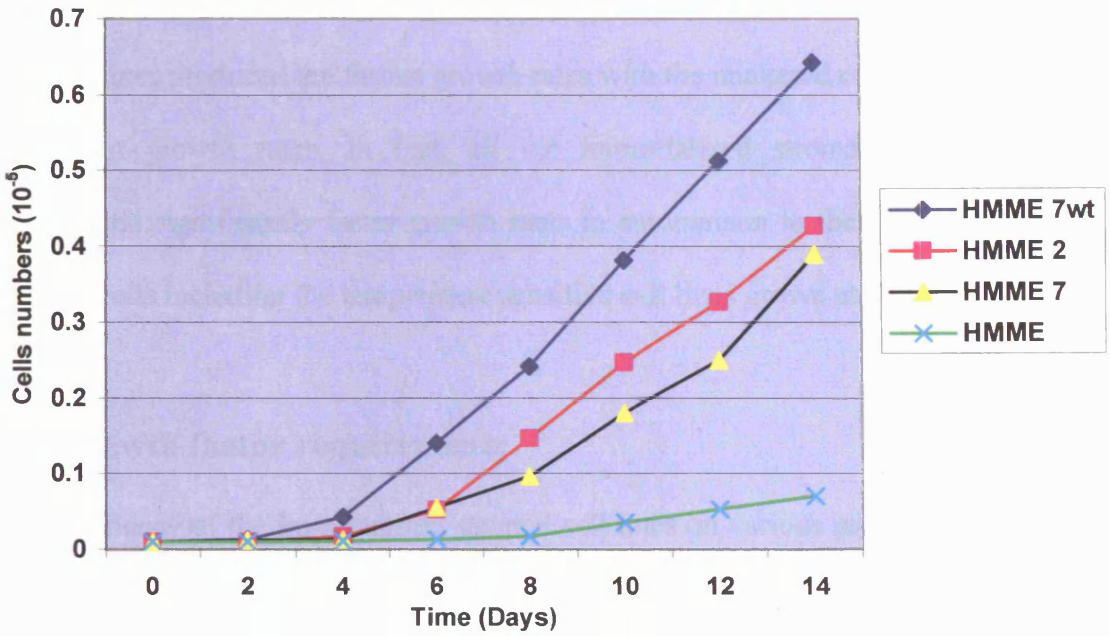


Figure 3.3. Multi-point graphical representation of the growth rates of the immortalized human mammary endothelial cell lines over a two week period. HMME and the HMME 7wt cell line were cultured at 37.5°C with HMME 2 and 7 cell lines grown at 33.5°C. Data shown are means +/- standard deviation (n=3) (shown as error bars) of triplicate measurements.

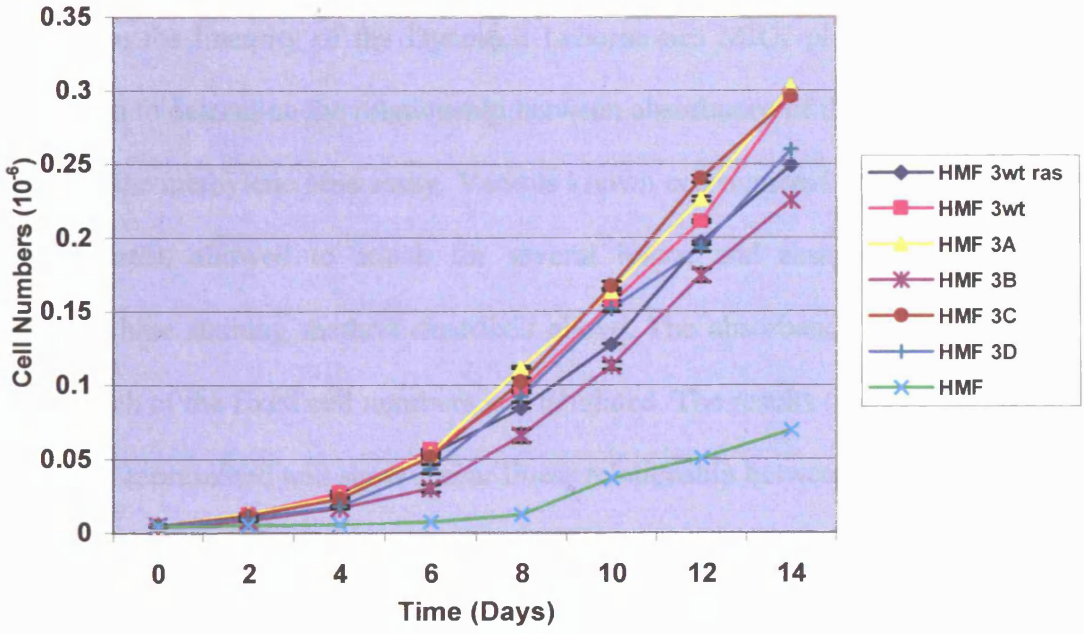


Figure 3.4. Multipoint graphical representation of the growth rates of the immortalized human mammary fibroblast lines over a two week period. HMF, HMF 3wt and HMF 3wt *ras* cell lines were cultured at 37.5 °C with HMF 3A-D cell lines grown at 33.5°C. Data shown are means +/- standard deviation (n=3) (shown as error bars) of triplicate measurements.

The wild type LT variants of both the human mammary endothelial cell and the fibroblast lines produced the fastest growth rates with the unaltered cells showing the slowest growth rates. In fact, all the immortalized stromal cell lines demonstrated significantly faster growth rates in comparison to their respective unaltered cells including the temperature sensitive cell lines grown at 33.5°C.

3.2.2 Growth factor requirements

The dependency of the immortalized stromal cell lines on various growth factors and supplements was also investigated as another criterion of transformation. This was analysed by monolayer growth in microwell plates performed as described in Chapter 2.5.2. Cell numbers were determined by the methylene blue staining assay also described in Chapter 2.5.2.

Calibration of the Dynatech Laboratories MRX plate reader

Prior to use the linearity of the Dynatech Laboratories MRX plate reader was investigated to determine the relationship between absorbance of the dye and cell number in the methylene blue assay. Various known cell numbers were plated in 96-well plates, allowed to attach for several hours, and assayed using the methylene blue staining method described above. The absorbance reading was taken at each of the fixed cell numbers and tabulated. The results (Figure 3.5) are graphically represented and show a near linear relationship between dye intensity and cell numbers over the range tested, which represented the range encountered in the actual experiments.

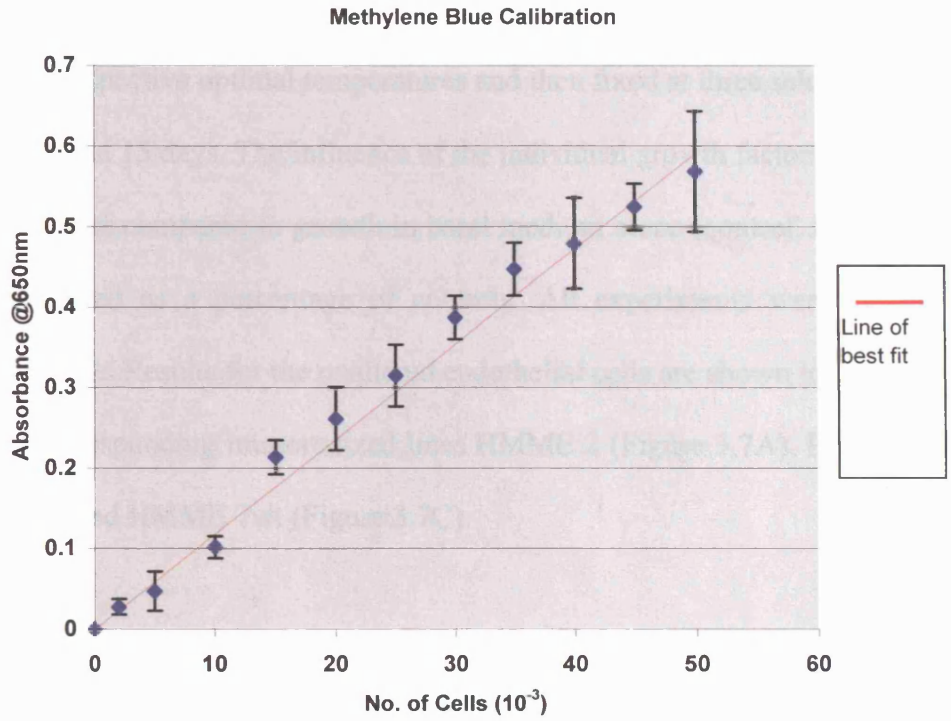


Figure 3.5. Calibration of the Dynatech Laboratories MRX plate reader. Error bars signify the standard deviation (n=3). Line of best fit calculated by the formula y = ax + b where a is the slope and b is the intercept (using Microscoft Excel).

Endothelial cells

The growth factors present in the commercially available endothelial cell growth medium EGM-2 Bullet kit (Biowhittaker) were used. These are the growth factors that have been identified as necessary for the optimal growth of normal human endothelial cells. These were heparin (hep), hydrocortisone (HC), human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), insulin like growth factor (IGF-1), ascorbic acid (AA) and human epidermal growth factor (hEGF). With the possible exception of VEGF and heparin the 'endothelial' growth supplements are also reported to act on many different cell types and were therefore also tested on the fibroblast lines.

1 x 10^3 cells were plated in 24-well plates in the basal endothelial cell medium (EGM-2) containing 2% (v/v) FBS with the addition of each of the

2% (v/v) FBS without any growth supplements added. The cells were grown at their respective optimal temperatures and then fixed at three selected time points; 7, 12 and 15 days. The influence of the individual growth factors on different cell lines was compared to growth in basal medium alone (control, EGM-2) and was calculated as a percentage of controls. All experiments were carried out in triplicate. Results for the unaltered endothelial cells are shown in Figure 3.6, with its corresponding immortalized lines HMME 2 (Figure 3.7A), HMME 7 (Figure 3.7B) and HMME 7wt (Figure 3.7C).

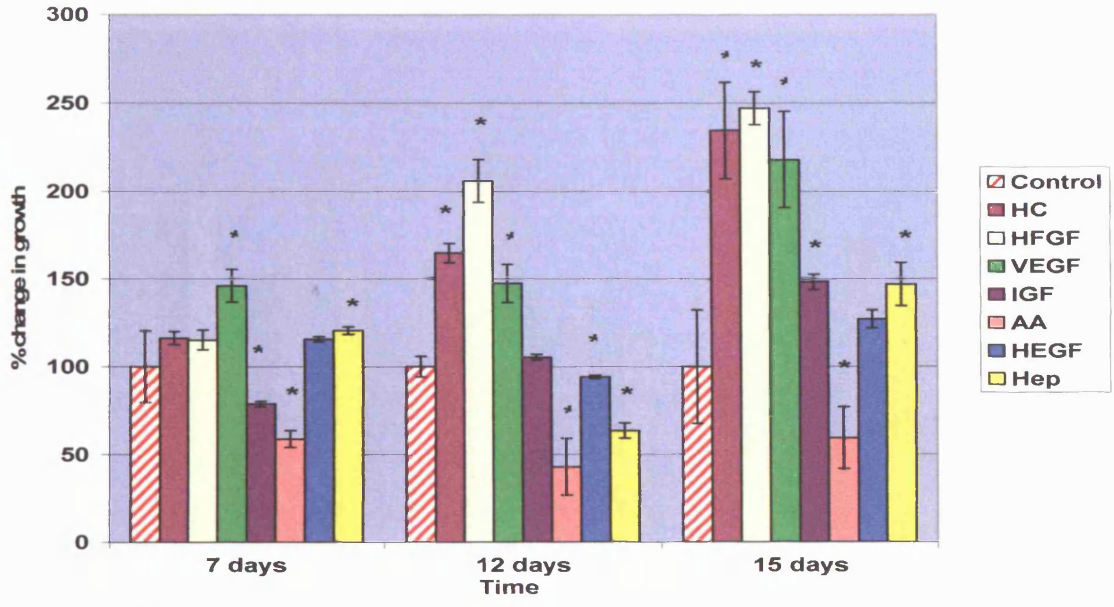
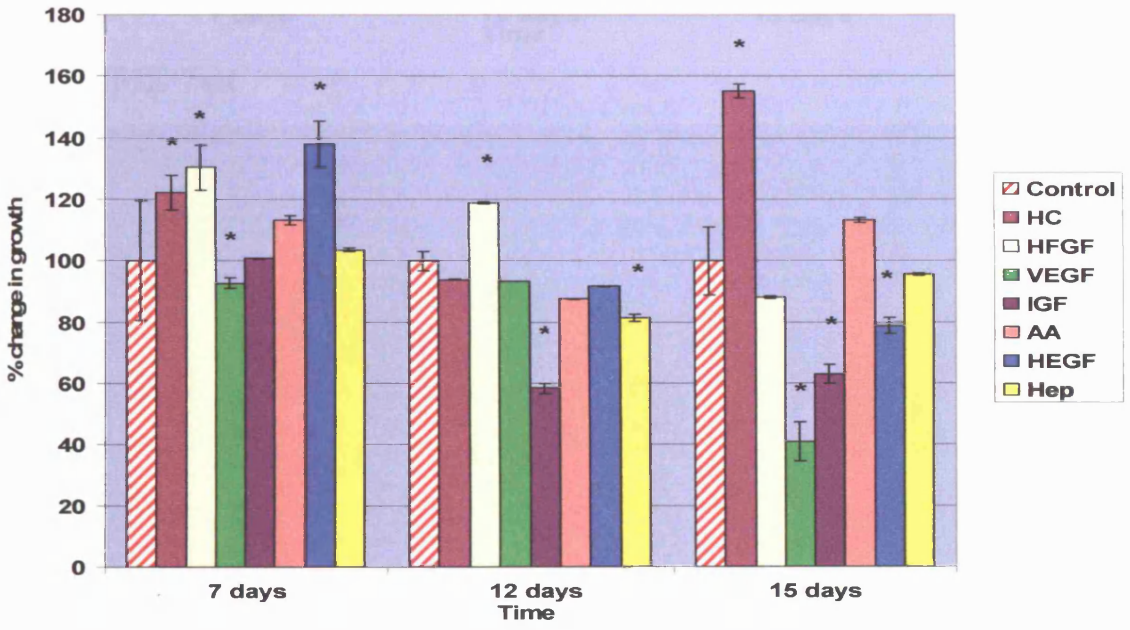


Figure 3.6. Effect of individual growth supplements on the growth of the unaltered human mammary endothelial cells. Control was defined as 100% (basal media containing serum with no supplements). The growth effect of the supplements was calculated as a percentage increase/decrease compared to control. Data shown as means +/- standard deviation (n=3) shown as error bars. Statistically significant results are marked with (*) where $p \leq 0.05$ using the Students t-test in Excel's statistical package.

Unaltered endothelial cells were stimulated by all the growth factors with HC, hFGF and VEGF being particularly potent (Figure 3.6). However, AA had an inhibitory effect as a single addition. The dependency of the unaltered cells to growth factor stimulation is in line with expectation as the commercial medium used (EGM-2) has been optimized for human endothelial cells.

Data for the corresponding experiments on the endothelial cell lines HMME 2, HMME 7 and HMME 7wt are shown in Figure 3.7.

A) HMME 2



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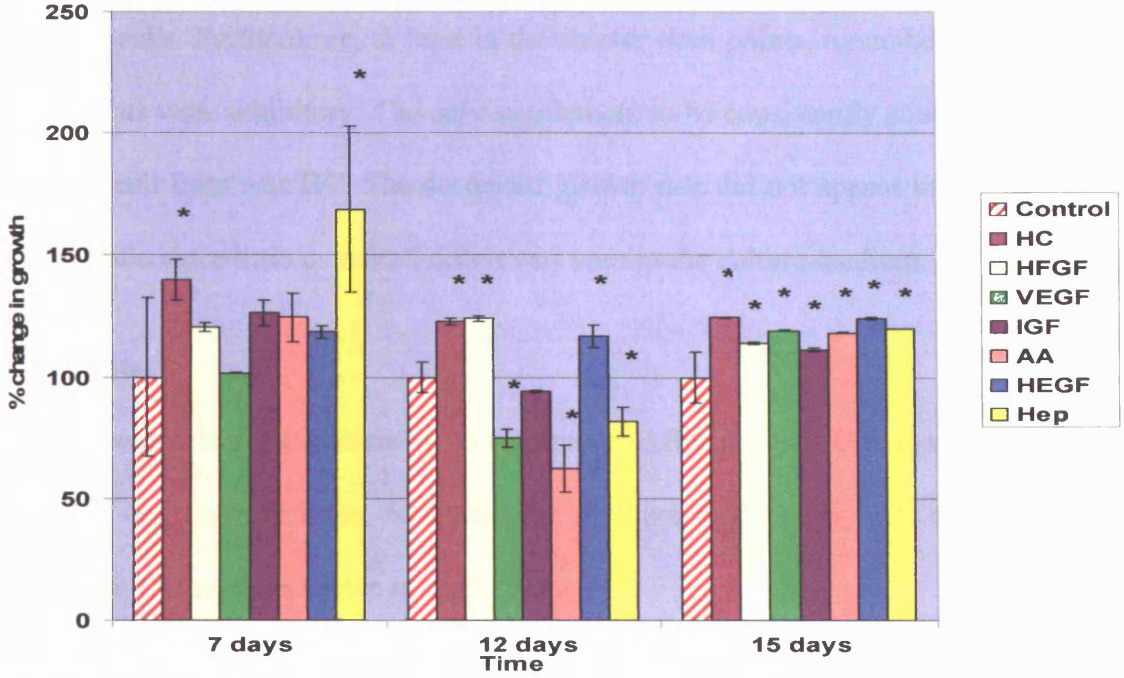
B) HMME 7



C) HMME 7wt

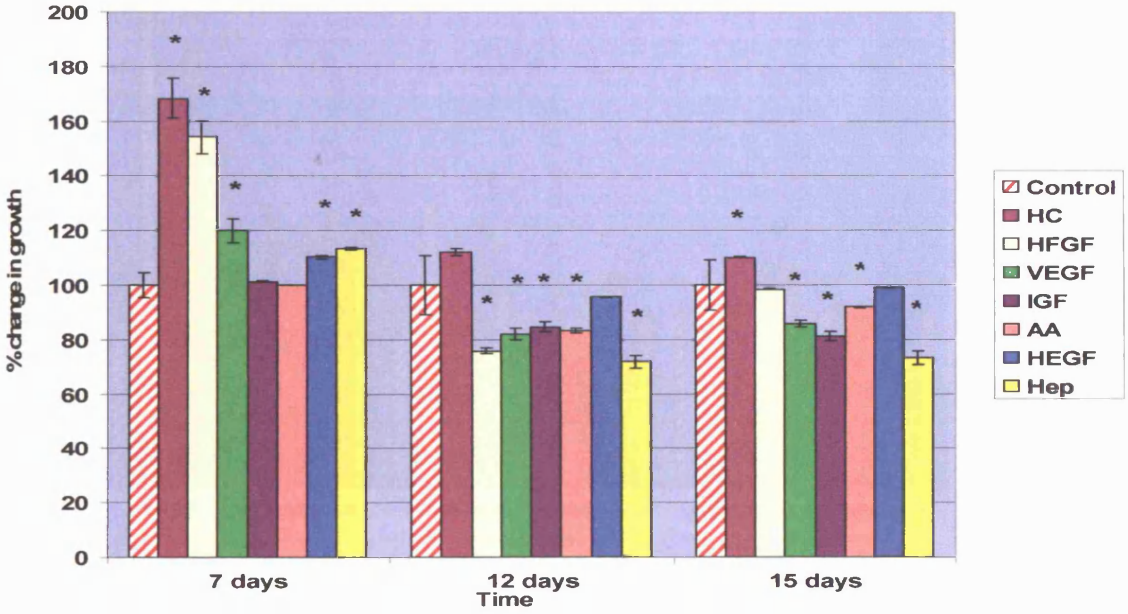


Figure 3.7. Effect of individual growth supplements on the growth of the immortalized human mammary endothelial cell lines (a) HMME 2, (b) HMME 7 and (c) HMME 7wt. Control was defined as 100% (basal media containing serum with no supplements). The growth effect of the supplements was calculated as a percentage increase/decrease compared to control. Data shown as means +/- standard deviation (n=3) shown as error bars. Statistically significant results are marked with (*) where $p \leq 0.05$ using the Students t-test in Excel's statistical package.

Overall, the effects of individual growth factors were much smaller than with the unaltered cells. Furthermore, at least in the shorter time points, a number of the supplements were inhibitory. The only supplement to be consistently stimulatory in all the cell lines was HC. The decreased growth rate did not appear to be due to cell death, since little or no cell debris was seen in the culture medium.

Fibroblasts

A similar experiment was carried with the unaltered fibroblasts and immortalized fibroblast cell lines with the exception that measurements were made a single time point. The result is shown in Figure 3.8.

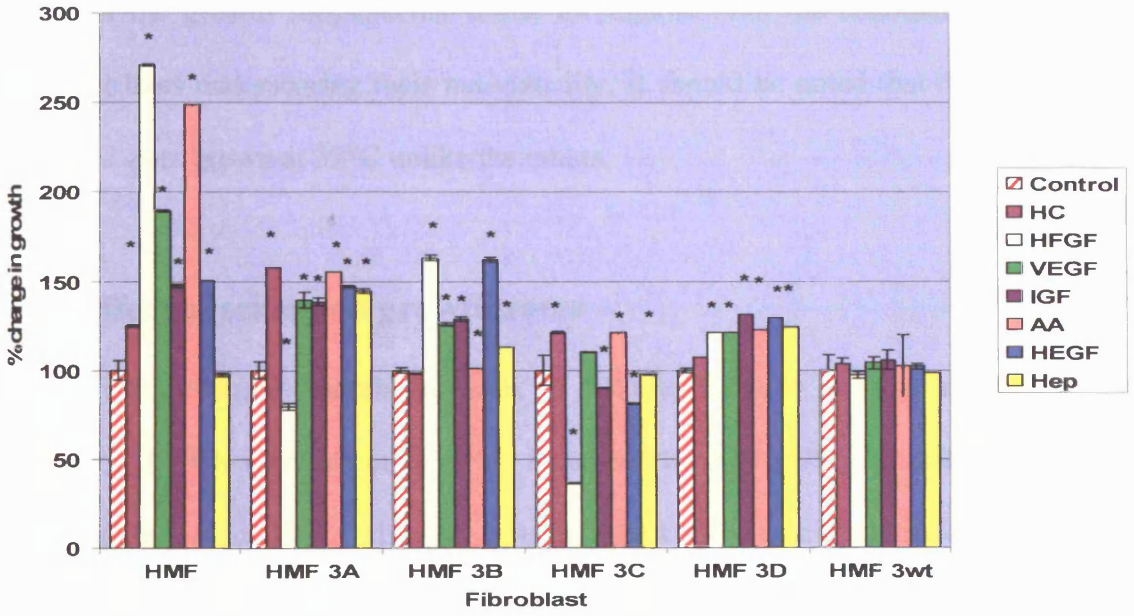


Figure 3.8. Effect of individual growth supplements on the growth of the human unaltered and immortalized mammary fibroblast cell lines. Cells were grown for 7 days at 37°C HMF. Control was (growth in basal media containing serum but no growth supplements) defined as 100%. The effect of growth supplements on cell growth is shown as a percentage increase/decrease in comparison to control. Data shown as means +/- standard deviation (n=3)-error bars). Statistically significant results are marked with (*) where $p \leq 0.05$ using the Students t-test in Excel's statistical package.

Most of the 'endothelial' growth supplements had dramatic stimulatory effects on the unaltered fibroblasts increasing growth rates considerably (Figure 3.8). The increase in growth produced by the various factors ranged from 50-150%. Even VEGF stimulated the cells and the only factor that did not was heparin. The fibroblast lines, by contrast all showed relatively minimal effects, compared with the unaltered cells. However, such mainly stimulatory effects as were noted were also somewhat greater than had been observed in the endothelial lines (e.g. experiments with HMF 3A and HMF 3B)

In summary the growth factor dependency of all the immortalized stromal cells was significantly less than that of the unaltered cells. The stimulatory effects of the growth supplements varied throughout both the endothelial and fibroblast lines underscoring their individuality. It should be noted that the wild type lines were grown at 37°C unlike the others.

3.2.3 Effect of serum on growth rates

Human microvascular endothelial cells in culture normally require a serum content of 10-20% (Iijimata et al. 1991, Hohenwarter et al. 1992, Lassalle et al. 1992, Fickling et al. 1992) when growing in relatively simple medium, or as in the case with EGM-2 medium 2% with specific growth factors.

Serum contains many endogenous growth factors. The fact that, as shown in the previous section, the immortalized endothelial cells have significantly reduced growth factor requirements compared to unaltered cells could be due to both the growth promoting activity of the SV40 LT and to the presence of endogenous growth factors in the 2% serum to provide optimal conditions. To ascertain whether any major growth factor responses were retained by the cell

lines, they were retested with growth factors at reduced serum concentrations. Growth assays in microwell plates were performed, as previously described.

Endothelial cell lines were grown in 3 different reduced serum concentrations: 0.2%, 0.4%, 1% and 2% FBS (i.e.10%, 20%, 50% and 100%) of the normal foetal bovine serum concentration (2% v/v) present in the basal endothelial cell medium. 1 x 10^3 cells were plated in wells of a 24-well plate in endothelial cell basal medium (EGM-2) containing one of the four serum concentrations with the addition of each of the growth factors provided in the EGM-2 bullet kit medium. Controls for each serum concentration consisted of basal medium containing the appropriate serum concentration without any growth supplements. The cells were grown at their optimal temperature and conditions for 7 days before being fixed in 4% (w/v) formaldehyde in PBS. Cell numbers were determined by the modified methylene blue staining assay as described in Chapter 2.5.2.

The effect of different serum concentrations on the growth rates of the unaltered endothelial cell HMME and immortalized cell lines HMME 2, HMME 7 and HMME 7wt are shown in Figure 3.9.

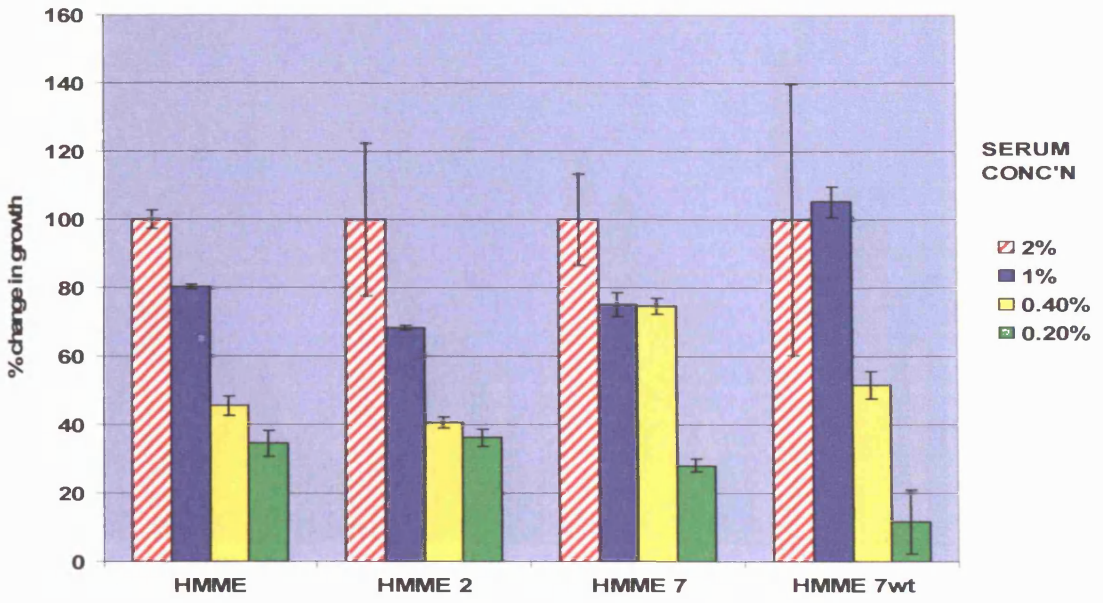


Figure 3.9. Effect of serum concentration on the growth of unaltered and immortalized human mammary endothelial cells in medium without growth factors. Control consists of the normal serum concentration of 2% v/v and is shown as 100%. The effect of serum on cell growth is shown a percentage increase/decrease in comparison to control. Data shown as means +/- standard deviation (n=3,-error bars) of triplicate measurements.

All the cells tested, both the unaltered and immortalized cell lines, were strongly dependent on serum concentration in the absence of the additional growth factors.

The effect of the individual growth factors at different serum concentrations on the unaltered endothelial cells is shown in Figure 3.10.

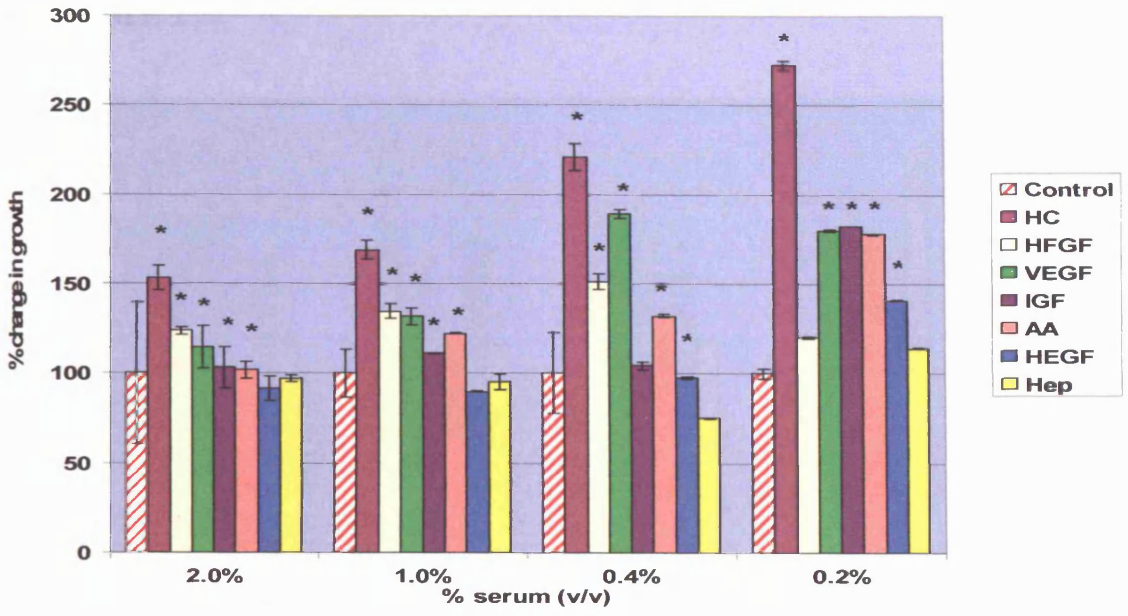
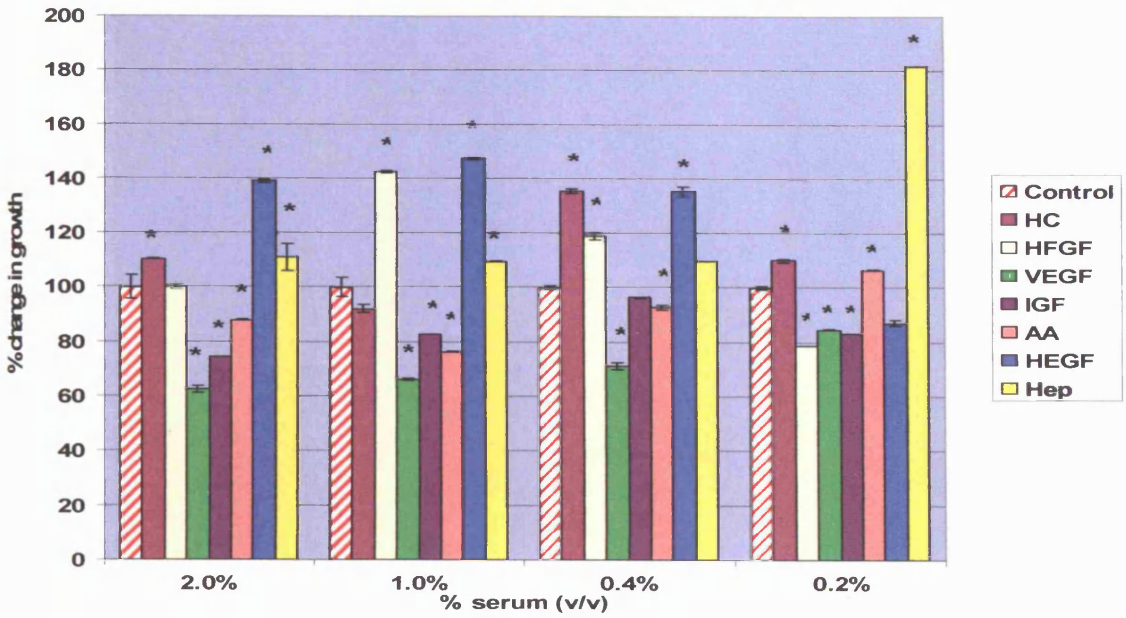


Figure 3.10. Effect of serum concentration on the influence of individual growth supplements on the growth of the unaltered human mammary endothelial cells. Cells were grown in 4 different serum concentrations in the presence of each of the growth factors for a period of 7 days. Data shown as means +/- standard deviation (n=3-error bars). Statistically significant results are marked with (*) where $p \leq 0.05$ using the Students t-test in Excel's statistical package.

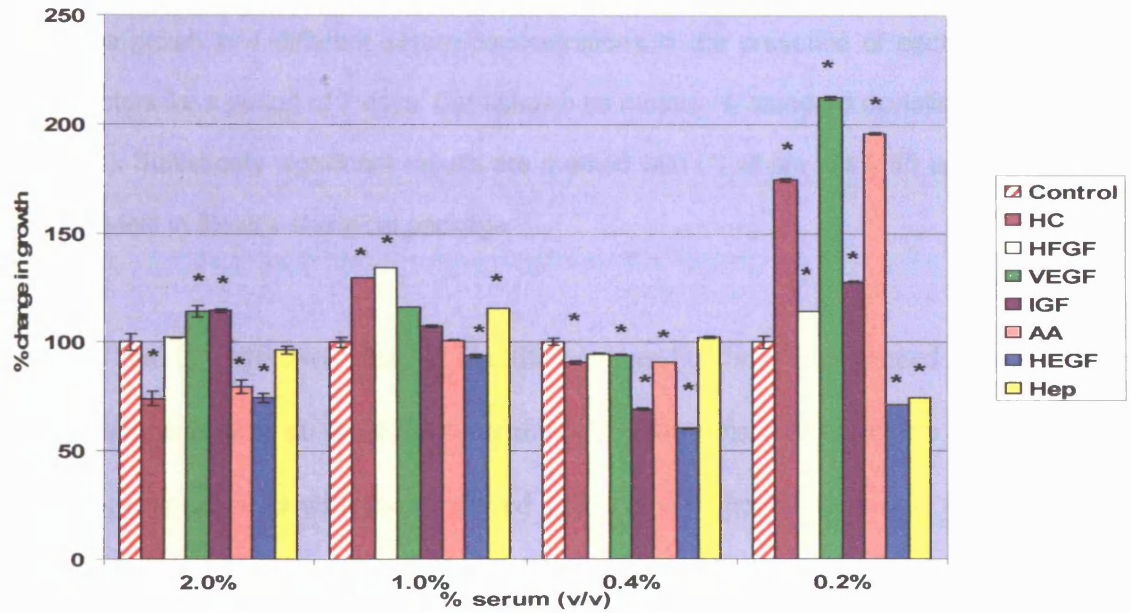
In the unaltered cells the responsiveness to individual growth factors was increased as the serum concentration was decreased (Figure 3.10). This effect was most noticeable with hydrocortisone.

The results of the endothelial cell lines HMME 2, HMME 7 and HMME 7wt are shown in Figure 3.11.

A) HMME 2



B) HMME 7



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C) HMME 7wt

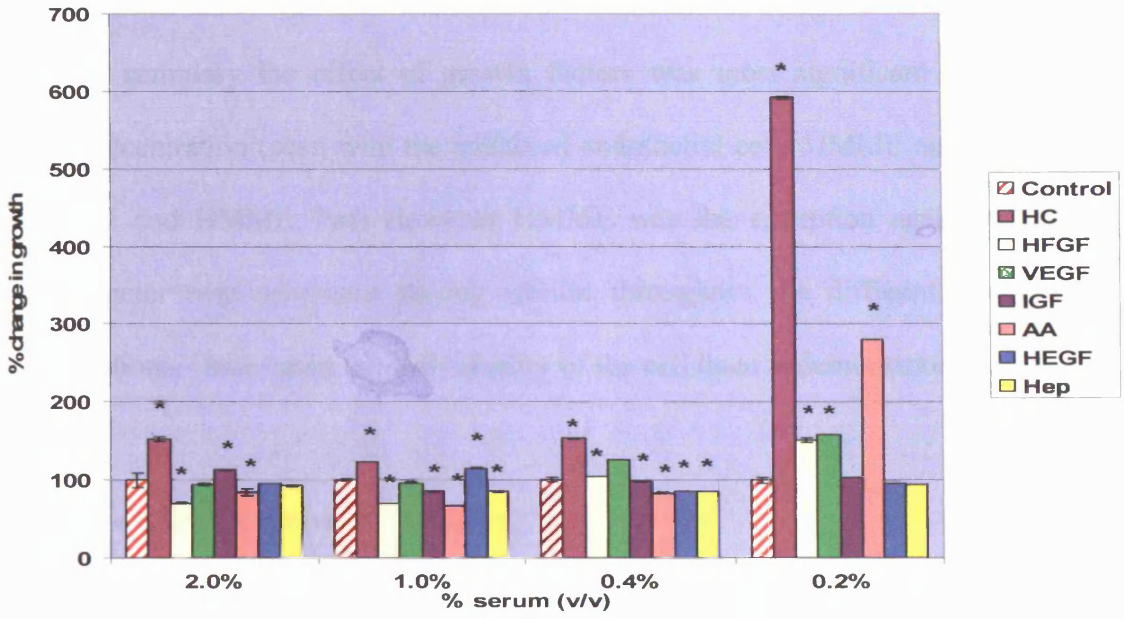


Figure 3.11. Effect of growth supplements in different serum concentrations on the growth of the (a) HMME 2, (b) HMME 7 and (c) HMME 7wt endothelial cell lines. Cells were grown in 4 different serum concentrations in the presence of each of the growth factors for a period of 7 days. Data shown as means +/- standard deviation (n=3-error bars). Statistically significant results are marked with (*) where $p \leq 0.05$ using the Students t-test in Excel's statistical package.

HMME 7 and HMME 7wt behaved like the unaltered cells with enhanced growth factor responsiveness at the lower serum concentrations, although the effects were not progressive as with the unaltered cells but only become strongly evident at the lowest serum level (0.2% v/v). Again hydrocortisone was the most consistently active individual growth factor. It was noteworthy that ascorbic acid did not promote growth under low serum conditions, in contrast to the results with 2% (v/v) serum. HMME 2 did not show consistent evidence of growth factor response even at the lowest serum concentrations. As shown previously

(Figure 3.11A) this line was the least growth factor sensitive at normal serum concentrations.

In summary the effect of growth factors was most significant at low serum concentration (seen with the unaltered endothelial cells HMME and lines HMME 7 and HMME 7wt) However HMME was the exception again with growth factor responsiveness staying similar throughout the different serum concentrations. Once again the individuality of the cell lines is demonstrated.

3.3 Co-culture growth assays

Tumour-stromal interactions are now emerging as a crucial factor in tumourigenesis. To investigate the effect of tumour-stromal interactions, in an *in vitro* setting, a series of co-culture experiments were performed using a cell culture insert and multiwell system (cells were separated into two compartments in each well). The method used is described in Chapter 2.5.3. The following interactions were studied: effect of tumour on stromal cell growth, effect of stroma on tumour growth and finally endothelial cell-fibroblast interaction.

3.3.1 Effect of tumour cells on the stromal cell growth

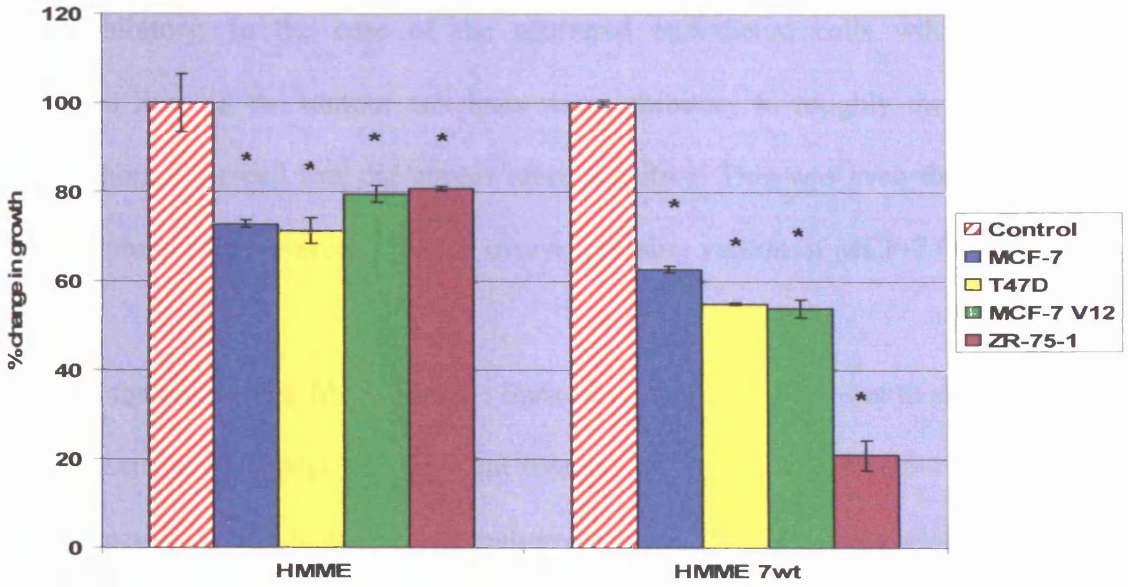
As a preliminary study the wild type (non-temperature sensitive cell lines) were tested with MCF-7 cells. Firstly the effect of different numbers of breast tumour cells (MCF-7) on the growth of immortal fibroblasts and endothelial cells was studied. Results are presented in Figure 3.12. The cells to be studied were plated on the bottom of the wells with the effector cells (i.e. MCF-7 cells) plated on the membrane of the cell culture inserts. The cell culture inserts consisted of 0.4μm pore sized polyethylene terephthalate membranes ($1.6x10^6$ pores/cm^2) with cells

deposited into two separate compartments in each well to prevent physical contact by inhibiting migration of cells from the inserts in the upper chamber to the lower chamber. Controls consisted of wells with cells seeded on the lower compartment and cell culture inserts without cells. The end point was determined when the cells, in the lower chamber below the inserts, in which ever experimental condition resulted in the most rapid growth to become 80-90% confluent. The temperature sensitive cell lines were not tested because they were unlikely to provide a satisfactory system for future *in vivo* studies, but the *ras* variant of HMF 3wt was included as a 'transformed' control.

In both endothelial cells and fibroblasts increasing numbers of MCF-7 tumour cells inhibited stromal cell growth. However the unaltered and wild types *ras* fibroblast demonstrated slight stimulation when cultured in 1×10^3 tumour cells before being progressively inhibited at higher tumour numbers (Figure 3.12b). HMF 3wt was inhibited with all the tumour cell numbers.

Next the unaltered HMME and HMF cells and their corresponding wild type lines were co-cultured with 4 different human breast epithelial tumour cell lines to determine whether the effects of tumour cells on the growth rates of the stromal cells were to any extent tumour cell type specific (Figure 3.13).

A) Endothelial cells



B) Fibroblasts

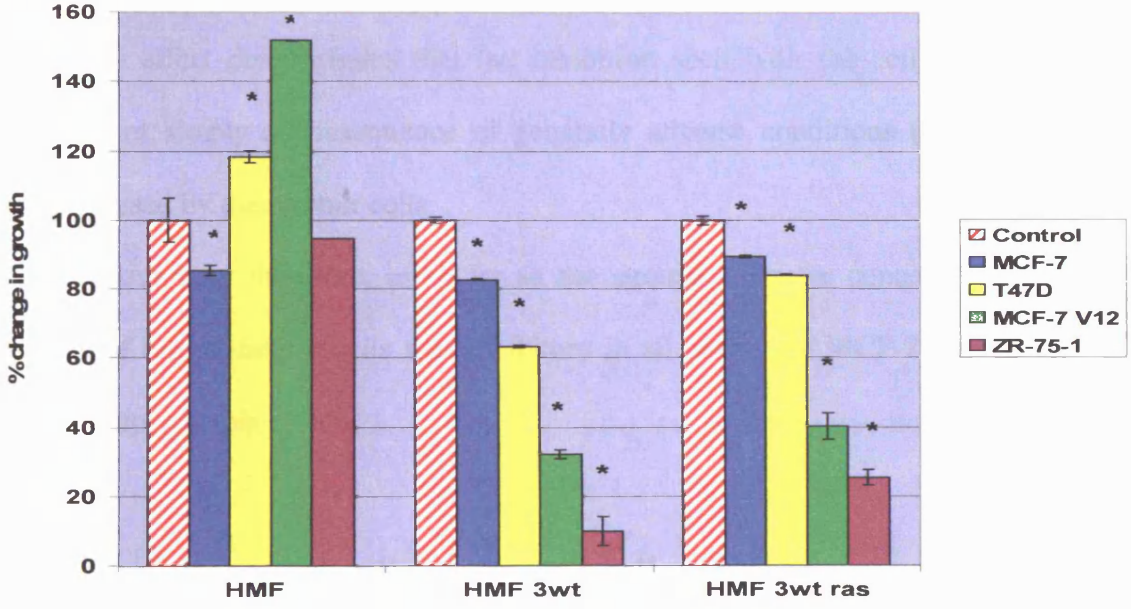


Figure 3.13. Endothelial cell (a) and fibroblast (b) growth rates in co-culture with 4 different breast tumour cell lines. Growth was measured over a 7 day period with 10^4 cells seeded in the top compartment of the microwell inserts. Results are calculated as a percentage inhibition/stimulation of control (culture inserts with no added cells). Data shown as means +/- standard deviation (n=3-error bars). Statistically significant results are marked with (*) where $p \leq 0.05$ using the Students t-test in Excel's statistical package.

Results (Figure 3.13) showed that the tumour-stromal interactions were on the whole inhibitory. In the case of the unaltered endothelial cells wild type endothelial line, all the tumour cell lines were inhibitory to roughly the same extent although the cell line did appear more sensitive. This was even the case when the tumour cells were the VEGF over-expressing variant of MCF-7 (MCF-7 V12).

In the case of the fibroblasts the lines were again inhibited but to slightly different extents in respect of individual tumour cell lines, with ZR-75-1 being the most active in this respect. The unaltered fibroblasts were less sensitive to inhibition and in some instances activities actually showed enhancement of growth, for example when co-cultured with MCF-7 V12 and T-47D. This stimulatory effect demonstrates that the inhibition seen with the cell lines is probably not simply a consequence of generally adverse conditions (i.e. over acidity) caused by the tumour cells.

In summary therefore, in so far as the stromal cells are concerned the presence of breast tumour cells was inhibitory in all cases, but MCF-7 was the least inhibitory in this system.

3.3.2 Effect of stromal cells on tumour cell growth

The unaltered HMME and HMF cells and their corresponding lines were next co-cultured with the 4 human breast epithelial tumour cell lines, following a similar protocol as Chapter 3.3.1 but with the compartments reversed (i.e. tumour cells in the bottom and HMME/HMF cells in the inserts, to determine the effects of stromal cells on the growth rates of 4 different tumour cells. In order to avoid potential problems of over-acidity and other toxic media effects the medium was

changed every day. Results for both endothelial and fibroblasts are shown in Figure 3.14.

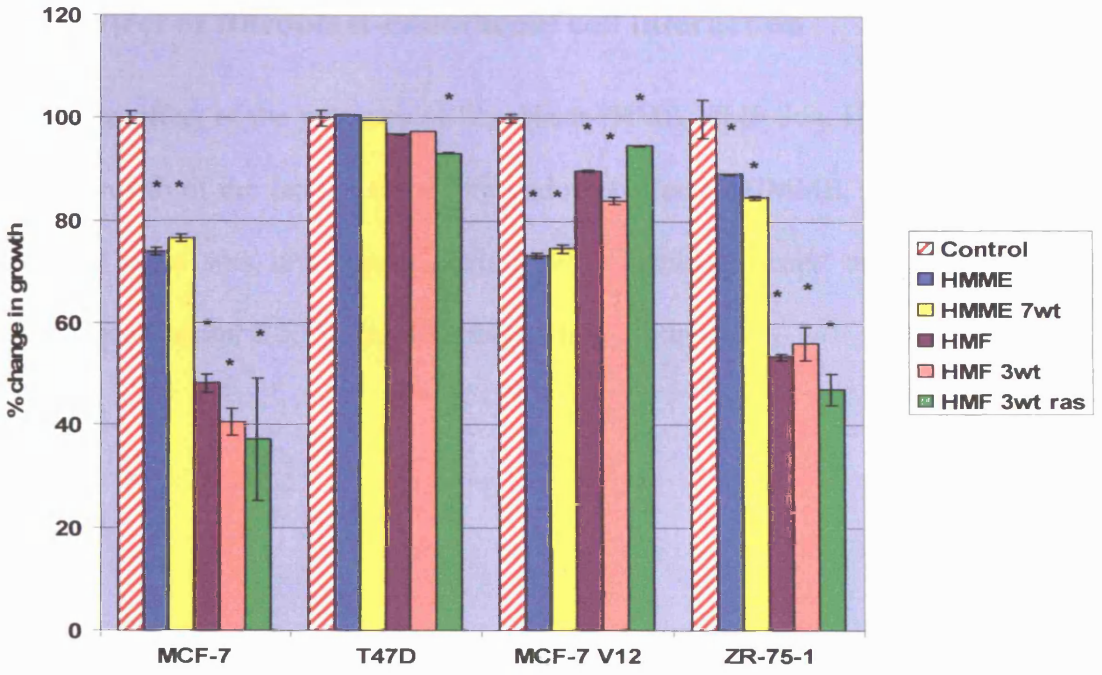


Figure 3.14. Breast tumour cell line growth rates in co-culture with human mammary stromal cells. Growth was measured over a 7 day period with 10^4 cells seeded in the top compartment of the microwell inserts. Controls were tumour cells alone. Results are calculated as a percentage inhibition/stimulation of control (culture inserts with no added cells). Data shown as means +/- standard deviation (n=3-error bars). Statistically significant results are marked with (*) where $p \leq 0.05$ using the Students t-test in Excel's statistical package.

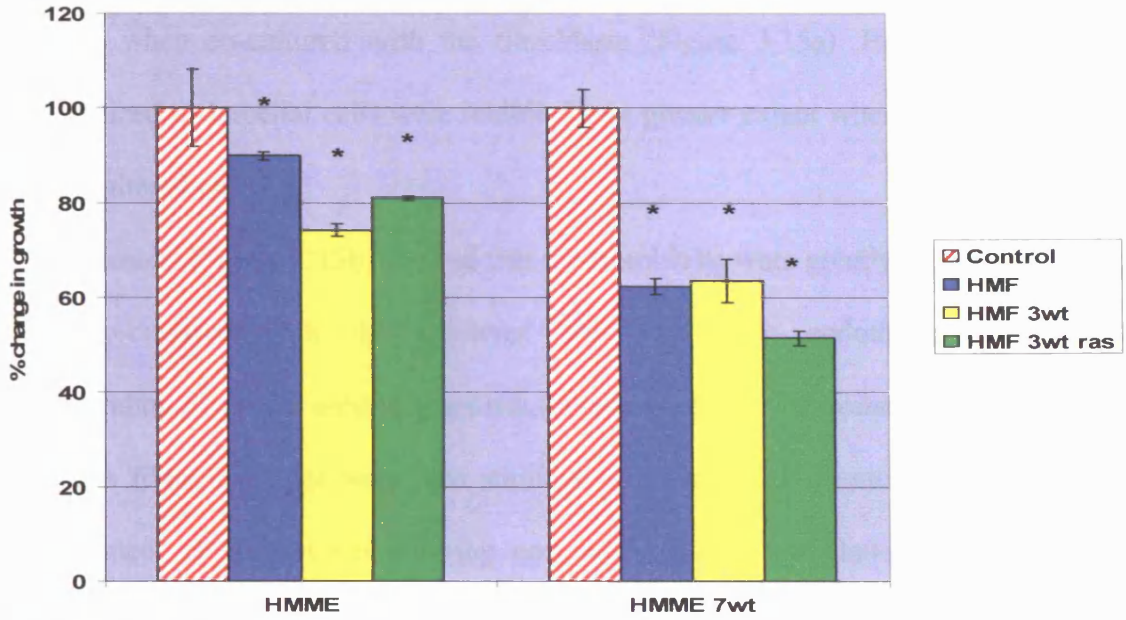
The results clearly demonstrated that the stromal cells tested, both the unaltered and their corresponding fibroblasts and endothelial cell lines, had inhibitory effects on the growth of all the 4 different breast tumour cell lines. The fibroblasts and endothelial cells inhibited the 4 different tumour cell lines to

different extents. The stromal cells inhibited the growth of the MCF-7 cell line most with T47D showing the least inhibition.

3.3.3 Effect of fibroblast-endothelial cell interaction

Lastly, the effect of the presence of fibroblasts (HMF, HMF 3wt, HMF 3wt *ras*) on the growth of the human mammary endothelial cells (HMME, HMME 7wt) and vice versa was investigated, using the cell culture insert and multiwell system (see Chapter 2.5.3). Results are shown in Figure 3.15.

A) Fibroblasts



B) Endothelial cells

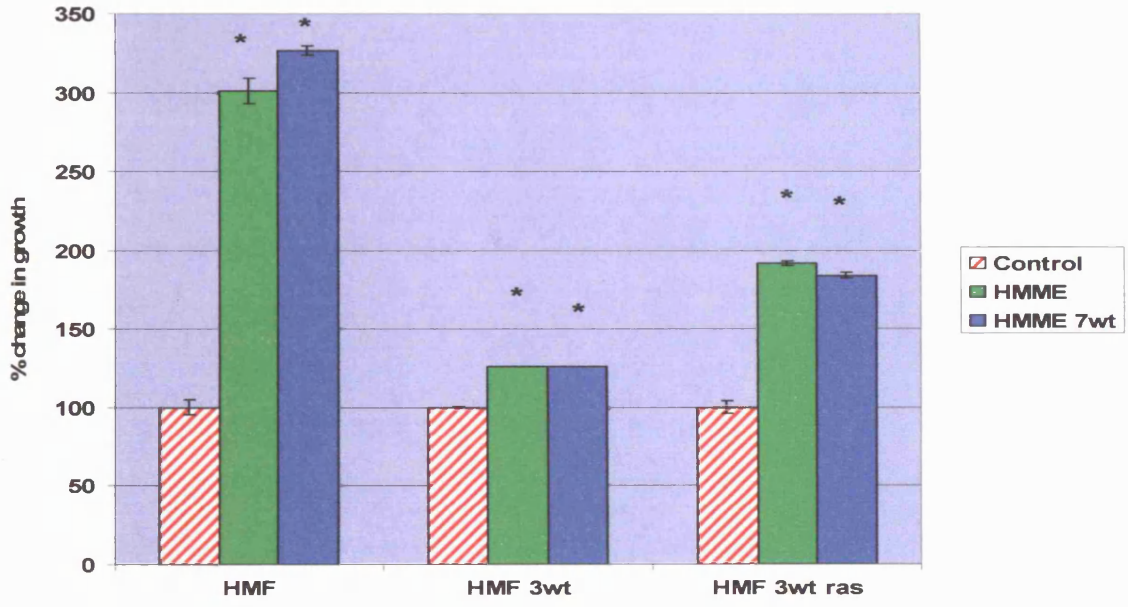


Figure 3.15. Growth rates of (a) human mammary fibroblasts in co-culture with human mammary endothelial cells and (b) endothelial cells in co-culture with human mammary fibroblasts. Growth was measured over a 7 day period with 10^4 cell seeded in the top compartment of the microwell inserts. Controls were endothelial cells or fibroblasts cultured alone. Results are calculated as a percentage inhibition/stimulation of control (culture inserts with no added cells). Data shown as means +/- standard deviation (n=3-error bars). Statistically significant results are marked with (*) where $p \leq 0.05$ using the Students t-test in Excel's statistical package.

Both the unaltered and its corresponding wild type endothelial cell line were inhibited when co-cultured with the fibroblasts (Figure 3.15a). However the immortalized endothelial cells were inhibited to a greater extent when compared to the unaltered cells.

Results (Figure 3.15b) showed that the fibroblasts were greatly stimulated when co-cultured with the unaltered and wild type endothelial cells demonstrating markedly enhanced growth, in excess of 200% in some cases. The wild type fibroblast lines were also stimulated but to a lesser extent with the 'transformed' HMF 3wt *ras* showing noticeably greater stimulation than the HMF 3wt line.

A summary of all the co-culture data is shown in Tables 3.3-3.5

	Effector	HMF 3wt	HMF 3wt ras	HMF	T47D	ZR-75-1	MCF-7	MCF-7 V12
Target	HMME	↓	↓	↓	↓	↓	↓	↓
	HMME 7wt	↓	↓	↓	↓	↓	↓	↓

Table 3.3. The effect of breast tumour cell lines and fibroblasts on endothelial cell growth

	Effector	HMME	HMME 7wt	T47D	ZR-75-1	MCF-7	MCF-7 V12
Target	HMF 3wt	↑	↑	↓	↓	↓	↓
	HMF 3wt *ras*	↑	↑	↓	↓	↓	↓
	HMF	↑	↑	↑	↓*	↓	↑

Table 3.4. The effect of breast tumour cell lines and endothelial cells on fibroblast growth

	Effector	HMME	HMME 7 wt	HMF 3wt	HMF 3wt *ras*	HMF
Target	T47D	—	—	—	—	↓
	ZR-75-1	↓	↓	↓	↓	↓
	MCF-7	↓	↓	↓	↓	↓
	MCF-7 V12	↓	↓	↓	↓	↓

Table 3.5. The effect of stromal cells on breast tumour cell line growth.

↓ **Inhibitory** * *P=0.013*
↑ **Stimulatory**
— = No change $p \geq 0.4$

All results $p \leq 0.01$ Calculated using the Students T–test assuming unequal variance-two tailed

3.4 Endothelial cell differentiation assays

The ability of human mammary endothelial cells to undergo terminal differentiation and morphogenesis can be determined by its growth on the basement membrane preparation, Matrigel. Basement membranes are thin extracellular matrices underlying epithelial cells and separating them from connective tissue. BD Matrigel™ Matrix is a solubulized basement membrane preparation extracted from EHS mouse sarcoma, a tumour rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin. Matrigel can be used to coat tissue culture surfaces, where cell growth can promote tissue like morphogenesis and tissue specific cell differentiation and function, thus providing an *in vitro* model for *in vivo* tissue morphogenesis. 'Transformed' cells can show reduced morphogenesis on Matrigel, apparent as a loss of organisation and the failure to produce any recognisable structure. Due to the differences observed between normal and transformed cells' differentiation and morphogenesis, a differentiation assay using matrigel was performed to assess endothelial cell phenotype.

The ability of the immortalized human mammary endothelial cells to proliferate, differentiate and form tubular structures *in vitro* was therefore investigated. In addition the effect of growth factors, either exogenous or those produced from tumours, on endothelial cell proliferation and differentiation was determined.

To investigate the ability of the immortalized endothelial cells to differentiate *in vitro* on extracellular matrix with minimal growth factor support, differentiation assays on GFR Matrigel were carried out (Figure 3.16).

A) B)

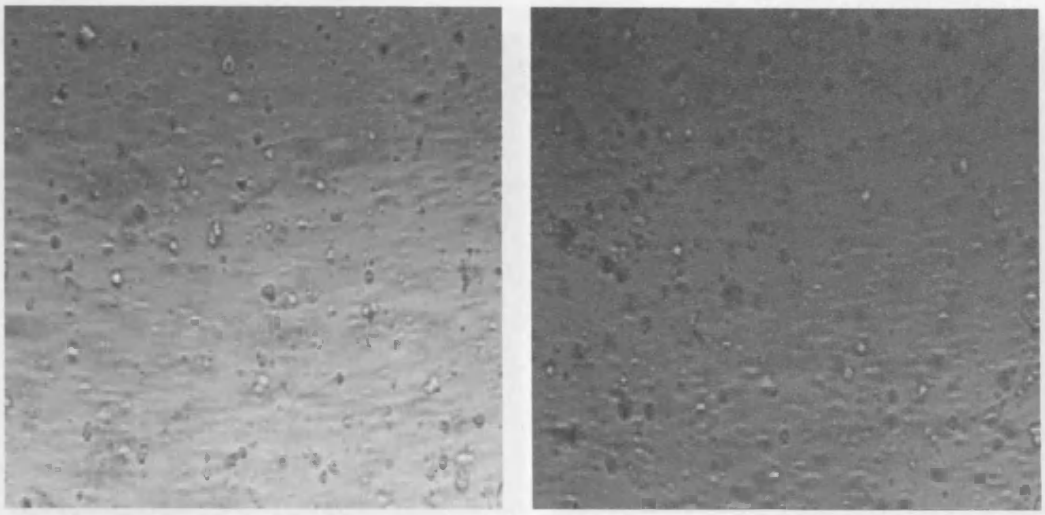


Figure 3.16. Phase contrast micrographs showing (a) unaltered and (b) immortal human mammary endothelial cells plated on GFR Matrigel with complete endothelial medium EGM-2 Bullit kit after 24 hours. No signs of primitive microcapillary like differentiation. Similar results were observed with the other endothelial cell lines including the temperature sensitive lines when grown at 33.5°C. Magnification ×80.

Both the unaltered and HMME endothelial cell lines failed to differentiate and produce primitive capillary networks on the GFR Matrigel (Figure 3.16).

To further investigate tumour stromal interactions, assays were repeated this time including in either fibroblasts or tumour cells with the endothelial cells when plating of GFR Matrigel. Results are shown in Figure 3.

A) B)

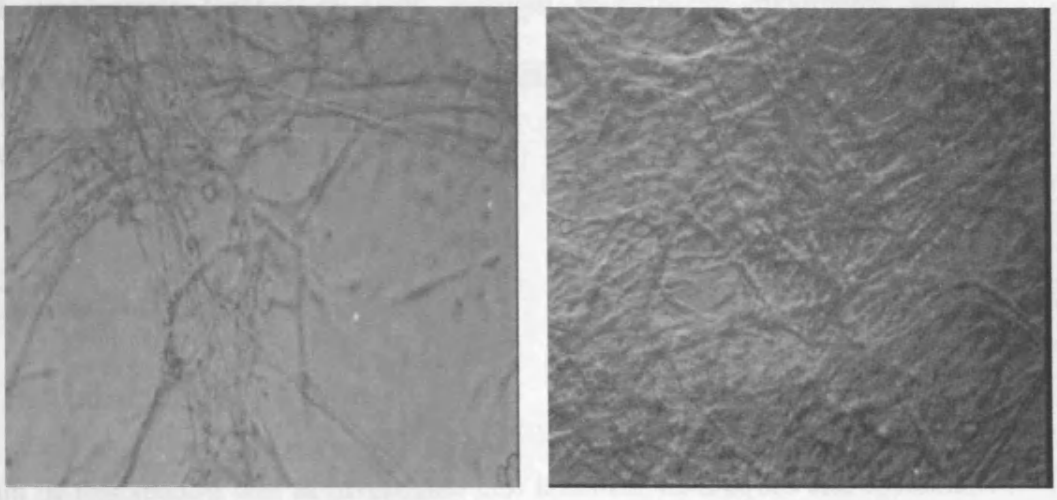


Figure 3.17. Phase contrast micrographs taken after 24 hours showing primitive microcapillary networks produced by the endothelial cell line HMME 7wt when incubated with (a) immortalized human mammary fibroblasts and (b) tumour epithelial cells (MCF-7). The unaltered endothelial cells, HMME 2 and HMME 7 produced very similar results. Taken at ×80 magnification.

In contrast both unaltered and immortalized endothelial cells differentiated and produced capillary structures when cultured on GFR Matrigel in the presence of either fibroblasts or tumour (Figure 3.17), suggesting that the addition of the tumour cells/fibroblasts provided the necessary factors to promote endothelial cell differentiation. Therefore, the presence of tumour cells/fibroblasts had a stimulatory influence on endothelial cell differentiation. A point of note is that in this experiment the tumour cells/fibroblasts were in direct contact with the endothelial cells.

In summary, the immortal human mammary endothelial cells displayed the same differentiation characteristics and properties as the unaltered endothelial

cells on Matrigel. Fibroblasts and tumour epithelial cells provided the necessary growth supplements lacking in GFR matrigel to promote endothelial cell differentiation. Tumour-stromal stromal interactions were key to providing the necessary support.

3.5 Cell labelling

3.5.1 Introduction

The aim of this section was to investigate cell labelling techniques and systems in order to select a suitable system for identifying live individual cell types in mixed populations of stromal and tumour cells in *in vitro* cell culture experiments. In addition to identifying different cell types, the dyes would be used to assist in the clarification of cell position in relation to each other. Success would allow us to follow the different live cell types in heterologous co-culture experiments, endothelial cell differentiation assays, heterologous co-culture spheroids and more importantly xenograft studies.

To serve as an effective tracer of cell morphology or location, a probe must have the capacity for localized introduction into a cell or organelle and subsequent long term retention within that structure. The tracer should also be biologically inert. Ideally cells should remain viable for at least 24 hours after loading and persist through several cell divisions so they can be utilised for *in vivo* studies.

The cellular labelling ability of two different classes of dyes was investigated. These were the cytoplasmic (CellTracker™) and membrane (Vybrant lipophilic) labelling dyes. To test the manufacturer's claims, together with a view to using in *in vitro* and *in vivo* location studies, a series of

experiments were devised to assess the duration of labelling and to determine whether, as claimed, all dyes would stably label individual cells and would not be transferred to adjacent cells by release and re-uptake. (See Chapter 2.4 for details of labelling)

3.5.2 CellTracker™ dyes

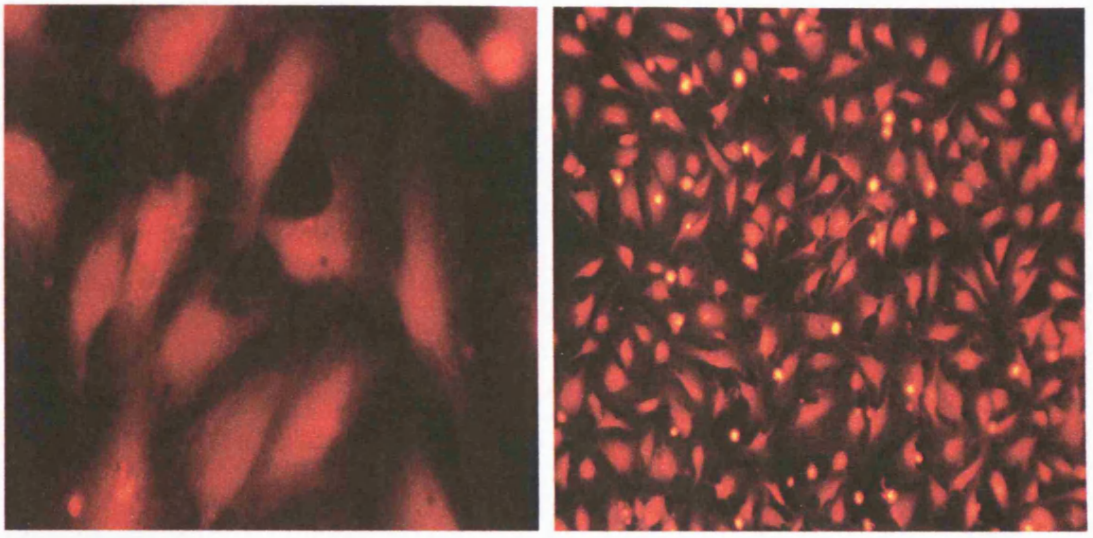
The first experiment used cytoplasmic CellTracker™ dyes as they should produce stronger fluorescence compared with the membrane labelling dyes. CellTracker™ dyes are reported by the manufacturer (Molecular Probes) to be retained in living cells through several generations and also inherited by daughter cells after cell division (Cumberledge and Krasnow 1993). These dyes contain a chloromethyl group that reacts with thiols, probably in a gluthathione *S*-transferase-mediated reaction. The reagents diffuse freely through the membranes of live cells. Once inside the cell, these probes undergo a reaction to produce a membrane-impermeant glutathione–fluorescent dye adduct, although other experiments suggest that they may also react with other intracellular components (Zang et al. 1992). Furthermore, unlike the free dyes, the peptide–fluorescent dye adducts contain amino groups and can therefore be covalently linked to surrounding biomolecules by fixation with formaldehyde or glutaraldehyde.

To investigate the ability of these dyes to provide a reliable method of cellular labelling for the tracing of the three cell types in mixed *in vitro* cell cultures and eventually in an *in vivo* setting the studies described below were performed.

Three different cell types, MCF-7, HMF 3wt and HMME 7wt, were each loaded with the orange CellTracker™ CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine)) or green CellTracker™ dyes CMFDA (5-chloromethylfluorescein diacetate), one at a time (see Chapter for 2.4.1). They were then cultured in their respective growth medium and examined at 1, 3, 5, 7, 10, 12 and 14 day time points to assess their fluorescence intensity. This was undertaken at two different cell densities: cells plated at confluency and non confluent rapidly proliferating cells to assess how much cell division diluted fluorescence intensity.

Using the fluorescein filter set on the fluorescence microscope, both green CellTracker™ and orange CellTracker™ could be detected with the cells showing diffuse cytoplasmic staining with both dyes (Figure 3.18).

A) Orange CellTracker



B) Green CellTracker

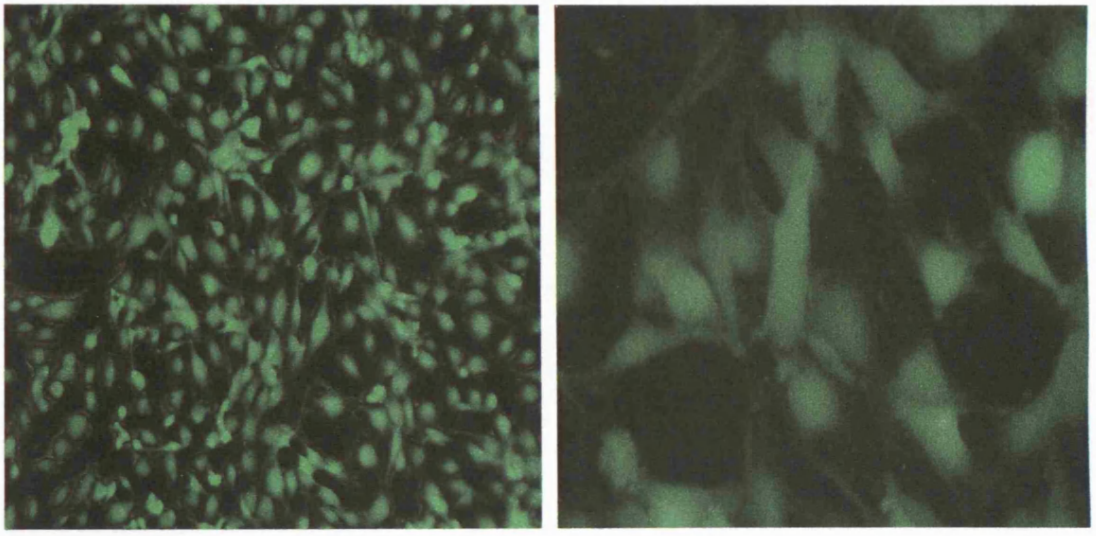


Figure 3.18. HMME 7wt endothelial cell line labelled with (a) the orange CellTracker™ and (b) HMF 3wt fibroblast line labelled with the green CellTracker™. Images taken at day 2 post labelling at ×80 and ×160 magnification.

Fluorescence intensity was stably maintained for 5 days but after this time staining decreased rapidly until by 14 days it was barely visible (Figure 3.19). Colour intensity decreased much more rapidly in non-confluent rapidly dividing cells in

comparison to slowly dividing confluent cells and by 5 days fluorescence intensities were very low, compared with confluent cells.

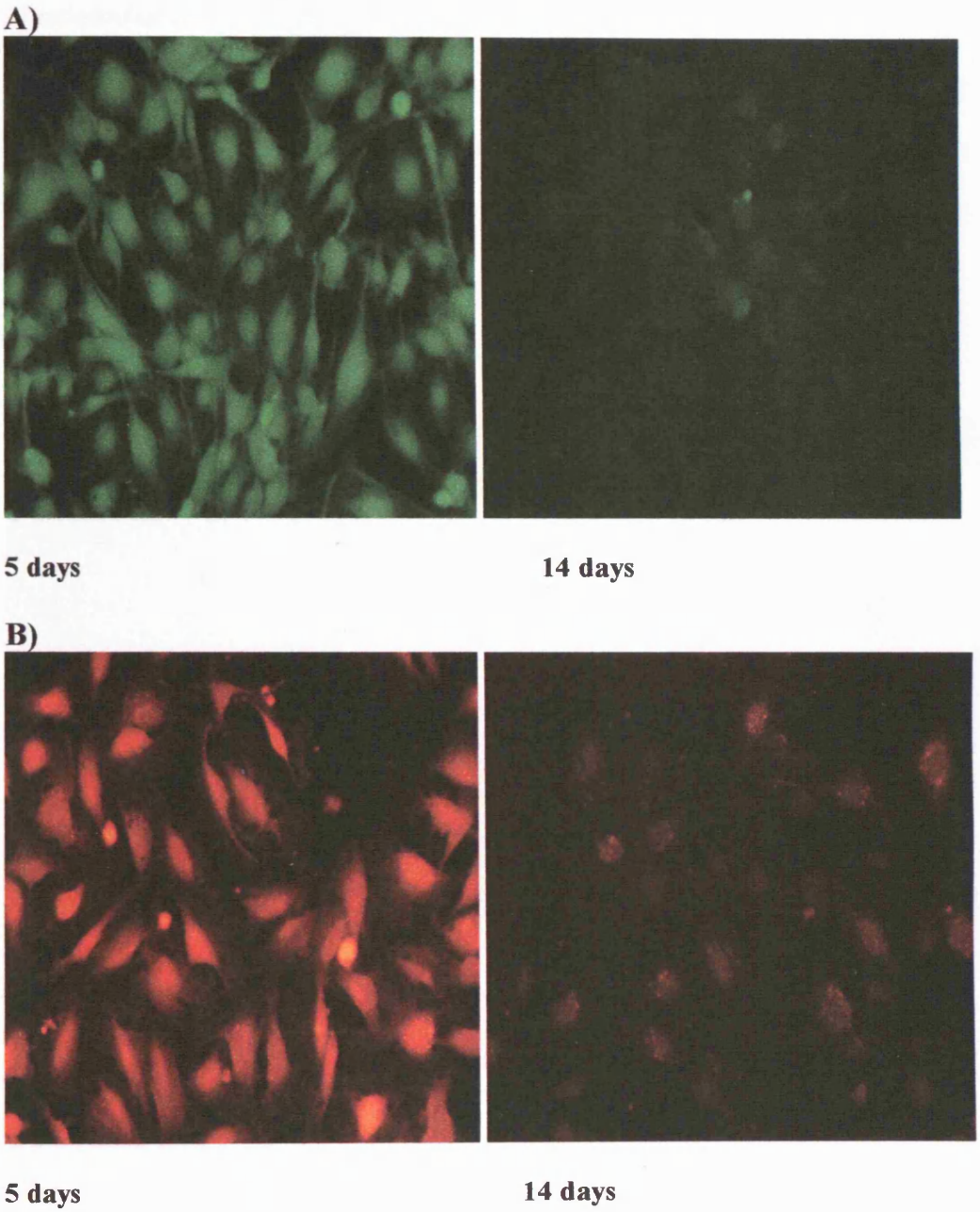


Figure 3.19. (a) Green CellTracker™ labelled HMF 3wt fibroblast line at different time points. (b) Orange CellTracker™ labelled HMME 7wt endothelial cell line at different time points. Difference in fluorescence intensity is dramatic with endothelial cells barely visible after 15 days. Images taken ×160 magnification.

The ability of the two CellTracker™ dyes to be retained without leakage or transfer in a mixed population of cells was tested as follows. Two different cell types (5×10^4 of endothelial cells and fibroblasts) were plated on a coverslip in 24-well plates, one previously labelled with a dye and the other unlabelled. The labelled cells had been thoroughly washed to remove any excess un-incorporated dye. The combined cells were cultured for 5 days and then examined. Each cell type could be identified by its characteristic morphology (Figures 18 & 19).

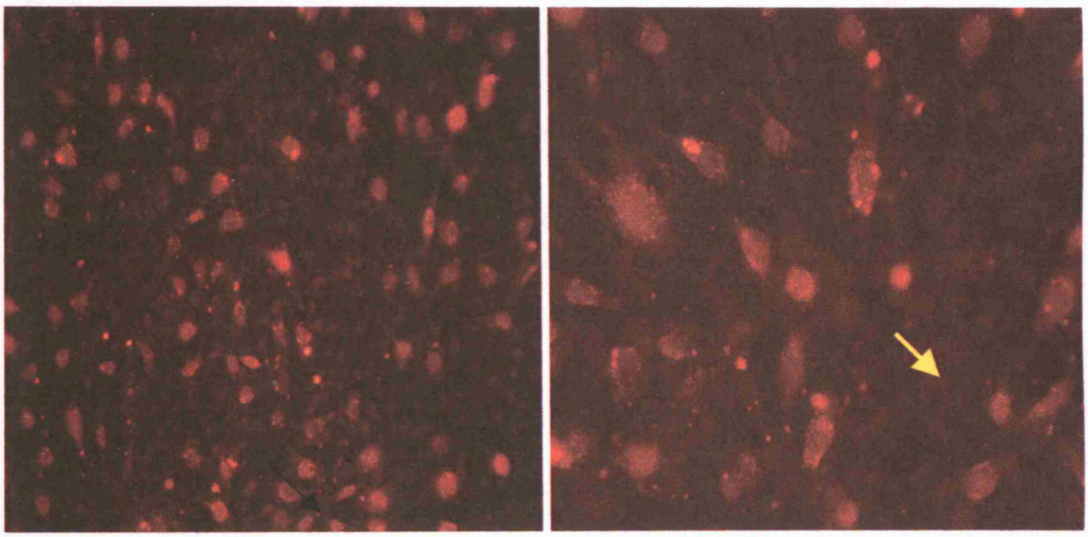


Figure 3.20. Leakage of the orange CellTracker™ from endothelial cells to surrounding unlabelled fibroblasts. Taken at ×80 and ×160 magnifications.

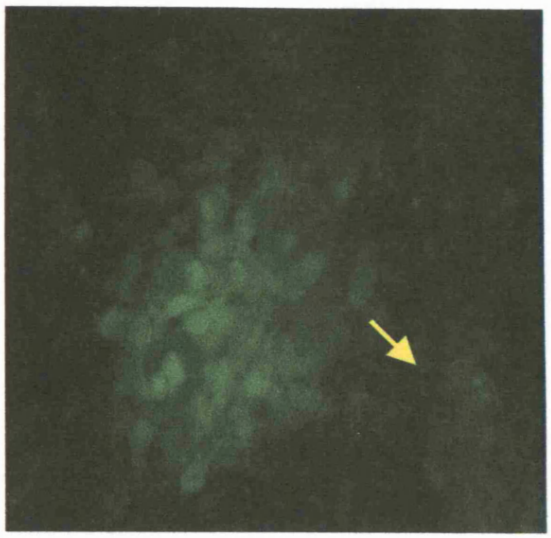


Figure 3.21. Leakage of the green CellTracker™ from fibroblasts to surrounding unlabelled endothelial cells. Taken at ×80 magnification.

The orange (Figure 3.20) CellTracker™ dye can be seen to be released from the endothelial cells labelling adjacent fibroblasts whilst the green CellTracker™ dye (Figure 3.21) can be seen labelling adjacent endothelial cells.

To examine whether dye transfer occurred by direct cell-cell contact or by simple leakage and re-uptake a second experiment was carried out. Medium from CellTracker™ labelled cells that had been cultured for 5 days were added to non-labelled cells and cultured for 2-3 days. These cells were then examined to determine whether they had taken up dye from the conditioned medium.

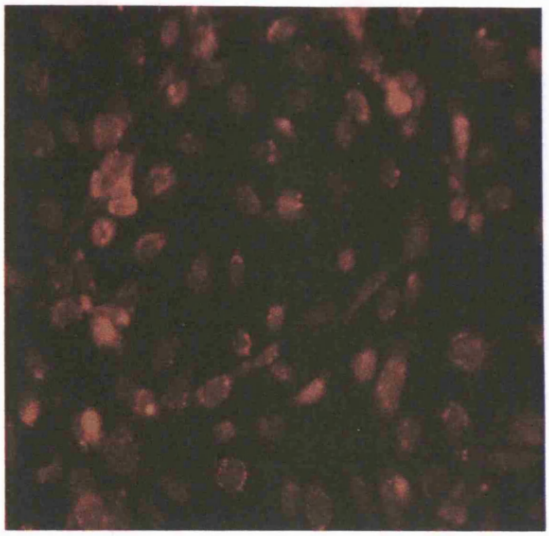


Figure 3.22. Staining of unlabelled endothelial cells from conditioned medium harvested from orange Celltracker™ labelled fibroblasts. Cells were incubated for 48 hours. Taken at ×80 magnification.

Medium harvested from orange Celltracker™ labelled cells did stain unlabelled cells, whereas medium from the green CellTracker™ labelled cells resulted in minimal staining.

In summary, therefore while green CellTracker™ did provide a stable marker for the cells tested, the orange CellTracker™ did not. Therefore, a combination of these two cytoplasmic dyes is not an effective means of following differently labelled cells for more than 5 days.

3.5.3 Fluorescent lipophilic membrane dyes

Cell membranes provide a convenient conduit for loading live and fixed cells with lipophilic dyes. Not only can cells tolerate a high concentration of dye, but lateral diffusion of the dye within the membrane can serve to stain the entire cell, even if the dye is applied locally. Lipophilic carbocyanine tracers like DiI (Vybrant Cell-Labeling Solutions) are claimed to be ideal labels for long-term cell tracing *in vivo* and *in vitro*, including studies of cell migration and transplantation. DiI labelling does not appreciably affect cell viability, development or basic physiological properties (Honig and Hume 1986, Honig and Hume 1989). A range of long-chain dialkylcarbocyanines with a variety of spectroscopic and cellular labelling properties; DiO, DiL and DiD were tested (emission spectre shown in Chapter 2.4).

The same methods used to assess the CellTracker dyes were adopted to assess the suitability of the lipophilic membrane dyes for labelling fibroblasts and endothelial cells. DiO (green) could be detected using the fluorescein optical filter set on the fluorescence microscope. DiI (orange) and DiD (red) dyes could be detected with the rhodamine optical filters. All cells showed homogenous membrane staining after 40-60 minutes staining with all these dyes. Again fluorescent intensity was stably maintained for 5 days but after this time staining decreased rapidly until by 14 days it was barely visible. Colour intensity decreased much more rapidly in non-confluent rapidly dividing cells in comparison to slowly dividing confluent cells.

All three tracers demonstrated equal stability displaying similar characteristics in regards to retention, leakage and re-uptake. MCF-7, HMF 3wt and HMME 7wt did show detectable uptake from dye present in the conditioned

medium from labelled cells, but to a much lesser extent compared with the orange CellTracker. No one dye emerged as being superior to the others. In conclusion the red emitting DiD tracer could be effectively used with the green CellTracker™ for *in vitro* co-culture studies of up to 5 days duration. The red/green combination was selected as it was more easily visualized than a green/blue pairing.

Dyes	**Duration of constant level of fluorescence**	**Retention**	**Leakage**
CMFDA CellTracker™ (green)	5days	+ + +	+
CMTMR CellTracker™ (orange)	5days	+	+ + +
DiD (red)	5days	+ + +	+
DiO (green)	5days	+ + +	+
DiI (orange)	5days	+ + +	+

Table 3.6. Table summarizing the properties of the various cell labelling dyes tested. Emission colours are shown in brackets.

3.6 Conclusion and discussion

3.6.1 Growth assays and transformation

Cells can become immortal as the result of transformation *in vivo*, spontaneous immortalization and by *in vitro* manipulation of normal cells to generate cell lines. However spontaneous immortalization of adult human cells is very infrequent (less than one in 10^9 cells). Therefore genetic manipulation of normal cells is necessary to generate human cell lines. The acquisition of an unlimited proliferative potential defined as true immortality is probably a critical step during tumourigenesis often associated with several genetic alterations (e.g. expression of the *ras* oncogene). The study of immortalization and other alterations associated with neoplastic transformation of mammary stromal cells is important to understanding the development of human mammary tumours. Because stromal cells are important regulators of associated epithelial cells, alterations in the regulation of stromal cell proliferation that influence epithelial cells may also contribute to the development of mammary carcinomas. To study the phenotypic alterations associated with immortalization on the growth properties of human mammary stromal cells, established human mammary stromal cells immortalized with SV40 Large T antigen and hTERT were used.

Role of SV40 LT antigen in cellular transformation

SV40 large T antigen (LT) is a powerful oncoprotein capable of transforming a variety of cell types (Hahn et al. 1999, Bryan and Reddel 1994). The transforming activity of LT is due in large part to its ability to bind and inactivate the retinoblastoma (pRB) and p53 tumour suppressor proteins (Ozer 2000). These two tumour suppressor pathways are also perturbed in the majority of human cancers through mutation and methylation of the genes controlling

various components of these pathways (Levine 1997, Sellers and Kaelin 1997). Consequently, expression of the LT antigen can lead to the transformation of a cell from a normal to a growth-deregulated state. Alterations in growth suggest modifications in growth regulation, caused by changes in growth factor sensitivity, or increased autocrine productions of growth factors (see Table 3.2). Changes in growth properties, *in vitro,* associated with transformation towards a tumourigenic phenotype are often indicative of a more robust phenotype. Weinberg (2002) reported that in addition to the disruption of pRB and p53 tumour suppressor pathways, complete transformation of some human cells required the additional perturbation of protein phosphatase 2A by small T antigen. Expression of ST in this setting stimulated cell proliferation, permitted anchorage-independent growth, and conferred increased resistance to nutrient deprivation. These facts begin to clarify and even delineate a set of intracellular pathways whose disruption, in aggregate, appears to be necessary to generate tumourigenic human cells.

Therefore, as SV40 LT immortalization is involved in the process of transformation, immortal cells would be expected to have altered properties (inactivation of pRB and p53 among other things). In addition, immortalization achieved with SV40 large T antigen can also introduce variability into cell lines both as a consequence the site of integration of the LT antigen gene into the host cell genome and also the number of copies integrated. Integration may disrupt or deregulate normal gene function, while the copy number determines the level of SV40 large T expression. SV40 large T antigen itself is transforming if present at high levels, and results in the genetic instability of cell lines (Chang 1986).

The fact that SV40 Large T antigen itself can be transforming dependent on the level of its expression is now well recognised. Low levels are required for

immortalization while high levels are required for transformation (Price et al. 1994, Stewart and Bacchetti 1991, Ray et al. 1990). Price et al. (1994) compared the effects of low and high levels of T antigen expression in human fibroblasts. High levels of LT antigen expression resulted in cultures with a high rate of proliferation, an extended *in vitro* life span, a loss of contact inhibition of growth and a morphology characteristic of SV40-transformed cells. Low but detectable levels of the LT protein was accompanied by a 50% or greater reduction in the proliferative rate and restoration of cell morphology and contact inhibition similar to that found in non-transfected cells. These results demonstrate that low amounts of LT antigen are sufficient to maintain cell viability and prevent the re-expression of the senescent phenotype seen in the absence of LT antigen. Similarly, the ability of LT antigen to extend the *in vitro* life span is not dependent on high level expression of LT antigen. In contrast, the rate of proliferation of human cells as well as the cell morphology and contact inhibition are dependent on the amount of LT antigen present. Many of the cellular effects can be minimised or reversed by reducing LT antigen expression.

Furthermore, LT binds to several other cellular factors, interacting with and perturbing the function of proteins that are components of other cellular regulatory circuits (Ali and DeCaprio 2001, Flint and Shenk 1997, Pipas and Levine 2001). These include the transcriptional co-activators p300 and the related protein CBP, which may contribute to its transformation function (Eckner et al. 1996, Avantaggiati et al. 1996). Several other features of LT that appear to contribute to its full transformation potential are yet to be completely understood. Therefore, as the human mammary stromal cells studied were immortalized by SV40 LT (with hTERT), a certain level of genetic instability and phenotypic variability is to be expected.

Growth assays

The main purpose of this chapter was to determine how closely the immortalized stromal cell lines behaved in comparison to unaltered cells derived from primary cultures. This was in order to assess which of the immortalized cell lines, alone or in conjunction with the tumour cell lines, could provide the best model system for future *in vivo* experiments.

The significantly increased growth rates, a criterion of transformation *in vitro*, of the immortalized cells in comparison to the unaltered cells suggest that they must have adopted or changed to a more robust phenotype. The immortalization process had partially transformed/altered the stromal cell lines (fibroblast and endothelial cells) enhancing their growth properties to provide a significant growth advantage *in vitro*. A possible reason for the difference in growth rates may be attributed to cells being grown at different temperatures and thus having different metabolic rates. However, even when the temperature sensitive cell lines were grown at a lower temperature (33.5°C) than the unaltered cells (37.5°C) they still had greater growth rates.

In addition to the increased growth rate, the growth factor dependency of all the immortalized endothelial cells was significantly less than the unaltered cells at normal serum levels. Furthermore, the wild type LT variant HMME 7wt was even less dependent than the two temperature sensitive cell lines HMME 2 and 7. However, at low serum concentrations (0.02% v/v) all the immortalized endothelial cells behaved in a manner very similar to the unaltered endothelial cells. With the absence of dead cells and cellular debris in the serum dependent growth assays on microscopic examination, the difference in growth rates between the cell lines can be confidently attributed to be differential growth rather than differential survival due to cell death. The immortalized fibroblasts

lines also demonstrated reduced growth factor sensitivity with growth factors producing a minimal effect in comparison to the unaltered fibroblasts at normal serum concentrations. In contrast, the immortalized stromal cell lines also displayed properties consistent with normal cells such as anchorage-dependent growth and endothelial cell differentiation on Matrigel.

To summarise the results, all the immortalized stromal cell lines tested had undergone partial transformation evident from the results of the growth assays. All the fibroblast and endothelial cell lines were passaged in excess of 100 population doublings without evidence of cell senescence or crisis. The enhanced ability of the immortalized stromal cells to grow *in vitro* may suggest a higher chance of survival as well as enhanced growth when xenografted *in vivo*.

In order to define the effect the immortalization process had on the transformation status of each of the cell lines an attempt at a semi-quantitative analysis was carried out using the *in vitro* criteria of transformation previously listed in Table 3.2. Each criterion was assigned an arbitrary numerical score dependent on the degree of transformation. The higher the score obtained, the closer the cells were deemed to be to the fully transformed phenotype. Thus, fully transformed cells with a tumourigenic phenotype *in vivo* would be assigned a score of 5, while cells deviating from normal with an enhanced growth rate would be scored as 1. Intermediate values were assigned to other *in vitro* transformation characteristics. In compiling the final score the highest numerical score was summated with all the lower scores as the acquisition of a strong transformation phenotype (i.e. anchorage independent growth) is usually associated with concurrent expression of all the weaker features. Although not tested in all cases this was seen to be the case with the cell lines tested, for example HMF 3wt *ras*.

Criteria of transformation *in vitro*	Transformation score
I) Increased growth rate	**1**
II) Growth factor insensitivity NORMAL SERUM	**1**
III) Growth factor insensitivity LOW SERUM	**2**
IV) Higher confluent density (loss of contact inhibition of growth)	**2**
V) Cellular overlapping (loss of contact inhibition of movement)	**2**
VI) Loss of serum dependence	**3**
VII) Acquisition of anchorage independent growth (high colony formation in soft agar)	**4**
VIII) Ability to produce tumours in immunodeficient mice	**5**

Table 3.7. Arbitrary numerical scoring system to grade transformation

Immortalized stromal cells	Transformation score
NORMAL CELLS Unaltered fibroblasts- HMME Unaltered fibroblasts- HMF	**0**
HMF 3A HMF 3B HMF 3D HMF 3wt HMME 7 HMME 7wt	**4** **(Positive for criteria I, II & IV)**
HMME 2	**6** **(Positive for criteria I, II III & IV)**
HMF 3C (at late passage >60PD) HMF 3wt *ras*	**15** **(Positive for criteria I-VII)**
TUMOUR CELLS	**20**

Table 3.8. Assessment of cellular transformation. Cells and ranked by summating the transformation score (Table 3.7).

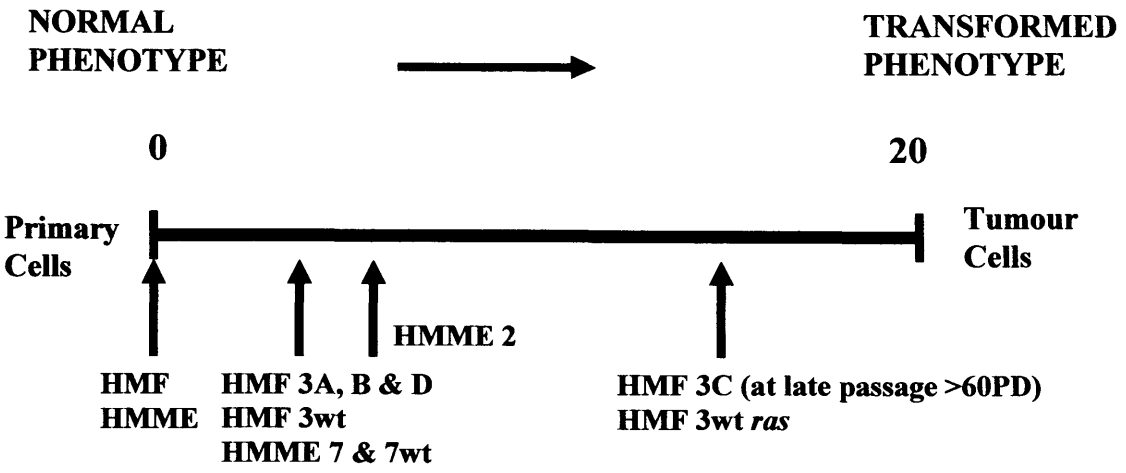


Figure 3.23. Diagrammatical representation of the transformation status of each of the immortalized human mammary stromal cells. The immortalized stromal cell lines demonstrated some of the hallmarks of transformed cells such as enhanced growth rates and reduced growth factor sensitivity at normal serum concentrations.

With the exception of HMF 3C and HMF 3wt *ras*, the remaining cell lines showed a similar transformation status; this phenotype being much closer to the normal phenotype than a fully transformed tumourigenic phenotype (Table 3.8 and Figure 3.23). However, although the remaining cell lines were transformed to a similar 'degree' they each also had subtle differences which made then unique. For example, HMF 3D lost their temperature sensitivity at late passage whereas HMF 3B did not express temperature sensitivity from the outset even though they were transduced with tsLT. HMF 3A and HMF 3wt, however, remained stable even at late passage (>100PD). HMF 3C as mentioned before was transformed at late passage to an anchorage independent phenotype. Also the cell lines differed in their growth factor sensitivities. For example, the wild type

LT variant HMF 3wt demonstrated reduced growth factor sensitivity in comparison to the tsLT transduced fibroblast lines.

With the endothelial cell lines the wild type LT variant HMME 7wt demonstrated significantly enhanced growth rates followed by the HMME 2 cell line with HMME 7 having the slowest growth rate. Both HMME 7 and HMME 7wt demonstrated reduced growth factor sensitivity at normal serum concentrations, however they behaved in a similar manner to the unaltered endothelial cells at low serum concentrations demonstrating growth factor responsiveness. HMME 2, by contrast, showed little evidence of growth factor responsiveness at normal and even low serum concentrations. These individual characteristics clearly demonstrate that these lines do differ even though the differences may be small.

In summary, the immortalized stromal cell lines displayed some of the hallmarks of transformation *in vitro*, although they also retained many of the characteristics of normal cells. Therefore, although they can not be regarded as normal cells they are also by no means fully transformed. In fact they were much closer phenotypically to normal cells than fully transformed cells. The results in this chapter also demonstrate that the each of immortalized human mammary stromal cell lines tested was unique, with each possessing slightly different phenotypic properties. Therefore one can conclude that the characteristics of the cell lines were not just a manifestation of LT antigen expression alone, but had gained other additional properties which made then individual.

Thus it seems we have useful cell lines: cells that possess an immortal growth potential which function essentially like normal cells without being fully transformed into tumourigenic phenotypes. They can therefore provide a readily available source of essentially normal human mammary stromal cells potentially

able to support a tumour microenvironment when xenografted *in vivo*. They thus provide a valid model for more complex culture systems and *in vivo* experiments. However, with the results available it was not possible to select a particular cell line as being better suited or a superior model for future *in vivo* studies. Their individual characteristics meant that each would have to be tested separately.

3.6.2 Tumour-stromal interactions

In the mammary gland, mesenchymal-epithelial interactions are of paramount importance during normal development and tumourigenesis (Cunha 1994, Silberstein 2001, Bissell and Radisky 2001). Positive regulation of normal and tumoural mammary epithelial cell proliferation by fibroblasts in co-culture are now well recognised. A number of published studies have provided evidence that stromal and epithelial components surrounding tumour cells are undoubtedly involved in tumour growth regulation and differentiation. The stromal-tumour co-culture experiments in this chapter were carried out to determine whether the immortalized stromal cells function like normal stromal cells in their interaction with tumour cells. As shown in the results (Chapter 3.3.1) the interaction of all the stromal cells with the tumour lines were similar, with no obvious distinction between either normal unaltered fibroblasts and immortalized lines, on the one other hand, or between immortalized lines and a *ras* transformed variant on the other.

Results from the co-culture experiments showed that the tumour-stromal interactions were on the whole inhibitory. Increasing concentrations of the breast tumour cell line, MCF-7, produced increasing inhibition of both the unaltered and wild type LT variants of the immortalized endothelial cells and fibroblasts.

Three different well established breast tumour cell lines acted in a similar manner also inhibiting both the unaltered and immortalized stromal cell lines. Even the VEGF over-expressing cell line MCF-7 V12 failed to stimulate the unaltered and immortalized endothelial cells. Furthermore, the stromal cell lines inhibited the growth of the 4 tumour breast cell lines when co-cultured in a similar manner. Both the unaltered fibroblasts and the transformed HMF 3wt *ras* fibroblast cell line inhibited tumour cell line growth. In addition, from investigation of fibroblast-endothelial cell interactions, it was found that the fibroblasts inhibited the endothelial cells whereas the endothelial cells greatly stimulated the fibroblasts (see Figure 3.15). The latter result demonstrates that growth stimulation can be detected with the chosen growth assay.

The results of the present study are contrary to current general perceptions which are that stromal-tumour interactions have a stimulatory effect on growth both *in vitro* (van Roozendaal et al. 1992, van den Hoof 1991) and *in vivo* (Shekhar et al. 2001). Several studies have shown that stromal-tumour interactions promote growth, differentiation and also invasion of human mammary tumour cells when studied in co-culture experiments similar to those carried out here (Ronnov-Jessen et al. 1995, Schor et al. 1994, Camps et al. 1990). Furthermore there are numerous studies comparing normal and tumour associated stroma, in particular fibroblasts, showing that in most cases both have a stimulatory influence on tumour cell proliferation and differentiation (van Roozendaal et al. 1996, Lefebvre et al. 1995, Gache et al. 1998, Ryan et al. 1993, Adam et al. 1994). Therefore the present results are not consistent with the majority of similar experiments. However it is worth noting that none of the above studies involved SV40 LT expressing stromal cell lines. In one study however (Dong-Le Bourhis et al. 1997), it was claimed that normal fibroblasts

inhibited tumour growth while tumour associated fibroblasts had no effect. Details of studies are shown in Table 3.9.

The experimental protocol used here was carefully defined to exclude factors and conditions which could have resulted in non-specific effects. For example cultures were inspected on a daily basis for signs of apoptosis, over confluency and extreme media conditions such as over-acidity. Medium was changed daily or as required. Experiments were repeated three times and gave similar results. In the present study the unaltered stromal cells (both fibroblasts and endothelial cells) from primary cultures also inhibited the growth of the mammary tumour cell lines and therefore it appears that the effects observed here are not solely associated with LT expressing cells.

	Reference	Target cell	Cell-cell contact	Effect
Fibroblast type				
Normal, immortalized & transformed cell lines	Present study	MCF -7, MCF-7 V12, T47D, ZR-75-1	No, co-culture insert system	All types inhibitory
Normal & tumour-associated	Valenti et al. 2001	MCF -7	No, conditioned media	Both types stimulatory
Normal & tumour-associated	Gache et al. 1998	MCF -7, MDA-MB231, T47D, and BT-20	No, co-culture insert system	Both types stimulatory
Normal & tumour-associated	Dong-Le Bourhis et al.1997	MCF -7	No, co-culture insert system	Normal-inhibitory, tumour associated-no effect
Normal & tumour-associated	Lefebvre et al. 1995	MCF -7	Yes	Both types stimulatory
Normal & tumour-associated	van Roozendaal et al. 1996	MCF -7	No, conditioned media	Both types stimulatory
Normal & tumour-associated	Adam et al. 1994	MCF -7	No, co-culture insert system	Both types stimulatory

Table 3.9. Studies that have examined fibroblast/mammary tumour cell interactions using co-culture systems. This study and those referenced are listed.

However, some studies do report inhibitory effects similar to the present study. Dong-Le Bourhis et al. (1997) demonstrated that fibroblasts originating from normal breast tissue inhibited the proliferation of MCF-7 cells in co-culture experiments, while serum free conditioned medium produced by primary stromal cells did not affect MCF-7 cell growth. These results suggest that epithelial cancer cells could induce, through the secretion of diffusible factors, normal

fibroblasts to produce tumour growth inhibitory factors. Indeed, it has been reported that TGF-ß released from tumour associated fibroblasts induces production of short-lived apoptosis-inducing factors in normal fibroblasts (Jurgensmeier et al. 1994, Wehrle et al. 1994). The apoptosis inducing signal in turn acts specifically on tumour cells leading to apoptosis. It was proposed that this complex and dynamic interaction may constitute a growth regulatory mechanism whereby normal stromal elements react to the presence of tumour cells and contribute to the repression of tumourigenesis.

The comparison in effect between normal and carcinoma associated fibroblasts on tumour cells are often carried out as part of the same experiment (see Table 3.9). Dong-Le Bourhis et al. (1997) reported that when fibroblasts from tumour tissue were used no effect was observed on the proliferation of MCF-7 cell in co-culture experiments. Lefebvre et al. (1995) used direct co-culture experiments allowing membrane contacts between the different cell types and showed stimulation of tumour growth. Thus, a possible reason for the discrepancy in results found in this chapter with those reported may be due to the fact that the immortalized stromal cells required actual physical contact in order to provide growth stimulation. The cells in the present study are not in direct physical contact but communicate through free exchange of growth and other soluble factors. These data suggest that, unlike normal tissue derived fibroblasts, tumour tissue derived fibroblasts as well as partially transformed fibroblasts may require cell-cell contact in order to act upon the proliferation of breast cancer epithelial cells. Direct contact may be a crucial criterion for promotion of growth and differentiation. Otherwise, as shown in our results, factors that inhibit tumour growth may be dominant.

However, Gache et al. (1998) studied the paracrine growth regulation of a variety of breast epithelial cells in co-culture with normal and tumour associated breast fibroblasts. They showed that all fibroblasts stimulated the proliferation of the hormono-responsive breast carcinoma MCF-7 cell line, suggesting that cell contacts were not indispensable for the paracrine stimulation of MCF-7 cell growth by fibroblasts. Moreover, the proliferation of a variety of other breast carcinoma cells (MDA-MB231, T47D, and BT-20) was also stimulated by fibroblasts. However, the amplitude of the proliferative response seemed to be dependent on the carcinoma cell line considered. Furthermore, the proliferative response of normal mammary epithelial cells to the presence of fibroblasts was shown to be significantly higher than the tumour cell response. The nature of the tissue of fibroblast origin, normal or tumour did not influence the growth response of the epithelial cells, in this study.

To investigate the disparity in results between the present study and the various other studies the method of co-culture was examined and compared. Factors such as length of experiment period, size of membrane pores, method of assessing cell numbers, medium used, cell numbers used and also the stage of cell growth could affect the outcome of the study. For example, the cell number used as well as the stage of growth, whether cells are proliferating or nearing senescence will affect cell-cell interactions. Gache et al. (1998) used a near identical cell co-culture method as the present study also using culture inserts with 0.4mm pore sizes. Cells were only co-cultured for 2 days in a serum-free or a 5% FBS-supplemented DMEM/F12 culture medium. Passage numbers were not stated, but the study suggests primary cultures with low passage numbers were used. A constant number of epithelial cells (5×10^3–1×10^4 cells/cm^2) was seeded either alone or in the presence of a constant number of fibroblasts

according to a fibroblast/epithelial cell ratio of 2. Cell proliferation was evaluated by measuring the incorporation of ^{3}H-thymidine.

Dong-Le Bourhis et al. (1997) who produced similar results as the present study plated 5×10^{3} epithelial cells on the membranes with 5×10^{3}- 5×10^{4} stromal cells in the lower chamber in a 10:1 fibroblast/MCF-7 ratio (same as present study). No pore size was stated. Both cell types were plated in DMEM/F12 culture medium containing 10% FCS which was changed every other day. Cell proliferation was evaluated by measuring the endogenous lysosomal hexosaminidase or the incorporation of ^{3}H-thymidine. Passage numbers were not stated, but the study suggests primary cultures of early passage were used. Cells were co-cultured for 8 days (similar experiment period). This co-culture experiment is much closer to the one used in this chapter. Hence it may explain why a similar set of results were obtained.

The effect of tumour cells on the growth of stromal components is not very well described in the literature. In one reported study Valenti et al. (2001) showed that conditioned medium (CM) from the MCF-7 cell line induced myofibroblastic differentiation as defined by increased smooth muscle actin formation, decreased cell proliferation, and increased apoptosis in cultured normal fibroblasts but not in fibroblasts from malignant breast tissue. These data suggest that structural and functional differences exist between stromal fibroblasts from normal breast and breast cancer with respect to the responsiveness to soluble factors present in the CM. They hypothesized that the lack of *in vitro* sensitivity to CM shown by tumour fibroblasts was the result of an *in vivo* inherent and stable phenotypic change on the fibroblasts surrounding breast tumour cells occurring via a paracrine mechanism. This study provides

evidence of tumour cell inhibition of normal fibroblast growth as found in the present study.

The present study also investigated tumour-endothelial growth interactions something, as far as we are aware, that has not been previously reported as distinct from effects on differentiation (i.e. tubule/vessel formation). Again mutual inhibitory effects were observed. Moreover, the investigations of stromal-stromal interactions described in this chapter involving fibroblasts and endothelial cells are unique. The growth of the unaltered fibroblasts HMF, immortalized fibroblast line HMF 3wt and the fully transformed *ras* transfected fibroblast cell line HMF 3wt *ras* were all stimulated by the presence of endothelial cells (both immortal and unaltered cells) in the co-culture experiments. However this stimulation was not bi-directional, as the unaltered and immortalized endothelial cells were inhibited by the 3 different fibroblasts mentioned above.

Overall the inhibitory effects observed in the study did not differ markedly between the different cell lines and therefore the experiments did not assist in selecting any specific line as more appropriate for *in vivo* studies.

3.6.3 Live cell labelling

Testing of cell labelling systems for the identification of different viable cell types in mixed populations of cells was not successful in that no one reliable long term labelling system was identified. The cellular labelling ability of two different classes of dyes, the cytoplasmic (CellTracker™) and membrane (Vybrant lipophilic) labelling system was investigated. Results showed that the all the dyes failed to provide an adequate level of cellular labelling *in vitro* after a

period of 5 days. However, the green CellTracker™ and red emitting DiD did provide a reliable method for labelling and separating two different cell types. Many previous studies using these dyes have been limited to hours or at most 2-3 days (Duffield et al. 2001, Kespichayawattana et al. 2000, Finnegan et al. 2001). As these dyes have been shown in the present study to be reasonably stable for up to 5 days, the problems encountered in the present study may have not occurred in the shorter time frame. However the longer term loss of staining intensity and transfer of dye noticeably with the orange CellTracker™ clearly precludes their use in longer term xenograft studies.

Chapter 4

Interaction of human and mouse endothelial cells *in vitro*

4.1 Introduction

For human tumour cell xenografts to survive and proliferate they must acquire a blood supply. It is well recognised that host derived vasculature provides this function. Recently, with the ability to incorporate human endothelial cells directly into xenograft models we can now determine whether these human endothelial cells can form the necessary vasculature alone or whether they cooperate with the host vascular system to form a chimeric vasculature. In other words do human endothelial cells interact with mouse endothelial cells *in vitro* to form organized structures – immature capillary networks. Successful integration of human and murine cells *in vitro* will allow us to further analyse the mechanism of complex vascular organization as well as develop pre-engineered vascular beds that can be used to perfuse transplanted human tissue.

It has been reported that human endothelial cell capillary networks/tubules formed *in vitro* when implanted *in vivo* (SCID mice) connect with mouse vessels to become perfused by the mouse circulation (Schechner et al. 2000, Nör et al. 1999, 2000, 2001). Human dermal microvascular endothelial cells transplanted into SCID mice on biodegradable polymer matrices differentiated into functional human microvessels that anastomosed with mouse vasculature (Nör et al. 2001). A matrix of collagen type 1 and human fibronectin supported the organization of human endothelial cells into primitive capillary like structures by 18-24 hours in culture and led to the development of human

endothelial cell lined microvessels perfused by mouse circulation after implantation. No significant mouse neo-vascularization or inflammation was associated with these constructs, indicating that a suitable balance had been achieved between sustaining human vessels and minimizing the host response. The human endothelial cells became invested by perivascular smooth muscle α-actin-expressing mouse cells 21 days after implantation. However, apoptosis of the primary endothelial cells was a problem in these studies. We therefore tested whether the potentially more stable immortalized human mammary endothelial cell lines could provide a better system to utilize in the formation of chimeric vasculature.

Mouse endothelial cells have in the past proven difficult to isolate and maintain in culture. The most useful techniques have either involved the infusion of target organs with digestive enzymes or the digestion and/or homogenization of an entire organ (O'Connell and Edidin 1990, DeBault et al. 1979). These methods are technically demanding and time consuming and generally yield relatively impure endothelial cells populations. Consequently, overgrowth of contaminating non-endothelial cells may occur in culture. In addition, a large number of mice are required for these endothelial cell preparations. Recently, methodological advances, such as the use of flow cytometry and Dynabeads together with monoclonal antibodies or appropriate lectins, have provided new ways to improve the purity of endothelial cell cultures (Sahagun et al. 1989, Jackson et al. 1990). However, these cell separation techniques have rarely been used to isolate mouse endothelial cells directly from tissue homogenates (Modzelewski et al. 1994). On top of this, long-term maintenance of normal mouse endothelial cells remains a difficult task. As an alternative, immortalization (e.g. with the polyoma middle-sized T antigen, PmT) has been

used to obtain mouse microvascular endothelial cultures from different tissues (Garlanda et al. 1994, Williams et al. 1988, Hahne et al. 1993). However, these immortalized mouse cells are transformed and tumourigenic and can differ considerably from their normal counterparts, as illustrated, for instance, by recent results of *in vitro* versus *in vivo* expression of the long pentraxin PTX3 (prototypic acute phase reactants that serve as indicators of inflammatory reactions). PTX3 expressed by normal mouse endothelial cells *in vitro* and *in vivo* in response to inflammatory cytokines/markers was not expressed by transformed endothelial cells (Introna et al. 1996). Immortomouse (Jat et al. 1991, Noble et al. 1995) endothelial cell lines could have been used in the construction of chimeric vessels *in vitro*, however it was decided to use normal mouse endothelial cells despite the labour isolating them, because they would be the best model for the interaction of human endothelial lines with hosts cells *in vivo*.

The aim of this section of the experimental programme, therefore, was to isolate fresh mouse endothelial cells to determine whether human endothelial cells, in the form of immortalized endothelial cell lines, can interact with mouse endothelial cells *in vitro* to form organized structures (i.e. immature capillary networks).

4.2 Isolation, culture and characterization of mouse endothelial cells

4.2.1 Isolation of mouse endothelial cells

A quick and efficient magnetic bead separation protocol outlined by Dong et al. (1997) was adopted and modified for the isolation of microvascular endothelial cells from mouse tissues as described in Chapter 2.3.1.

In brief, the lungs and hearts from 8 mice were dissected out, minced into small pieces (1 × 1mm squares) and digested in collagenase. The digested mix was then filtered to obtain a cell suspension ready for Dynabead immunomagnetic separation. The cell suspension was mixed with sheep anti-rat IgG coated Dynabeads bound to a rat anti-mouse PECAM-1 monoclonal antibody (MEC13.3, BD Pharmingen) for affinity binding.

Although precise cell numbers were not determined at this stage the size of the visible cell pellet isolated indicated that $\geq 10^6$ cells were potentially present. The isolation procedure was attempted 3 times with similar results. Each time the lungs and hearts of 8 mice, using mouse specific anti-CD31 conjugated Dynabeads, provided a small yield of approximately 1×10^4- 5×10^4 rosetted cells representing $\leq$1% of the initial digested cell suspension. The rosetted cells were lightly trypsinised to remove the Dynabeads. The immunomagnetically sorted cells were then cultured.

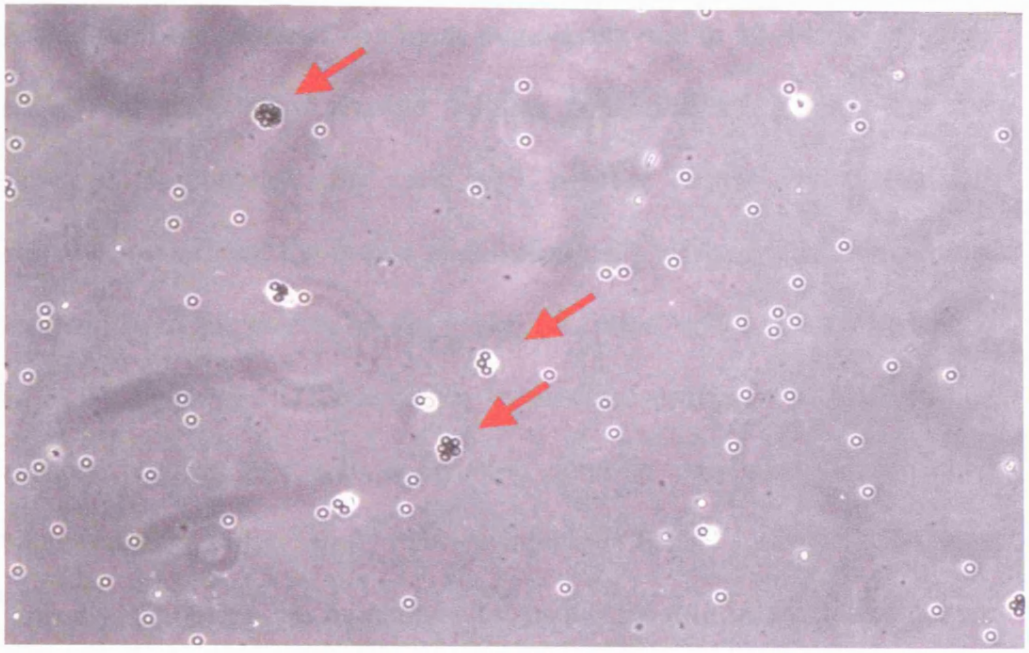


Figure 4.1. Dynabead rosettes of mouse microvascular endothelial cells labelled with an anti-mouse CD31 (arrows). Magnification ×220.

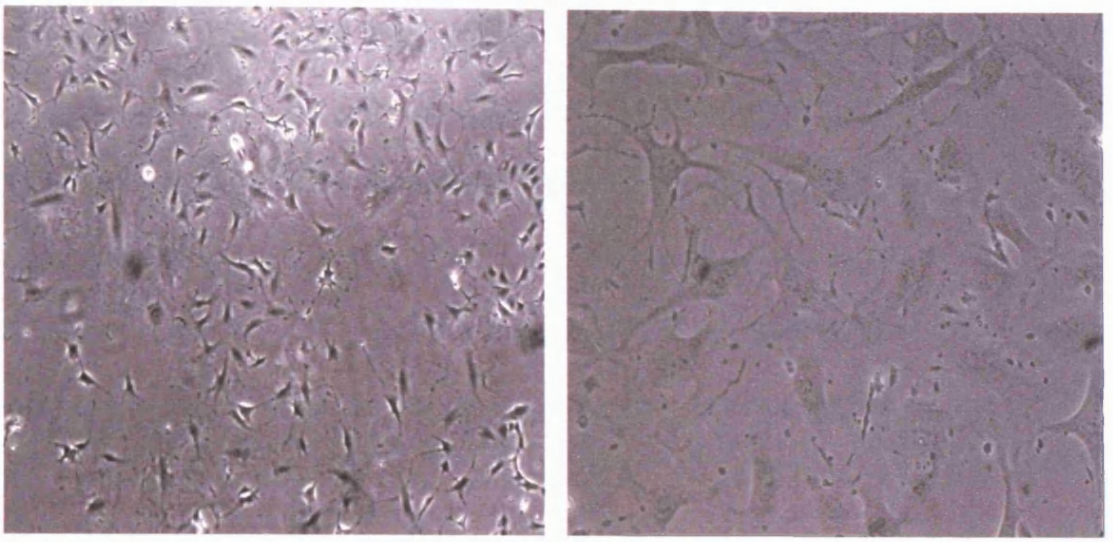
4.2.2 Culture of freshly isolated mouse endothelial cells

Immunomagnetically separated cells using the Dynabead separation method were plated in a 25cm^2 tissue culture flask containing 7ml of a specialized medium consisting of DMEM supplemented with 2mM glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 1mM sodium pyruvate, 20mM HEPES, 1% non-essential amino acids, 50mM 2-mercaptoethanol, freshly added 20% (v/v) heat inactivated FCS, 150µg/ml endothelial cell growth supplement and 12U/ml heparin (Marelli-Berg et al. 2000). They were cultured in either fibronectin (1mg/ml) or 2% (w/v) gelatin coated tissue culture flasks (see Chapter 2.2.1) at 37°C in a humidified atmosphere with 5% (v/v) CO_2.

Following isolation, trypsinisation and culture the cells initially attached and formed clusters which then spread out over 24-48 hours (Figure 4.2a) and

proliferated until they formed confluent monolayers within 10–14 days (Figure 4.2b). At this stage they showed contact inhibition of growth and the 'cobblestone' morphology also seen with primary human endothelial cell cultures. When confluent the mouse endothelial cells were serially sub-cultured by detaching from the culture flasks using a solution of 0.125% (w/v) bovine trypsin in 0.02% (w/v) EDTA and then re-passaged at a ratio of 4:1. In some experiments, enzyme free cell dissociation solution was used. This method involved washing twice with PBS containing 0.02% (w/v) EDTA and mechanically disrupting the monolayer by gentle tapping of the tissue culture flasks. In total the cells lasted for 3-4 months in culture, arresting after about 10 passages.

A) 2 days



B) 14 days

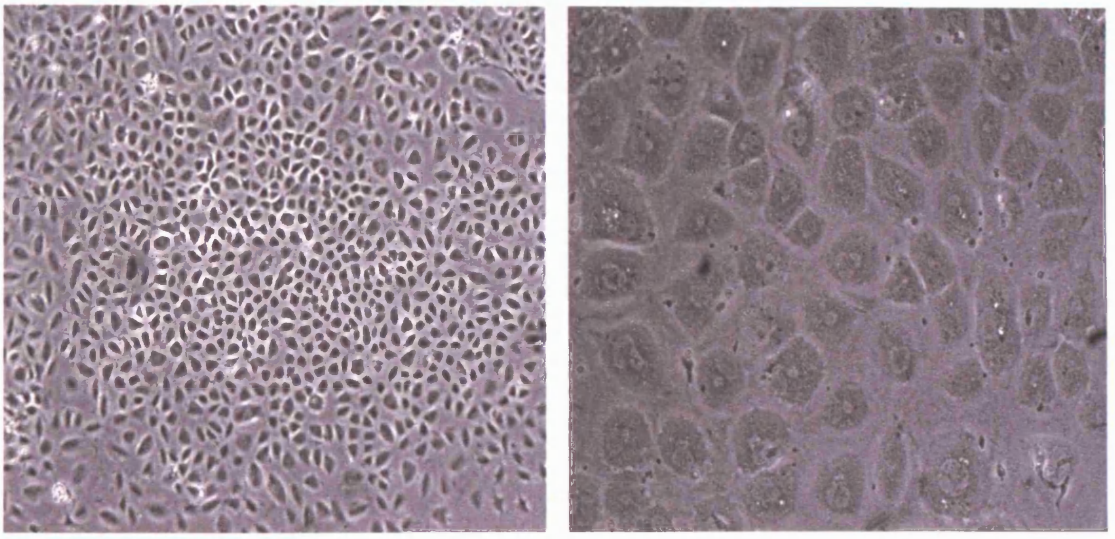


Figure 4.2. Phase contrast micrographs of the Dynabead sorted mouse microvascular endothelial cells in culture at 2 (a) and 14 (b) days. Following plating and two days in culture in the medium described above mouse microvascular endothelial cells formed characteristic 'spindle shaped' cells (a) before a typical cobblestone morphology was observed at confluence 14 days (b), also seen with primary human endothelial cells. Magnification ×80 (left) and ×160 (right).

4.2.3 Characterization of mouse endothelial cells

To confirm that the cells were truly differentiated endothelial cells and not simply contaminated with other cell types, cells harvested between passages 4-10 were immunophenotypically characterized. The mouse specific endothelial marker PECAM-1/CD31 that was originally used to sort the cells was used to label the mouse endothelial cells after they been in culture for between 14 and 50 days. Visualization was carried out by indirect immunofluorescence using secondary antibodies conjugated with either a FITC or TRITC fluorophore. Results are shown in Figure 4.3. Similar positive labelling of almost all cells (>95%) was observed with all cultures tested between passage 4 and 10. Previous chapters have demonstrated that this mouse specific anti-CD31 antibody was a reliable method of identifying mouse endothelial cells and vasculature *in vivo*. As confirmation of their mouse origin, Hoechst 33258 nuclear stain produced the characteristic punctuate staining pattern of this species (Figure 4.3d).

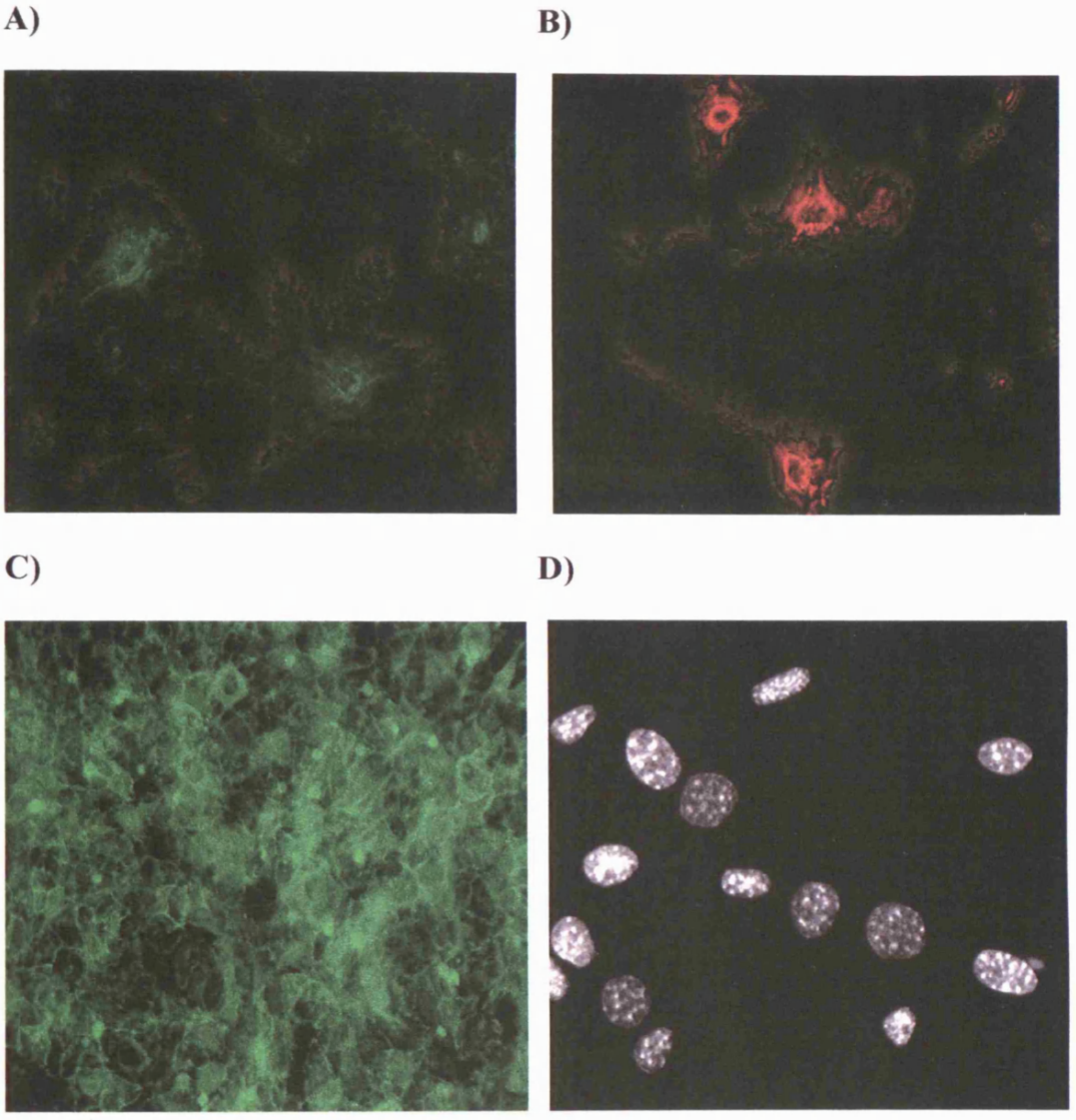


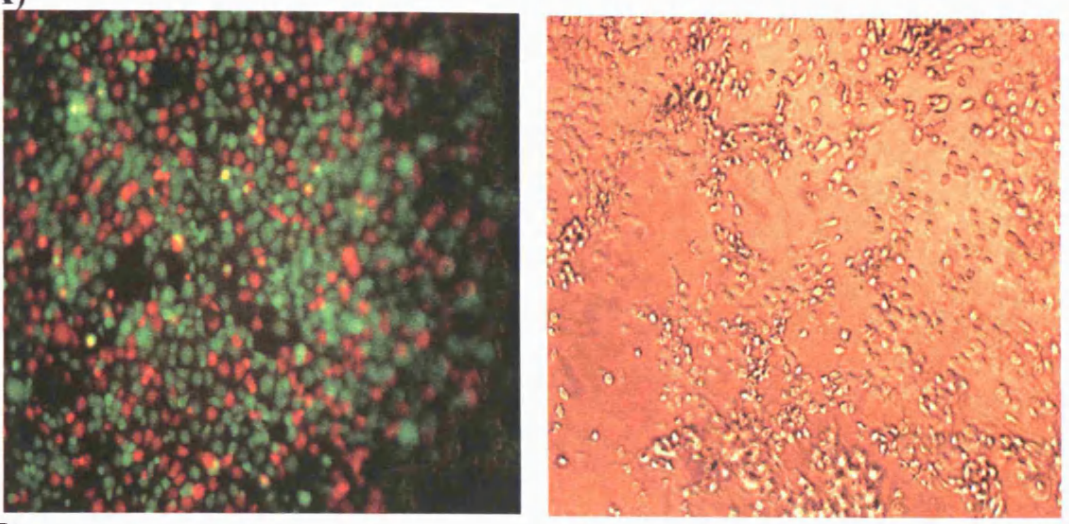
Figure 4.3. Immunofluorescence labelling of mouse endothelial cells using the mouse specific anti-CD31 antibody. Cells that had been in culture for 21 days (passage 1) were labelled using a rat anti-mouse CD31 monoclonal antibody (FITC & TRITC secondaries) (a-c). Hoechst 33258 nuclear staining of mouse endothelial cell (d) - note the punctuate nuclei which are unique to mouse cells differentiating them from human cell nuclei. Magnification a)×220, b)×220, c)×180, d)×220.

4.3 Human and mouse endothelial cell differentiation assays

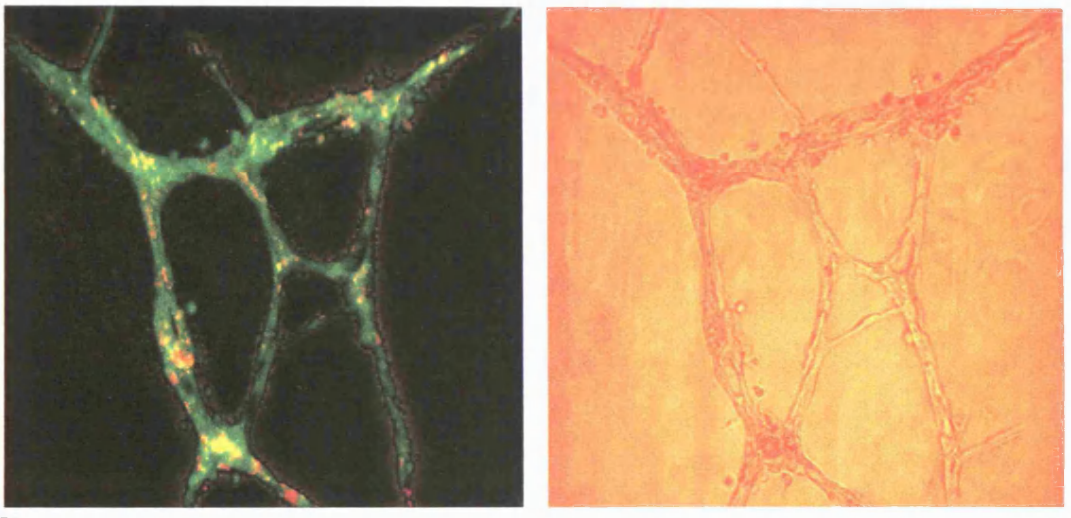
In order to investigate the interaction between human and mouse microvascular endothelial cells in an *in vitro* environment a simple differentiation assay was performed. Mouse endothelial cells that had been isolated from mouse tissues and cultured *in vitro* for between 21-35 days (as described in Chapter 4.2) were labelled using an orange CellTracker™ dye (see Chapter 2.4.1). The wild type human mammary endothelial cell line HMME 7wt was labelled with the green CellTracker™ dye. Although these dyes (in particular the orange CellTracker™) were not suitable for long term studies (see Chapter 3.5.2) the present experiment involved labelling of short term cultures only. Roughly equal numbers (5×10^4) of both labelled cells were then plated on prepared Matrigel in 24 well-plates and incubated at 37°C in a humidified atmosphere with 5% (v/v) CO_2 under a light microscope. Sequential images were then captured at 30 minute intervals for over a 24 hour period using an automated time lapse camera.

Collation of all the images showed that the human and mouse endothelial cells do interact and integrate with each other to form primitive micro-capillaries. Results are shown in Figures 4.4a-d.

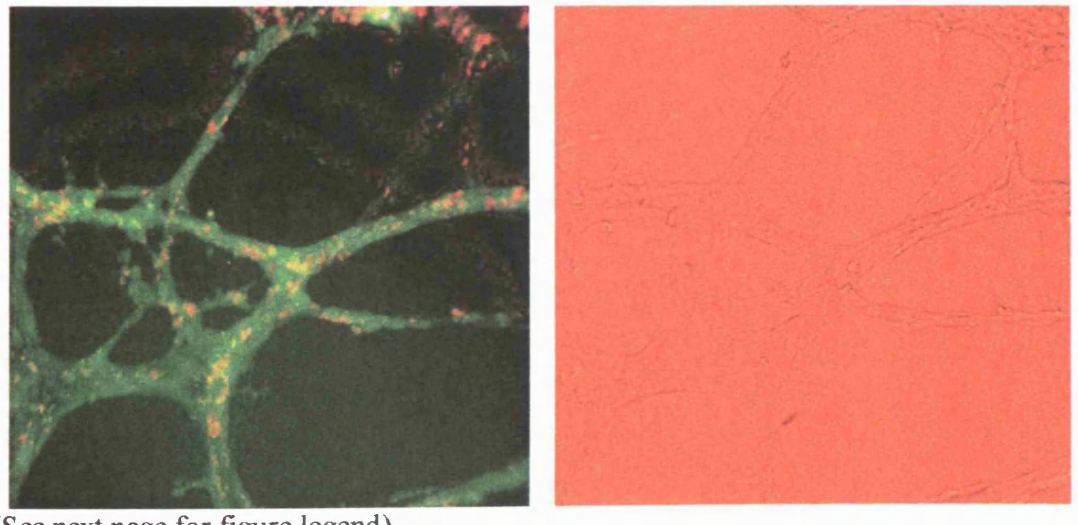
A)



B)



C)



(See next page for figure legend)

Figure 4.4. Immunofluorescence and brightfield images of chimeric capillary like networks formed by human and mouse endothelial cells. Human mammary endothelial cell line HMME 7wt was labelled with the green CellTracker™ dye whilst mouse endothelial cells were with labelled orange CellTracker™. Images on the left are fluorescence and on the right their corresponding brightfield images. (a) shows the two differently labelled cells plated on Matrigel. (b) & (c) are images taken after 24 hours of incubation on Matrigel. The two cell types have clearly integrated to form primitive micro-capillaries. Magnification ×80.

When initially plated the individual cells could be seen labelled green or orange (Figure 4.4a). Both cell types commenced aggregation within 1 hour and by 12 hours had formed long capillary like extensions (Figures 4.4b and c). Intermingling of both cell types followed by combined capillary network formation was then evident. On formation of capillary like networks, mouse and human endothelial cells could be clearly identified throughout the capillary networks as separately coloured cells. This experiment was repeated three times using mouse endothelial cells at a similar passage number from three different Dynabead sorts. Unfortunately as expected from previous results (Chapter 3.5.2) clear separation of labelling disappeared soon after this time, although the primitive capillary like structures did persist for several days (Figure 4.5).

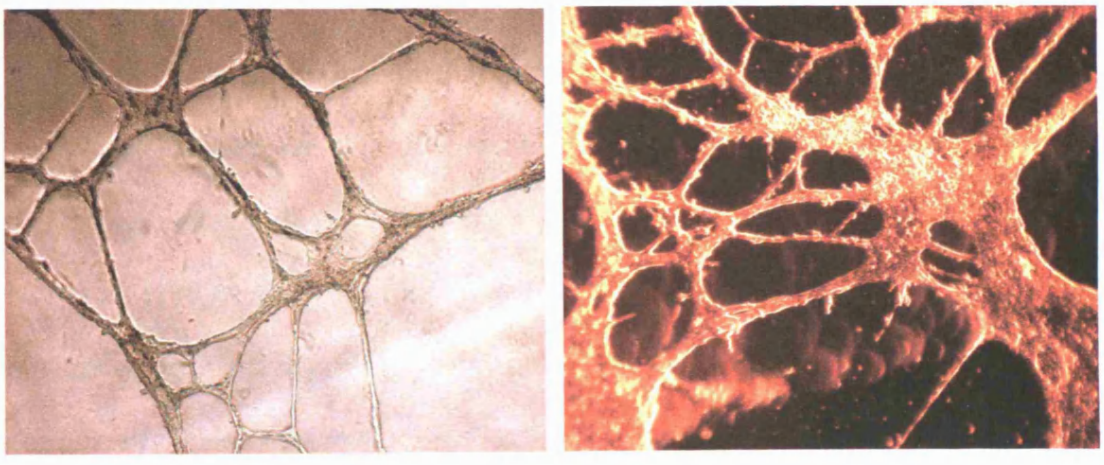


Figure 4.5. Phase contrast and darkfield images of primitive networks of microcapillaries consisting of both human (HMME 7wt) and mouse endothelial cells after 3 days of culture on Matrigel. Magnification ×80.

4.4 Conclusion and discussion

A modification of Dong et al. (2000)'s method for isolating mouse microvascular endothelial cells from mouse tissue provided a quick and an efficient method of producing a pure population of mouse microvascular endothelial cells. The resulting endothelial cells produced a good source of viable cells for further *in vitro* studies.

Results from the differentiation assays on matrigel showed that human and mouse endothelial cells do interact *in vitro* to differentiate and form micro-capillary like networks. These results are very promising as they suggest that the xenografted immortalized human mammary endothelial cell lines could interact with mouse endothelial cells *in vivo* and hopefully integrate into the vasculature of the proposed heterologous xenograft models.

The major limitation encountered with the construction and maintenance of human synthetic microvessels *in vitro* and *in vivo* has been the apoptosis of cultured human endothelial cells (Ilan et al. 1998). Both Schechner et al. (2000) and Nör et al. (1999) noticed the increasing presence of mouse microvessels in the implants over time. This was probably attributable to the fact that some of the endothelial cells underwent apoptosis, because transduction of endothelial cells with a modified (caspase-resistant) form of Bcl-2 protein delayed apoptosis allowing the consistent incorporation into the mouse circulatory system with an increased density of human cell-lined vessels. With our readily available source of immortalized human mammary endothelial cells this problem could be reduced by the greater robustness of the cell lines as compared with primary cells. Furthermore, it would be relatively straightforward to introduce a stable Bcl-2 over-expression in such lines if necessary. Due to the immortality of the

endothelial cell lines they should persist indefinitely as immortal cells. Incorporation of such human endothelial cell lined microvessels into our proposed 3-D organotypic breast tumour xenograft model may therefore improve tumour viability and proliferation. Successful integration of human and mouse cells will allow us to further analyse the mechanism of complex vascular organization as well as develop engineered vascular beds that can be used to perfuse transplanted human tissue.

Chapter 5

Three-dimensional heterologous co-culture models

5.1 Introduction

To investigate the pathobiology of human breast cancer, it is necessary to maintain or recreate the characteristic three-dimensional (3-D) architecture of the tumour tissue in culture. More specifically, to create 3-D heterologous *in vitro* co-cultures involving tumour cell interaction with stromal cells; endothelial cells, fibroblasts and immunocompetent cells. In previous years the study of normal human breast physiology and breast cancer has been significantly impaired by limitations inherent to available model systems, specifically two-dimensional models (2-D).

2-D monolayer culture models are easy and convenient to set up with good viability of cells in culture but they lack the 3-D microenvironment of intact tissue. In contrast organ cultures have a ready made tumour microenvironment which remains intact. This is of vital importance as cell–cell, cell–matrix interactions and interstitial fluid affect differentiated cell function within the 3-D environment. However organ culture is not without its own problems; with difficulty in obtaining specimens and poor viability of the tissues in culture being major obstacles.

The concept of the multicellular tumour spheroid was developed to overcome this very problem. Spheroid cultures mimic both the 3-D organization and differentiated function of intact tissues to a much greater extent than cell monolayers (Becker et al. 1993). Multicellular tumour spheroids were

specifically manufactured for cancer research and for the testing of the effects of radiation (Wheldon 1994) and new chemotherapeutic agents (Mueller-Klieser 1997, Yuhas et al. 1978). They were generally formed from robust tumour cell lines which aggregated homotypically to produce solid tumour cell spheroids (Yuhas et al. 1977). They probably grew well in avascular conditions as they were originally from anoxic tumours.

With recent advances in 3-D cell culturing techniques, spheroid-like 3-D heterologous *in vitro* co-culture systems can now be manufactured. With these individually designed models many important variables can be accurately controlled (e.g. cell types, numbers and experiment duration). These model systems can in principle reproducibly reflect the distinct invasive behaviour of breast tumour cells, mimic the tumour-stromal cell interactions of breast carcinomas and allow for systematic investigation into the multiple unknown regulatory feedback mechanisms between breast tumour and stromal cells in a well defined 3-D environment (Petersen et al. 1992, Bergstraesser and Weitzman 1993, Kunz-Schughart et al. 2001). Multicellular tumour spheroids have a well organized spherical symmetry of morphological and physiological features including complex cell-cell and cell-matrix interactions resembling avascular tumour sites and or micrometastatic regions *in vivo* and thus are a very useful model in tumour biology (Sutherland 1988).

In principle, 3D spheroids can be manufactured either by exploiting spontaneous cell aggregation, generating more or less spherical cellular conglomerates, or by culturing cells on artificial substrates that induce cellular differentiation and maintain cellular function. Cells can either adhere to each other (homotypic aggregation) or other cells (heterotypic aggregation) as well as to endothelial cells and exposed basement membrane. Some breast cancer and

breast cell lines will spontaneously aggregate during cell culture, to form tissue-like spheroids. For example, it is well documented that the human breast carcinoma cell line MDA-MB-435 undergoes homotypic cell aggregation (Glinsky et al. 2000) and the breast carcinoma cell line DU4475 grows in suspension to form clusters and cords which are attached chiefly by desmosomes (Langlois et al. 1979).

The aim of this chapter was to investigate which of the available tissue culture techniques was most suitable to manufacture a 3-D heterologous spheroid using the immortalized stromal cells as components. Therefore having selected, tailored and optimised the most suitable technique the final goal was to recreate a heterologous spheroid model closely resembling a 'metastatic avascular *in vivo* tumour' to xenograft into immunodeficient mice. If successful the advantages of this specific model will be two-fold. Firstly the spheroid will consist of both human tumour and stromal cells and secondly, the problem of cell dispersion on injection *in vivo* can be overcome by maintaining cells in close proximity. Furthermore tumour-stromal interaction within these heterologous tumour spheroids should provide the immortalized human mammary stromal cells with the best possible chance of survival and proliferation, not only *in vitro* but also *in vivo*.

To assist in the manufacture of heterologous spheroids in the present study, using immortalized human stromal cell lines, several different techniques were successively tested. These were a) use of microcarrier beads, b) liquid overlay cultures, c) 3-D biodegradable pre-engineered scaffolds and d) the Rotary Cell Culture System (RCCS). It may also be advantageous to use a combination of techniques to produce the best results.

Microcarrier beads were introduced to provide support as the tumour and stromal cell lines used depended on attachment and did not spontaneously aggregate. Also they provided an effective vehicle to improve the culture of sensitive cell lines (e.g. endothelial cells) (Bing et al. 1991, Davies 1981) which requires specialized conditions to grow. In addition microcarrier beads provided an effective way in which to culture different cell types in close proximity. The large surface area to volume ratio together with their variety of substrate coatings allows cells to proliferate on microcarrier beads in effect creating mini-spheroids. As will be shown these seeded microcarrier beads can then adhere to each other and form larger spheroid-like structures.

Liquid overlay culture is a relatively old technique which has been widely used in the past to generate and study a small numbers of spheroids. By culturing cells on an attachment limiting surfaces such as agar, cell-cell aggregation is promoted over cell-substratum attachment thus promoting spheroid formation (Yuhas et al. 1977). It therefore provides the simplest way of culturing a small number of spheroids.

The RCCS is a new and innovative form of optimized suspension culture which is designed to mimic microgravity to maintain cells in fluid suspension under very low-shear stress conditions (Hammond and Hammond 2001). Accordingly, the RCCS largely solves the problems of suspension culture: to suspend cells and microcarriers without inducing turbulence, or high degrees of shear, while providing adequate nutrition and oxygenation. Due to its low-shear and low-turbulence environment it minimizes mechanical cell damage and allows dissociated cells to assemble into high fidelity 3-D tissue aggregates, several millimetres in size which are largely devoid of necrotic cores, thereby promoting

cellular differentiation (Hammond and Hammond 2001, Unsworth and Lelkes 1998).

3-D pre-engineered scaffold serves as a 3-D physical support matrix for *in vivo* cell culture. In essence it simulates the structural and functional role of the ECM and its molecules. This method is not a technique to produce spheroids as such but it constituted an alternate way of manufacturing a 3-D heterologous tumour model for xenograft experiments. In addition, while microcarrier beads are inert and stable, these 3-D scaffolds are biodegradable once xenografted *in vivo* and so can help support the creation of a 3-D structure before eventually disappearing.

5.2 Microcarrier beads

5.2.1 Introduction

The use of microcarrier beads has provided many advantages to cell culture (Clark and Hirtenstein 1981). Conventionally microcarrier bead cultures involve growth in rotating vessels to assist in mixing and providing nutrition. A higher surface area to volume ratio can be achieved which can be varied by changing the microcarrier bead concentration. This leads to high cell densities per unit volume with the potential for obtaining higher cell concentrations. Cell propagation can be carried out in a single high productivity vessel instead of using many low productivity units, thus achieving a better utilization and a considerable saving of medium (Gao et al. 1997). Since the microcarrier culture is well mixed, it is easy to monitor and control the different environmental conditions such as pH, pO_2, pCO_2 etc. In addition, given that the beads settle

down easily cell sampling, cell harvesting and downstream processing of products are easy.

Several factors affect cell adhesion and attachment. The surfaces on which the cells are grown as well as the cells themselves are at a physiological pH and have either a net negative or a positive charge. The charge density on these surfaces rather than their polarity is responsible for attachment and spreading of cells. Cell adhesion also depends on a functional contractile system and is mediated by specific cell surface receptors (Hirtenstein et al. 1980). It involves multiple contacts with the surface where numerous filopodia are formed. They fit into a lattice structure formed by the glycoproteins on the substratum. This is followed by cytoplasmic webbing and flattening of the cell mass. In this way the cells get attached onto the substratum (Hirtenstein et al. 1980). Once the cells have attached onto the microcarriers, they grow using the nutrients provided in their culture medium. The complexities of this culture system are mainly due to the large number of parameters affecting cell yield (Clark et al. 1980). Medium composition now assumes importance since it contains the carbon and nitrogen source, the energy source, growth factors and dissolved oxygen and other gases. Besides nutrient limitation, growth of cells is also affected by the accumulation of toxic metabolites. Other important considerations are environmental factors like pH and temperature and shear sensitivity of the cells, especially in case of microcarrier cultures employing spinner bottles (Clark et al. 1980, Clark and Hirtenstein 1981).

A large variety of microcarrier beads are currently available (eg. positively-charged DEAE or trimethyl-2-hydroxyaminopropyl groups) in different sizes ranging from 95-210μm. The dextran Cytodex 3 (size 133-215μm) microcarrier beads coated with denatured porcine-skin collagen bound to

surface was used. This type was specifically chosen as it was designed to culture hard-to-grow cells.

5.2.2 Results

For microcarrier bead seeding, 5 x 10^6 cells of each type (MCF-7, MHF 3wt and HMME 7wt) were added to 5ml of bead containing solution (1.5 x 10^5) separately. The beads and cells were transferred to a 125ml Wheaton Magna Flex Spinner Flask, containing 75ml of complete medium, and incubated under constant stirring at 30 revolutions per minute (rpm) in a humidified atmosphere of 5% (v/v) CO_2 at 37°C overnight to attach. The medium was replaced with fresh medium every fourth day by letting the beads settle and changing 50ml of medium. To add cells in free suspension 5 x 10^6 of the required cells were counted and added to the pre-seeded microcarrier beads in the rotating Spinner flask.

To create heterologous co-culture spheroids, cells were added either in free cell suspension, as pre-seeded microcarrier beads or using a combination of both methods. All the combinations summarized in Tables 5.1a-c were cultured for 14 days. In addition a further experiment was carried out in which each cell type was used separately to coat a microcarrier bead suspension, and all three cell coated microcarrier beads were then mixed in equal proportions and cultured in the spinner flask. The following observations were made following their preparation by examining them microscopically at daily intervals.

A)

	MCF-7 (CS)	HMF 3 wt (CS)	HMME 7wt (CS)
MCF-7 (MCB)	N/A	+	+
HMF 3 wt (MCB)	+	N/A	+
HMME 7wt (MCB)	+	+	N/A

B)

	HMME 7wt (CS) + HMF 3wt (CS)	HMF 3 wt (CS)+ MCF-7 (CS)	HMME 7wt (CS) + MCF-7 (CS)
MCF-7 (MCB)	+	N/A	N/A
HMF 3 wt (MCB)	N/A	N/A	+
HMME 7wt (MCB)	N/A	+	N/A

C)

	MCF-7 (MCB)	HMF 3 wt (MCB)	HMME 7wt (MCB)
MCF-7 (MCB)	N/A	+	+
HMF 3 wt (MCB)	+	N/A	+
HMME 7wt (MCB)	+	+	N/A

Tables 5.1A-C. Table summarizing the combinations of cells used to form spheroids. Cells were added either as a free cell suspension (CS) or as cells seeded on microcarrier beads (MCB).

Cells when added as a free suspension to the rotating Spinner flasks containing pre-seeded microcarrier beads attached to the beads within 12-24 hours. Cells originally observed to be in free suspension in the liquid medium at one hour post seeding were not visible after 12-24 hours, indicating that they had attached to other cells or to microcarrier beads (Figure 5.1).

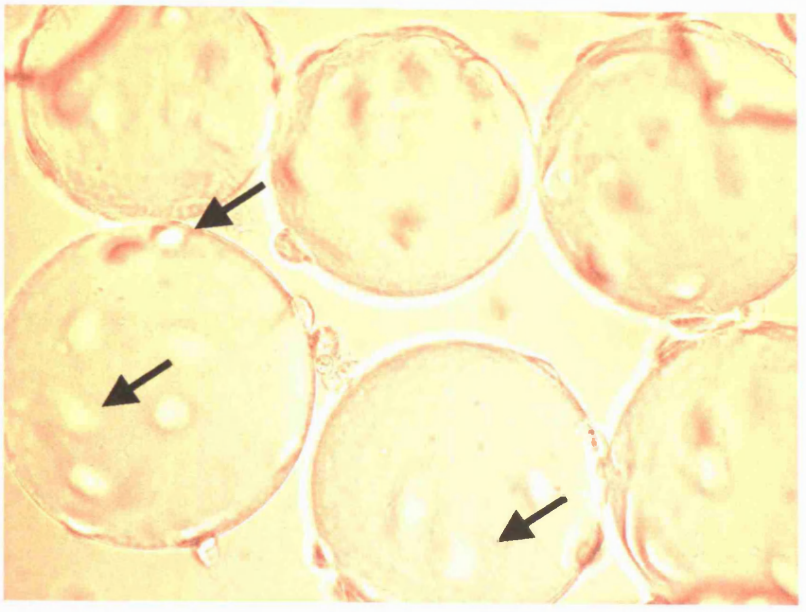


Figure 5.1. Microcarrier beads (Cytodex 3, size 133-215 µm). Brightfield mage shows microcarrier beads seeded with fibroblasts (HMF 3wt). Arrows point to cells attached to the surface of the microcarrier beads. Taken at ×320 magnification.

No one particular combination resulted in a noticeable difference in the rate of growth or size of spheroids produced. Initially single or small clusters of pre-seeded microcarrier beads adhered to other microcarrier beads resulting in progressively larger clusters of seeded microcarrier beads (Figure 5.2). Progressive increase in the size of these spheroid clusters, when observed daily over the 14 day experiment period, under the microscope, indicated that at least one of the cell types proliferated when cultured on microcarrier beads. However the spheroids broke up into smaller fragments at variable time points leading to smaller spheroids. This continued in a cyclical manner, with repeated aggregation followed by separation.

As a result of repeated aggregation followed by separation the number and size of spheroids obtained after 14 days of culture was too small to utilize as

xenografts. Thus in an attempt to stabilize the aggregation of microcarrier bead they were next used in combination with liquid overlay cultures, rather than in free suspension.

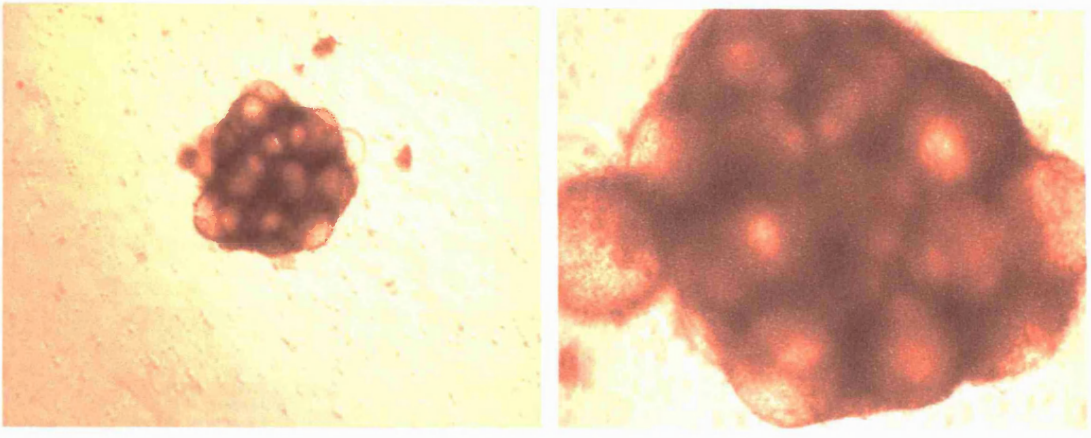


Figure 5.2. Heterologous spheroids manufactured from co-cultures of microcarrier beads separately seeded with MCF-7 cells, HMF 3wt and HMME 7wt cell lines at 14 days. Note the cells binding the microcarrier beads together. Brightfield images taken at ×80 (left) and ×160 (right) magnifications.

5.3 Liquid overlay cultures

5.3.1 Introduction

There has been extensive research describing the mechanisms by which the structural architecture of spheroids induces cell differentiation; increased cell-cell and cell-matrix interactions, changes in cell shape, and the development of a rich interstitial fluid within cell multilayers which are essential in this regard (Durand 1990, Sacks et al. 1989). In contrast, the underlying mechanisms governing spheroidal formation remain poorly understood. Many seemingly

promising cell lines are later found to be unable to self-assemble into spheroids (Sutherland 1988).

The simplest method of promoting spheroidal growth is liquid-overlay cultures. For cells to grow as 3-D aggregates or spheroids they require conditions in which the adhesive forces between the cells are greater than for the substrate it is plated on. Cell suspensions are grown in a static culture over an attachment limiting surface as agarose or agar (Yuhas et al. 1977). Liquid overlay techniques achieve this condition by reducing matrix deposition stimulated by contact with a flat surface. This promotes cell-cell attachment over attachment to the substratum. With this technique many tumour cell lines will undergo spontaneous homotypic aggregation. Spheroid formation can even be induced in tumour cells that do not spontaneously aggregate or are difficult to aggregate when cultured in free suspension. Essentially, spheroid formation in liquid-overlay cultures follows a biphasic process; firstly cells migrate towards each other on the agar and aggregate into spheroids. Secondly cell growth results in an increase of spheroid size. Cells do not adhere to the substratum but grow and aggregate on it.

Although liquid overlay culture is a relatively old method of manufacturing spheroids, with more advanced forms of culturing techniques available, they were utilized because they may be more advantageous in culturing individual 3-D heterologous spheroids. It was envisaged that a single carefully cultured spheroids may be adequate to produce a tumours when xenografted into mammary fat pads.

5.3.2 Results

Two different substrates were used to were used to construct the liquid overlay cultures; agar which inhibits substrate attachment and reconstituted basement membrane extract matrigel which allows attachment and movement of cells. The three cell types were cultured either by plating on or mixing in with one of the two different substrates. In a second series of experiment cells were pre-seeded on microcarrier beads prior to culture as described in Chapter 5.1. Again these were cultured both on and in the two different substrates.

For cultures with cells seeded in the substrates, cell suspensions (approx. 1×10^4 cells) or pre-seeded microcarrier beads (1×10^4 cells seeded on 5×10^3 beads) were added and mixed with the substrates (500µl) before being applied to a 24-well plate and allowed to gel. Then 2ml of growth medium was added to produce the liquid overlay. Following an overnight incubation, medium were replaced daily thereafter. For extended cultures (>2 weeks); a further 2 ml of fresh medium was added on the second day. This allowed for replacement of spent medium at the air liquid interface without disturbing the cellular layer on the gel surface.

For culture on the surface of the substrates the above method was repeated this time with cells or seeded microcarrier beads plated on the surface rather than mixed with the substrates. Both groups were cultured for 14 days.

Following culture several attempts, using various different methods, were made at harvesting these 3-D heterologous co-cultures to analyze their cell composition and architecture.

Liquid overlays using agarose

The three cell types when plated on agarose commenced aggregation after 48 hours by forming loose cell aggregates/clusters. They aggregated very slowly taking between 3-7 days to form loose cell clusters, however they did not resemble the tight compact architecture of spheroids. The use of microcarrier beads did not significantly enhance cell aggregation. Liquid overlays on agarose did not produce suitable spheroids for harvesting or xenografting.

Liquid overlays using Matrigel

As an alternative to agar Matrigel was used. This is not an attachment limiting substrate but it did promote the aggregation of cells as described. Cells when cultured on Matrigel as a mixed cell suspension commenced spheroid formation within 24-48 hours. Cells moved along the Matrigel to aggregate into solid spheroid like structures with very few free cells remaining un-aggregated. Although spheroids did form with and without microcarrier beads, spheroids formed with the aid of microcarrier beads were in general larger, faster growing and more structurally robust (Figure 5.3).

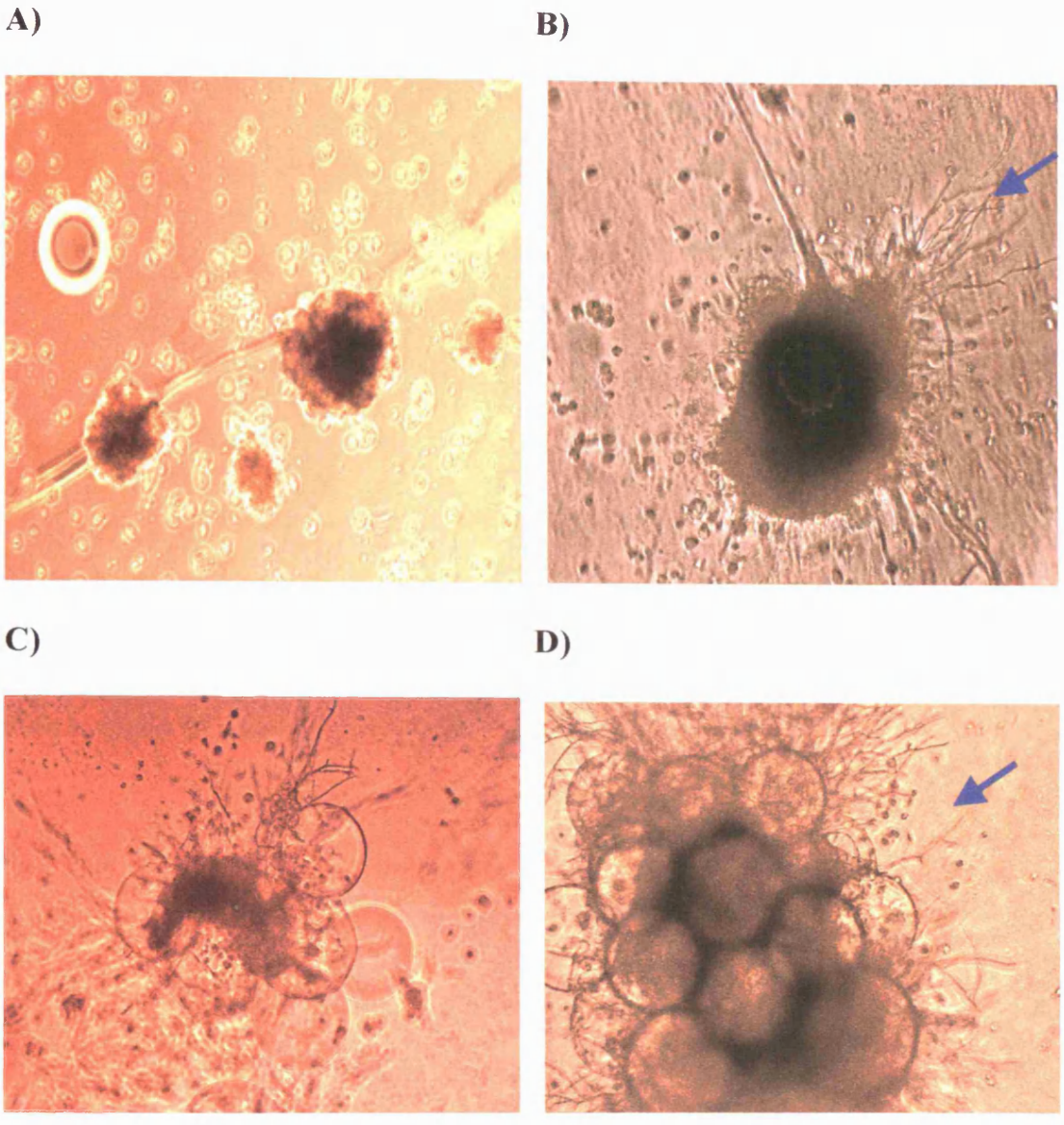


Figure 5.3. Brightfield images of heterologous tumour spheroids (containing MCF-7, HMF 3wt and HMME 7wt cell lines) manufactured in liquid overlay cultures (A & B) and with the aid of microcarrier beads (C & D). Images taken at ×80 and ×160 magnifications at various time points. Spheroids manufactured with microcarrier beads are larger at similar time points as well as being more differentiated demonstrated by larger visible sprouting of capillary like vascular structures. Arrows point to capillary like structures growing out from the spheroids formed from differentiated human mammary endothelial cells.

Culturing cells, with and without microcarrier beads, in Matrigel produced spheroids earlier (24-36 hours), compared to agarose, with larger overall spheroid sizes reached. However as expected, the total overall number of spheroids produced per well, in both groups, were low (≤10/well). Only spheroids grown in Matrigel showed evidence of endothelial cell differentiation by the large number of capillary like sprouting from the spheroid edges.

Harvesting spheroids

Three different methods were devised to harvest the heterologous spheroids from the matrigel cultures for more detailed histological analysis. The first attempt involved emptying the microwells of medium and fixing in formaldehyde followed by manually scooping out visible spheroids using a small spatula. This method was very labour intensive. However, the greatest problem faced was in handling these very fragile and unstable structures. Even the slightest trauma would disrupt and disaggregate the spheroids. The second method involved cryopreserving the microwell plates, containing the spheroids in the substrate, by placing in an -80°C freezer. Although both the agarose and matrigel froze into a solid form they were too soft to obtain good quality frozen sections. The third method involved encasing the spheroids in hot agarose which gelled to form a very hard solid block and then cryopreserving this block to cut under a microtome. In order to obtain a sufficiently hard agarose block for sectioning, a greater weight per volume of agarose was required (>35% w/v agarose). To solubilize such a high agarose content high temperatures (>95°C) were required which resulted in thermal damage to the spheroids.

To summarize liquid overlay cultures are well recognised as a method to study small numbers of spheroids as opposed to an efficient method of manufacturing lots of spheroids. However spheroids manufactured in this way did not have the

necessary density of cells and/or the structural stability to be used as xenografts. Therefore, although relatively simple to manufacture, liquid overlay culture did not represent an efficient way of manufacturing 3-D heterologous spheroids for xenografting.

5.4 Prefabricated 3-D scaffolds

5.4.1 Introduction

Recently 3-D, biodegradeable, pre-engineered scaffolds have been introduced as an improved method of simulating the extracellular matrix (ECM) and providing physical/structural support (Figure 5.4). These structures consist of natural molecules (collagen composite) or synthetic polymers (D, D-L, L polylactic acid) that can be used as a 3-D physical support matrix for *in vitro* cell culture (Martin et al. 1998, Wei Tan et al. 2001) as well as *in vivo* tissue regeneration (Evans et al. 1999, Saito et al. 2001). They function as temporary scaffolds that maintain transplanted cells in a defined space and therefore guide new tissue growth and organization. They bring great potential in recreating the natural physical and structural environment of living tissue (Wei Tan et al. 2001). This is of great advantage as ECM molecules have been shown to promote signalling pathways that influence key cell functions such as migration, proliferation and differentiation. Currently they present one of the most promising experimental approaches for regenerating the native structural and functional properties of living tissue.

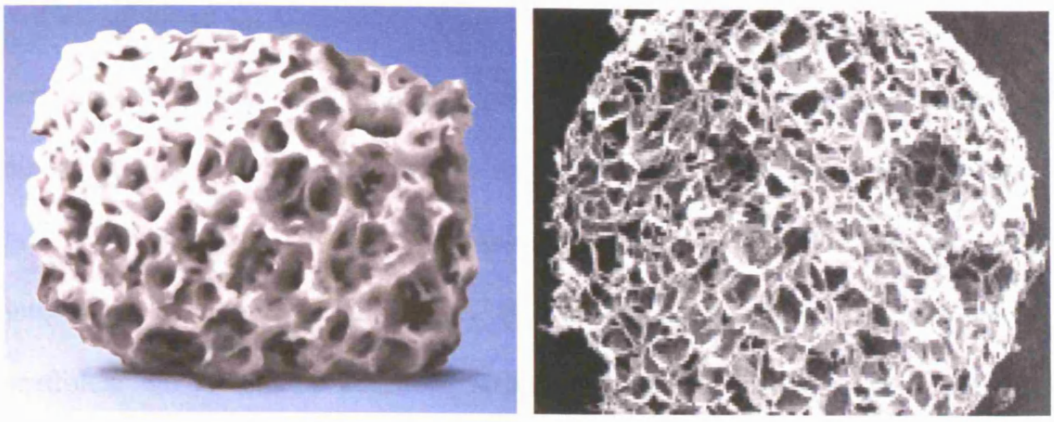


Figure 5.4. 3-D biodegradable pre-engineered scaffolds. Images are of a BD™ Three Dimensional Open-Cell Polylactic Acid (OPLA®) Scaffold. An open-cell facetted architecture is effective for culturing high density cell suspensions. They are approximately 5mm x 3mm x 0.039 cm^3 in size with an average pore size of 100-200 µm. They weigh 3.5mg when dry and have the capacity to absorb 25µl to give a final wet weight of 45 mg. (Images taken from BD Biosciences)

These scaffolds have been reported to be beneficial in the long-term growth and differentiation of a variety of cell types, including epithelial cells e.g.hepatocytes (Takezawa et al. 2000), neurons, endothelial cells (Sheridan et al. 2000), osteoblasts (Malekzadeh 1998), chondrocytes, (Chu et al. 1997) fibroblasts, and smooth muscle cells (Kim et al. 1999). Since tissue and organ function are dependent on the presence of an appropriate population of differentiated cells, a considerable amount of research has been focused on the development of 3-D culture systems for the expansion and differentiation of pluripotent stem cells (Solchaga et al. 1999). Recent studies have shown that human microvascular endothelial cells transplanted in sponges into SCID mice have been shown to organize and differentiate into functional microvessels that anastomose with mouse vasculature and become functional human microvessels containing mouse blood cells (Schechner et al. 2000, Nör et al. 2000).

As far as we are aware, there have been no reported studies of human breast epithelial cells or tumour cell lines being cultured in these scaffolds. The aim of this section was to culture human breast tumour cell lines together with human stromal cells in these scaffolds to promote cell differentiation and interaction. If successful the ultimate goal was again to transplant these seeded scaffolds, which should be more structurally stable than aggregates of cell suspensions with and without microcarrier beads, into the mammary fat pads of immunodeficient mice to create more realistic xenograft models. Once the seeded scaffolds had established the scaffolds should then degrade slowly leaving a well differentiated organotypic tumour.

5.4.2 Results

Scaffolds were seeded using two methods: still and dynamic. For the still seeding method a cell suspension containing approximately $5.0 \times 10^4 - 2.5 \times 10^5$ cells (of each cell type) in 250µl of growth medium was added to a scaffold (see Table 5.2 for combinations). The seeded scaffolds were transferred to 96-well plates containing 250µl of growth medium. In some experiments the seeded scaffolds were coated in matrigel or serum. For dynamic seeding, the seeded scaffolds were incubated with gentle agitation (~50–100 rpm) on an orbital shaker at 37°C in a gassed incubator for between 2-24 hours depending on the cell type. After incubation with agitation was completed, the seeded scaffolds were gently placed into a well of a 96-well plate containing 250µl of medium for the remainder of the experiment.

Seeded scaffolds were also placed in Matrigel, to further promote growth and differentiation. 5.0×10^4 cells were seeded onto the scaffolds using the

dynamic seeding method and then mixed with 300μl of Matrigel before being solidified in wells of a 48-well plate. Liquid overlays were made as described in Chapter 2.6.2. Medium was changed every other day. The cells seeded in the scaffolds are shown in Table 5.2. At fixed time points (1 and 3 weeks) scaffolds were removed and fixed in 4% (w/v) formaldehyde for sectioning and histochemistry (Figure 5.5).

Combination of cells	**In growth medium**	**In Matrigel**
1	MCF-7	MCF-7
2	MCF-7 + HMF 3wt	MCF-7HMF 3wt
3	MCF-7 + HMME 7wt	MCF-7 HMME 7wt
4	MCF-7 + HMME 7wt + HMF 3wt	MCF-7 + HMME 7wt + HMF 3wt
5	MCF-7 V12	MCF-7 V12
6	MCF-7 V12 +HMF 3wt	MCF-7 V12 +HMF 3wt
7	MCF-7 V12 + HMME 7wt	MCF-7 V12 + HMME 7wt
8	MCF-7 V12 + HMME 7wt + HMF 3wt	MCF-7 V12 + HMME 7wt + HMF 3wt

Table 5.2. Table summarizing the different combinations of cells seeded on the scaffolds. Scaffolds were either cultured in growth medium or in Matrigel for 1 or 3 weeks. The experiment was repeated at least 3 times with similar results.

Results from H&E labelling of formaldehyde fixed paraffin embedded scaffolds showed no significant difference in cell density between the different combinations (Figure 5.5).

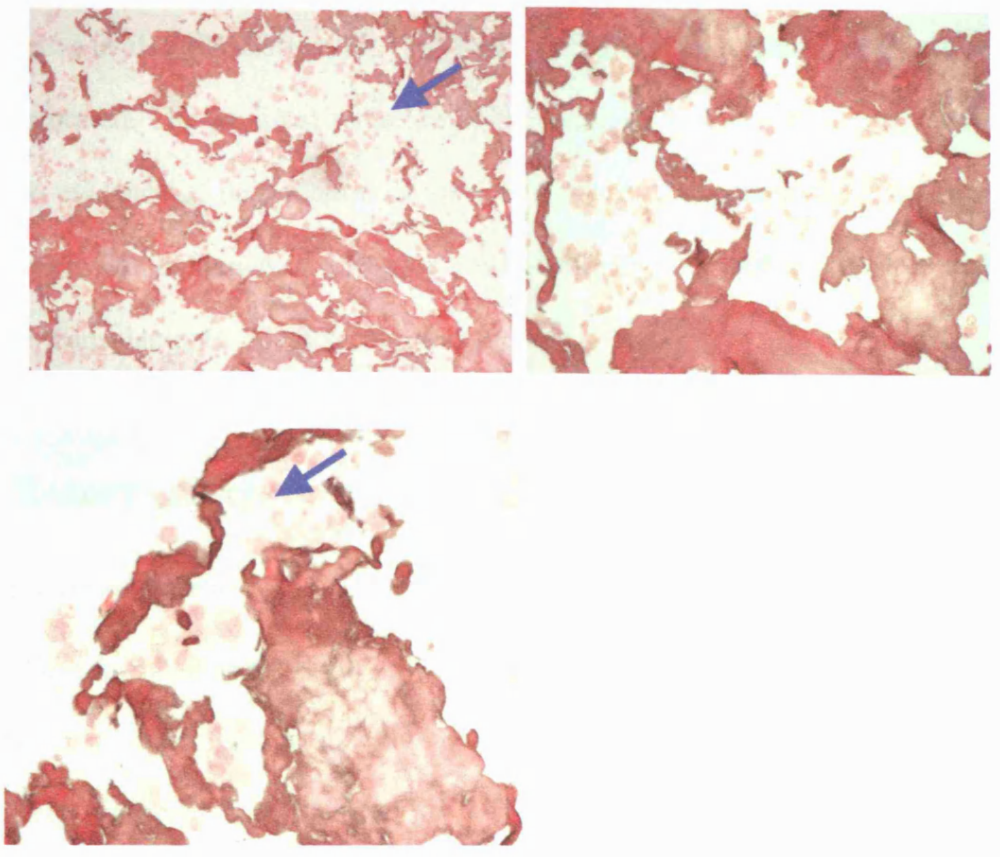


Figure 5.5. H&E sections of 3-D scaffolds, seeded with MCF-7, HMF 3wt and HMME 7wt cell lines, after 3 weeks in culture. H&E staining showed that the cells proliferated in the scaffolds. Cells are clearly visible in the centre of the scaffolds (arrows). Taken at ×80, ×160 and ×320 magnifications respectively.

Furthermore, Matrigel did not provide an observable promotion of cell proliferation. H&E sections showed that both in Matrigel and in liquid medium, cells were located in the centre of the scaffolds. This was evident by the roughly even distribution of cells throughout the scaffold. The densities of cells present in

the 1 week and 3 week harvested scaffolds were very similar indicating that the cells had not significantly proliferated. The density of cells observed in the H&E sections at 3 weeks was not as great as that had been reported in other cell types (Takezawa et al. 2000, Kim et al. 1999) expected although a quantitative test was not performed. The physical structure of the scaffold was still very much intact with no evidence of degradation *in vitro* at 3 weeks. In summary, the scaffolds did not provide evidence of adequate cell proliferation or cell aggregation to be used as transplantable 3-D heterologous models.

5.5 Rotary cell culture system

5.5.1 Introduction

The NASA developed Rotary cell culture system (RCCS) introduces a revolutionary concept in tissue engineering (Figure 5.7). This device provides a relatively quiescent environment for 3-D spheroid cell culture with adequate mixing for mass transport. By simulating microgravity, cells are maintained in a dynamic fluid suspension in liquid media mixed by minimal hydrodynamic forces. A culture flask (vessel) rotates whole on its horizontal axis, providing end over end mixing of the cells. Fluid turbulence and shear forces are minimized by the vessel being completely filled with media and providing aeration through a semi-permeable membrane which eliminates bubbles producing hydrodynamic forces. Therefore this system successfully integrates cellular co-localization, 3-D stromal/epithelial/matrix interactions, and low shear forces.

Figure 5.6. Operation of the RCCS. The cylindrical culture vessel is filled with culture medium, and the cells or tissue particles are added. All air bubbles are removed from the culture vessel. The vessel is attached to the rotator base and rotated about the horizontal axis (power supply not shown). Cell aggregate particles establish a fluid orbit within the culture medium in the rotating vessel. They do not collide with the walls or any other parts of the vessel. As 3-D tissues grow in size, the rotation speed is adjusted to compensate for the increased settling rates of the larger particles. The tissue particles do move enough within the fluid culture medium to exchange nutrients, wastes, and dissolved gases and make contact with other tissue particles. The cells and/or tissue particles join to form larger tissue particles that continue the differentiation process. Oxygen supply and carbon dioxide removal are achieved through a gas-permeable silicone rubber membrane.

Studies have shown that reduced turbulence has profound effects on the physical properties of a culture. The spheroids produced were not only larger in size, but they were significantly more differentiated than those produced in spinner flasks (Goodwin et al 1993, Cherry and Kwon 1990, Croughan and Wang 1991). This is a great advantage as higher fluid turbulence in the spinner flask has been shown to damage fragile animal cells affecting membrane integrity and metabolism (Goodwin et al. 1993). Another advantage of the rotary cell culture system is the ability to co-culture multiple cell types in forming 3-D *in vitro*

structures. To assist in 3-D structuring, inert scaffold material such as microcarrier beads coated in various substrates can be used (Meaney et al. 1998). By co-localizing cells in 3-D for an extended period of time, there is an opportunity to build tissue with characteristics and differentiation phenotypes that are very much different to that grown in 2-D.

5.5.2 Results

Once again, human mammary tumour (MCF-7, T47D, MCF-7 V12) and stromal cell lines (HMME 7wt, HMF 3wt HMF 3wt *ras*) were either pre-seeded on microcarrier beads or mixed as cell suspensions, before being placed in the RCCS.

To culture the three different cell types as a cell suspension, 5×10^6 cells of each cell type were inserted into either a 50ml or 10ml cylindrical culture vessel. The combinations used are summarized in Table 5.3. This was then filled completely with growth medium (EGM-2 bullet kit) and then rotated about the horizontal axis. Cells were cultured for fixed time points (7 or 14 days) or alternatively until the desirable spheroid sizes were manufactured. Spheroids meeting the size criteria (1-4mm) were carefully removed from the culture vessel through access ports using a syringe. Spheroids were either fixed in 4% (w/v) formaldehyde or snap frozen for histology. They were also used for further cultures in liquid overlay systems and *in vivo* xenograft studies. The cryopreserved and formaldehyde fixed spheroids were processed for immunohistochemistry as described in Chapter 2.7.

	HMF 3wt	HMF 3wt *ras*	HMME 7 wt	HMF 3wt + HMME 7 wt	HMF 3wt *ras* + HMME 7 wt
MCF-7	+	+	+	+	+
MCF-7 V12	+	+	+	+	+
T47D	+	+	+	+	+

Table 5.3. Table summarizing the different combinations of cells added to the RCCS. Cells were added as mixed free cell suspensions.

To pre-seed the microcarrier beads, 5×10^6 cells of each cell type were attached to microcarrier beads. The order of cell type seeding was varied to determine the most efficient method of manufacturing the heterologous spheroids. As the endothelial cell lines were the most sensitive cells of the three they were seeded on the microcarrier beads first so that they could establish before the more robust fibroblast and tumour cell lines were added. Alternatively the endothelial cells were seeded last after the other cell types so that they would benefit from the growth factor and matrix provided by the other cells and also because they would be closest to the nutrients in the matrigel and medium. All the combinations tested are shown in Table 5.4. These cells were cultured as above and harvested at 7 and 14 days. The position of the individual cell types within the spheroids were identified using cell specific antibody labelling. As described in Chapter 3, a pan-cytokeratin antibody was used to label the tumour cells, an anti-vimentin antibody for fibroblasts and both anti-CD31/CD34 antibodies to label the endothelial cells.

Combinations	1st	2nd	3rd
1	HMME 7 wt	HMF 3wt	MCF-7
2	HMME 7 wt	HMF 3wt *ras*	MCF-7
3	HMME 7 wt	HMF 3wt	MCF-7 V12
4	HMME 7 wt	HMF 3wt *ras*	MCF-7 V12
5	HMME 7 wt	HMF 3wt	T47D
6	HMME 7 wt	HMF 3wt *ras*	T47D
7	MCF-7	HMF 3wt	HMME 7 wt
8	MCF-7	HMF 3wt *ras*	HMME 7 wt
9	MCF-7 V12	HMF 3wt	HMME 7 wt
10	MCF-7 V12	HMF 3wt *ras*	HMME 7 wt
11	T47D	HMF 3wt	HMME 7 wt
12	T47D	HMF 3wt *ras*	HMME 7 wt

Table 5.4. Table summarizing the different order and combinations of cells seeded on the microcarrier beads prior to culture in the RCCS. Equal numbers of cells were seeded on microcarrier beads in the order described above.

When the combination of cells, as simple cell suspensions, outlined in Table 5.3 were inserted into the RCCS, spheroid formation was visible macroscopically after 48 hours. All of the combinations tested were roughly equal in the rate and size of spheroid produced. The maximum size of spheroids harvested at 7 days was ~1-1.5mm in diameter. At 14 days the largest spheroids were ~ 2-2.5mm in diameter. It is important to note that in this case the spheroids produced were true spheroids consisting of cells only.

When cells pre-seeded on microcarrier beads were inserted into the RCCS heterologous spheroids commenced formation within 18-24 hours, with small spheroids being visible macroscopically. Two different types of spheroids were produced; true spheroids consisting of cells only and mini-spheroids consisting of cells and microcarrier beads (Figure 5.7). The true spheroids formed after separating from larger mini-spheroids. Similar to the liquid overlay

cultures the latter group were significantly larger in size. These mini-spheroids increased progressively in numbers and size reaching sizes in excess of 5mm in diameter over a period of 7 days (Figure 5.7).

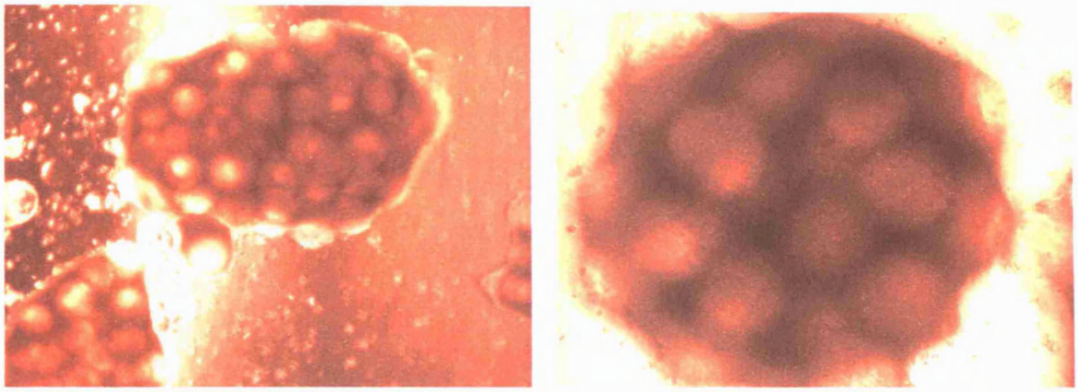


Figure 5.7. Brightfield images of heterologous tumour spheroids (containing MCF-7, HMF 3wt and HMME 7wt cell lines) manufactured in the RCCS. Microcarrier beads are clearly visible embedded in a cellular matrix. Magnification at x160 and x320.

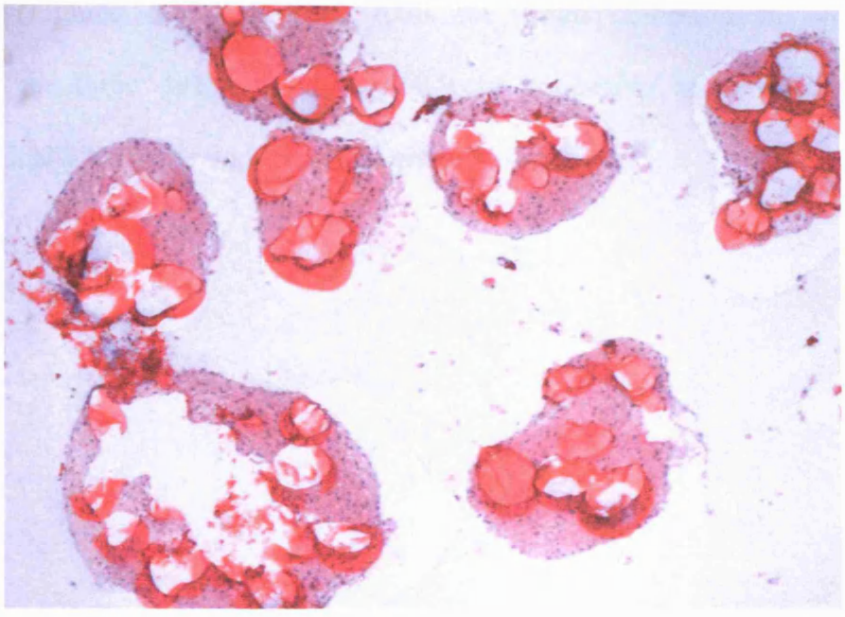
Microcarrier beads were clearly visible in the tumour spheroids with cells proliferating and dividing as indicated by the increasing size of the spheroids. Spheroids were harvested either for further culture in matrigel or for processing for Immunohistochemical analysis.

Heterologous spheroids of various sizes were harvested after 7 and 14 days of culture in the RCCS, fixed in formaldehyde and processed for sectioning and immunohistochemistry.

Hematoxylin and eosin (H&E) staining

Paraffin embedded sections of heterologous spheroids (all combinations) were harvested at 7 and 14 days and stained with H&E (Figures 5.8 & 5.9).

A)



B)

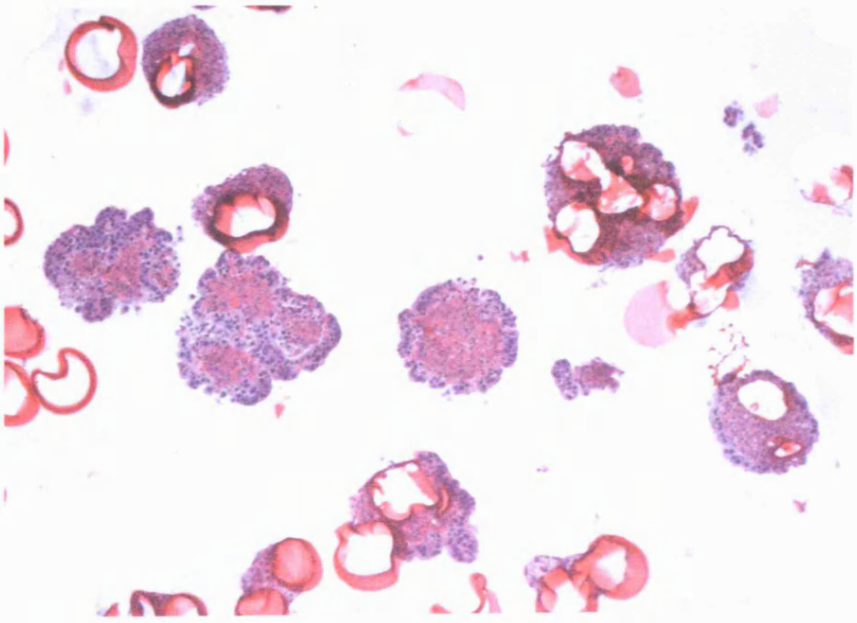


Figure 5.8. 3-compartment spheroids harvested at (a) 7 days and (b) 14 days and stained with H&E. Note the difference in morphology between spheroids harvested at 7 and 14 days; spheroids at 14 days have a well organised structure. Taken at x160(a) and x80(b) magnification.

The morphology of the heterologous spheroids, consisting of MCF-7, HMF 3wt and HMME 7wt cell lines, varied considerably at 7 and 14 days. Spheroids at 14 days contained more differentiated cells and were more morphologically organized (Figures 5.8 and 5.9). Also the three compartment spheroids containing the three different cell types were noticeably more differentiated morphologically than one and two compartment spheroids.

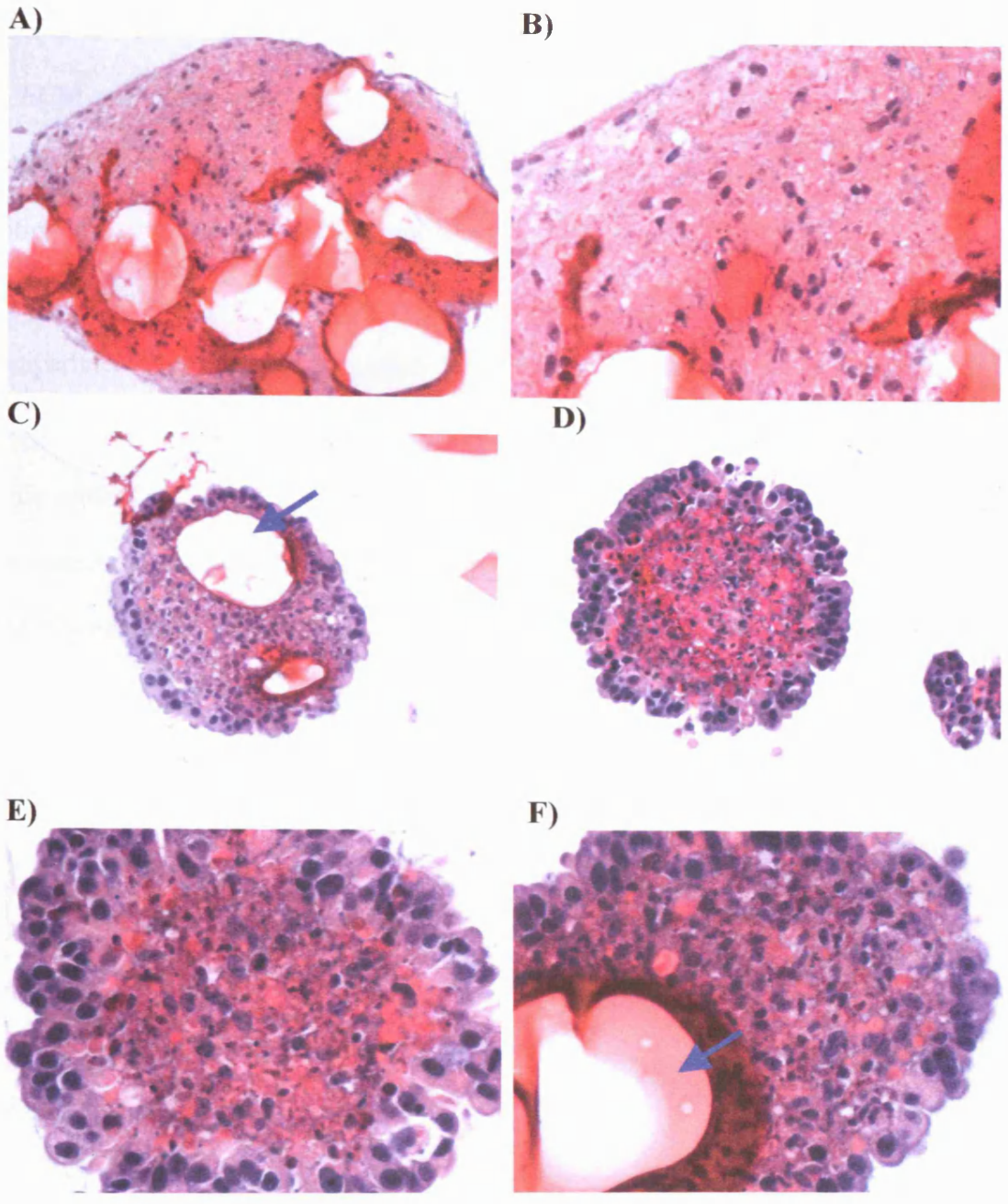


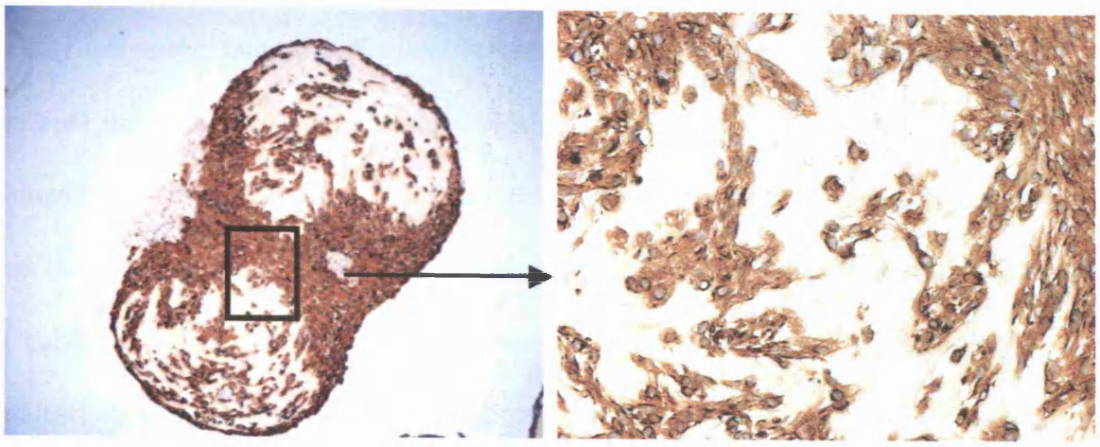
Figure 5.9. H&E sections of heterologous tumour spheroids (containing MCF-7, HMF 3wt and HMME 7wt cell lines) manufactured in the RCCS and harvested at 7 and 14 days. Note the lack of architecture in spheroids harvested at 7 days (a & b) compared to those harvested at 14 days (c-f). Spheroids formed on their own and on microcarrier beads - arrow points to microcarrier beads .Note the difference in architecture at 7 and 14 days. Figures a-d taken at x160, e-f taken at x320 magnification.

Epithelial like cells were visibly organized around the periphery of the tumours with ECM and other cell types located in the centre. At 7 days there was no evidence of structural organization with cells arranged in a random fashion resulting in an unorganised mass (Figure 5.9a & b).

Immunohistochemistry

3 compartment heterologous spheroids harvested at 7 and 14 days, containing MCF-7, HMF 3wt and HMME 7wt cell lines, were now labelled using cell specific antibodies. These were anti-pan-cytokeratin antibody for MCF-7 cells, anti-vimentin for fibroblast line HMF 3wt and an anti-CD31 antibody for the HMME 7wt endothelial line (Figures 5.10 & 5.11).

A)



B)

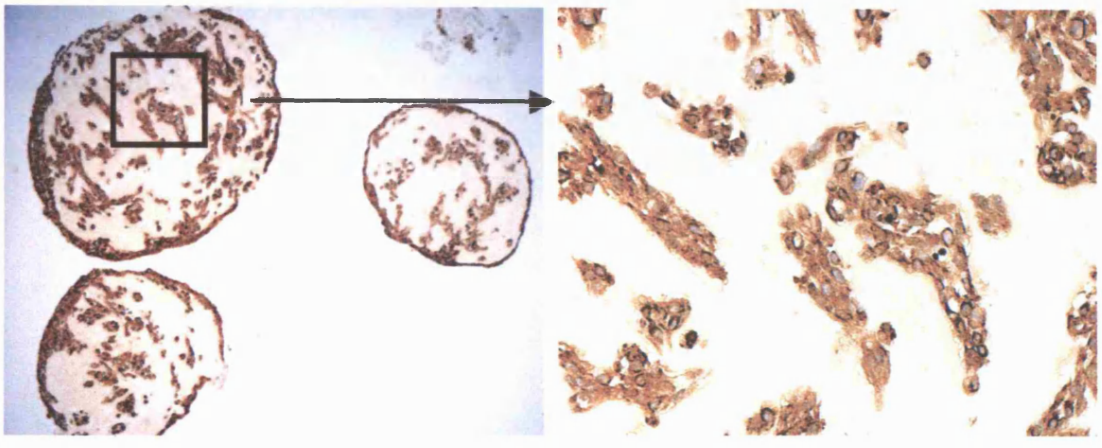


Figure 5.10. Immunohistochemical staining of RCCS manufactured spheroids (containing MCF-7, HMF 3wt, HMME 7wt cell lines) for (a) human mammary fibroblasts (HMF 3wt) using an antibody against vimentin and (b) for human endothelial cells (HMME 7wt) using an antibody against CD31 after 7 days of culture. The stromal cells are clearly seen to be located centrally as well as peripherally suggesting movement and subsequent proliferation of stromal cells in the central core of the tumour spheroids. Taken at x80 (left) and x320 (right) magnifications.

Staining of the heterologous spheroids containing MCF-7, HMF 3wt and HMME 7wt cell lines using human cell specific antibodies (see Chapter 6.2 for list of antibodies) demonstrated that the fibroblasts and tumour cells survived in all combinations. However, the endothelial cells only survived when they were seeded last after the tumour and fibroblasts had established (combinations 7-12 from Table 5.3) (see Figure 5.11). There was no evidence of survival when they were attached to the microcarrier beads first prior to the other cells being added (combinations 1-6 from Table 5.3). Cross-sectional staining of the 3-compartment spheroids showed that both the endothelial cells and fibroblasts migrated within the spheroids and so were located in central and peripheral areas.

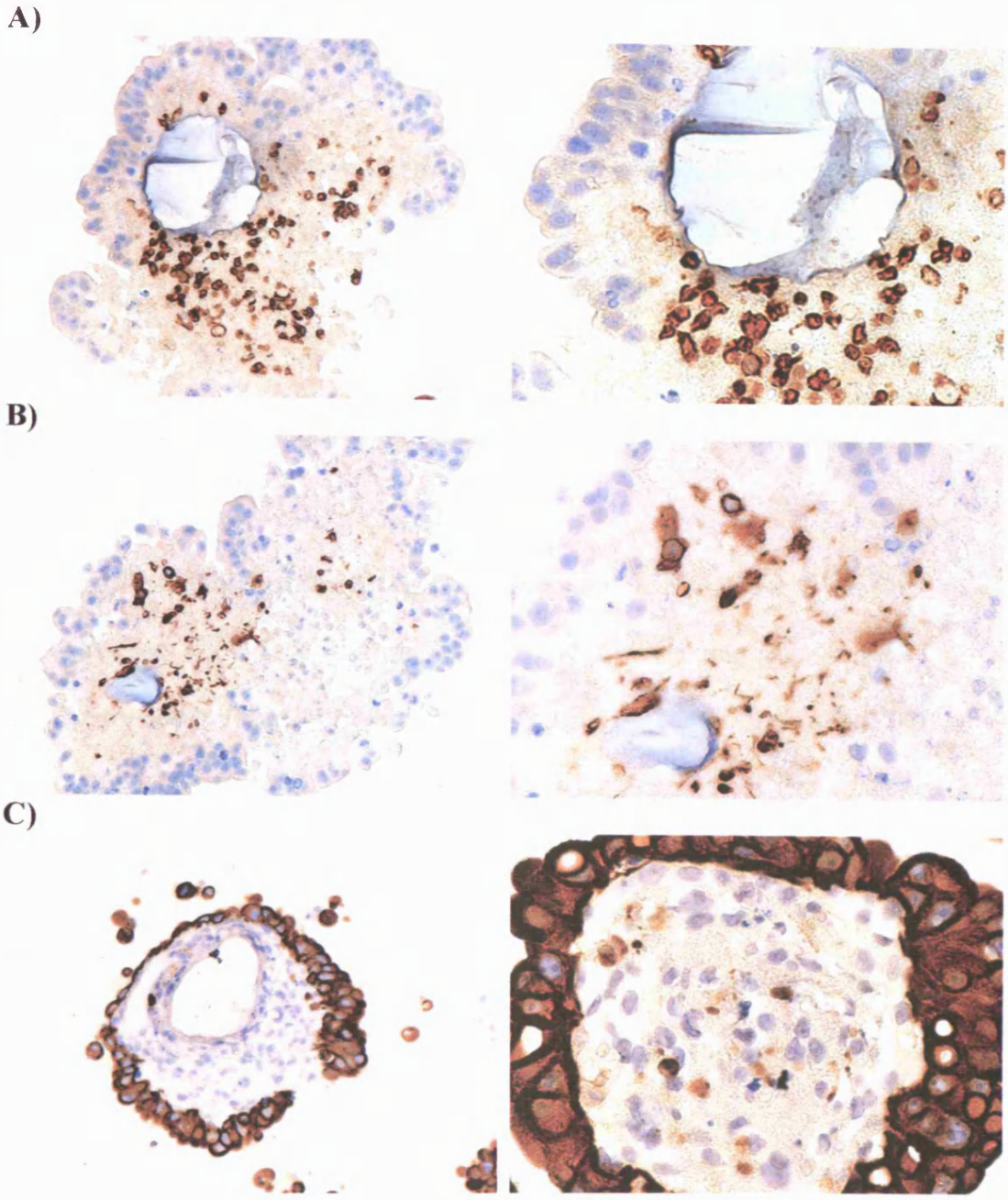


Figure 5.11. Staining for individual cell types in the 3-compartment heterologous tumour spheroids after 14 days of culture. Images demonstrate immunoperoxidase staining of the three-compartment heterologous spheroids using cell specific markers. Figures (a), (b) and (c) represents staining of HMF 3wt, HMME 7wt and MCF-7 cells within the heterologous tumour spheroids with antibodies against vimentin, CD31 and a pan-cytokeratin respectively. Taken at x160 (left) and x320 (right) magnifications.

After 14 days of culture the 3 compartment spheroids had developed a well organized morphology. MCF-7 cells stained positive only peripherally (Figure 5.11c). The centre of the spheroids contained the stromal cells shown by positive stained HMF 3wt and HMME 7wt stromal cell lines (Figure 5.11c & d). The remainder of the spheroid consisted of a non cellular ECM.

Growth of RCCS manufactured heterologous spheroids in Matrigel

To promote differentiation further, heterologous spheroids of different sizes (0.5-3.0mm) harvested after 7 of culture, in the RCCS, were mixed with liquid Matrigel before being plated in microwell growth plates. The hope was that 3-D Matrigel cultures could induce morphological differentiation of breast cancer cells in the spheroids. Once the Matrigel had gelled appropriate growth medium was added and the plates were incubated in the appropriate condition.

Heterologous spheroids that had been in culture for 7 days in RCCS with the aid of microcarrier beads were harvested and cultured further in Matrigel. The spheroids commenced sprouting of capillary networks 24 hours after seeding in Matrigel (Figure 5.12).

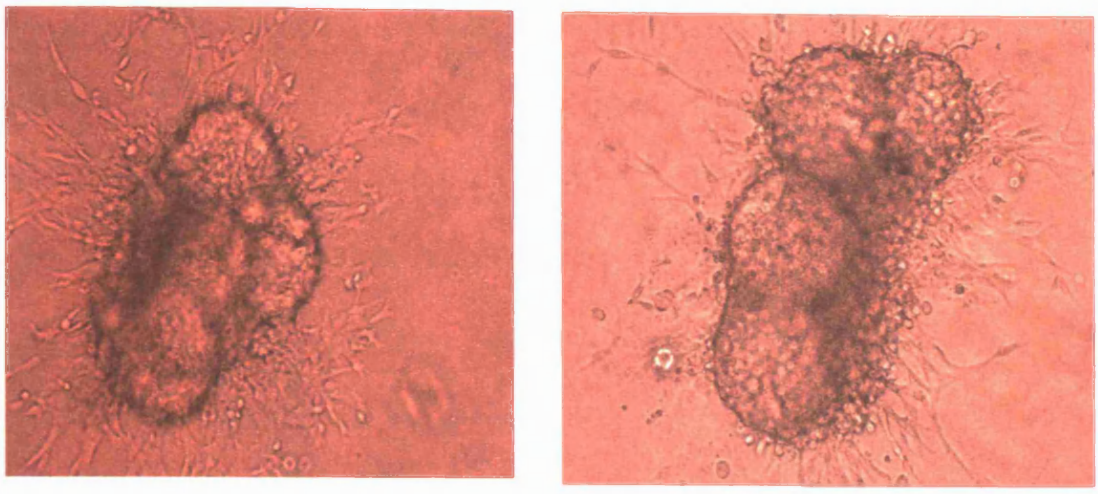


Figure 5.12. Growth of heterologous tumour spheroids (containing MCF-7, HMF 3wt and HMME 7wt cell lines), previously manufactured in the RCCS, in Matrigel. Growth of cells and capillary like vessels are visible extending from the spheroids. Magnification x320.

Lots of capillary like structures as well as cells were visible growing out away from the spheroids. Brightfield images were taken after 5 days in culture (Figure 5.8). NB: Sometimes tumour cells are known to produce hyphae like tubules. However, spheroids formed from tumour cells only (MCF-7) did not form capillary like structures in matrigel. As the concentration of growth factors and nutrients in the matrigel and or growth medium is considerably higher than in the spheroids it is thought that the capillary like networks grew outwards into the matrigel rather than inwards to the spheroids.

To summarize, 3-D heterologous spheroids manufactured in the RCCS with the aid of microcarrier beads were well differentiated, structurally robust and in sufficient supply to be used in future xenograft studies (see Chapter 6 for xenograft studies of these spheroids). Moreover, when cultured in 3-D ECM

conditions they demonstrated good viability with the ability to differentiate and form capillary like networks.

5.6 Discussion and conclusion

In recent years breast cancer models, based on 3-D cell culture methods. have greatly increased in number due to new culturing techniques emerging from the field of tissue engineering, which has lead to improved models. Previously spontaneous aggregation, liquid overlay cultures and spinner flasks were the most popular methods used to produce 3-D *in vitro* breast cancer models. More recently prefabricated scaffold based cultures, the NASA developed Rotary Cell Culture System (RCCS) and pre-engineered collagen scaffold have been developed. The design of the RCCS is a vast improvement on the spinner flask in that larger more morphologically and functionally differentiated breast cancer spheroids can be produced (Goodwin et al. 1993, Cherry and Kwon 1990, Croughan and Wang 1991). The scaffold based culture system is gaining in popularity as the 3-D matrix is used to promote multilayer growth of cells derived from epithelial cancers.

With the many advances in the methods of tissue culturing and engineering the purpose of this chapter was to utilize this technology and develop a 3-D heterologous organotypic tumour spheroid model *in vitro* composed of human breast cancer and stromal cells. Once a suitable and viable *in vitro* model had been established the aim was to xenograft these heterologous spheroid models into immunodeficient mice to produce a self contained human breast tumour model.

A variety of 3-D cell culture techniques were tested to determine their suitability to manufacture 3-D heterologous co-culture models. Microcarrier beads were useful in that they not only allowed the culture of anchorage dependent cells in suspension but they supported the growth of more sensitive 'harder to grow' cells such as endothelial cells. However microcarrier beads in suspension culture did meet the necessary requirements as the number of spheroids produced was insufficient with those produced being too small to contain adequate number of cells. Liquid overlay cultures were primarily employed to construct a small number of carefully constructed heterologous spheroids so that a single spheroid could be transplanted into one *in vivo* site (e.g. mammary fat pad). However, the spheroids produced did not support the density of cells required in addition to being too fragile for *in vivo* transplantation. Finally the 3-D scaffolds represented a potentially good method for tissue construction however initial studies did not provide evidence that the human tumour or stromal cell lines proliferated in them. Therefore these methods did not meet the criteria required.

The Rotary Cell Culture System was chosen as the best method for manufacturing heterologous tumour spheroids because it's many advantages. The primary advantage of the RCCS had over either dynamic or static tissue culture systems is that its low shear environment allows cells to aggregate, grow three-dimensionally, and differentiate. This advantage results in cells or tissues that very closely resemble the *in vivo* tissue equivalent (Hammond and Hammond 2001, Unsworth and Lelkes 1998). The vessel also supports co-culture efficiently by bringing different cell types of different size and density together simply and efficiently. It is very simple to expose the suspension cultures to reagents in the vessel. Finally, it was simple to use resulting in high productivity of spheroids

when used with microcarrier beads (Meaney et al. 1998). The rotating-wall vessel has been reported to be successful in engineering prostate organoids (O'Connor 1999, Zhau et al. 1997), colon carcinoma (Jessup et al. 1997), and cartilage (Freed et al. 1997, Vunjak-Novakovic et al. 1999), among other tissues (Unsworth and Lelkes 1998).

In the heterologous spheroids manufactured in the RCCS, the three cell types grew, multiplied, migrated into clusters, and produced an intracellular matrix via the functional interrelationship of cell-to-cell contact. The cells differentiated and grew along boundaries characteristic of normal functional tissue.

In the present study, we have developed a novel 3-D multi-compartment heterologous co-culture system in which human mammary epithelial cells, mammary fibroblasts and endothelial cells, seeded as a mixed single cell-suspension with the aid of reconstituted basement membrane matrix (Matrigel), retain their inherent ability to segregate (or compartmentalize) and organize in a 3-D manner with formation of a central stromal core composed of fibroblasts and functional endothelial cells from and around which epithelial buds emerge. This situation bears resemblance to the *in vivo* situation because, in the mammary gland, terminal ductules or acini are set within a rich and specialized stroma that defines the lobular unit. This lobular connective tissue is usually loose, possesses many capillaries, and is sharply demarcated from the surrounding fat and denser fibrous tissue of the structural rather than the functional portion of the breast (Page and Anderson 1987). It is interesting to note that the epithelial cells in this model demonstrate an example of early morphogenic movement referred to as epiboly; which is the process whereby epithelial cells flatten perpendicular to their apico-basal axes, accompanied by the lateral expansion of the sheet

spreading as a unit over deeper layers. The term epiboly is generally used to describe the movement of epithelial sheets (usually ectoderm cells) which spread as a unit (rather than individually) to enclose the deeper layers of the embryos (endoderm) (cells move over the surface toward the region of invagination or involution) seen in ectoderm formation of amphibians (Keller 1980), sea urchins (Trinkaus 1988) and tunicates.

The co-culture system, in the present study, bears physiological relevance because not only does it permit the mammary epithelial cells to exhibit a homotypic affinity for itself and form an interface with adjacent stromal core or compartment as observed *in vivo* but it also facilitates an arrangement, observed *in vivo,* that is most suitable for functional paracrine interactions between epithelial-fibroblast and epithelial-endothelial cells. It must be noted that, although the structural and functional organization observed in this model system mimics several characteristics of the mammary gland, it is representative not of normal mammary gland morphology but rather of alterations occurring during early breast cancer. It is important to note that organ specific stromal cells produced this effect as a similar study by Shekhar et al. (2001) demonstrated a requirement for organ-specific fibroblasts in the induction of epithelial morphogenesis.

The tumour and stromal cell lines present in the 3-compartment heterologous tumour spheroids interacted with each other to provide *in vitro* tumours that not only survived but proliferated and differentiated. Furthermore these spheroids were viable when re-cultured in Matrigel demonstrating the production of microcapillary like networks. The latter result is very promising as the ability to form differentiated capillary like structures *in vitro* may suggest that these structures could anastomose with host vasculature when xenografted *in*

vivo. Having established these viable heterologous tumour spheroids, *in vivo* experiments were set up to xenograft these self contained *in vitro* tumour units into immunodeficient mice (for results see Chapter 6).

Chapter 6

Xenotransplantation

6.1 Introduction

The concept of the human breast cancer xenograft model is simple; all that is involved is growth of human breast cancer cells and cell lines heterotopically (subcutaneously) or orthotopically (mammary fat pad) in immunodeficient mice. The main advantage xenograft models confer over other animal models is that it represents a model system of human origin. As with any model the similarity with the original is of paramount importance. However xenograft systems differ markedly from human breast cancer in several ways. They contain fewer stromal cells and the stroma that does exist is murine in origin, resulting in a chimeric tumour. The biology of chimeric rodent/human tumours can differ significantly from that of humans resulting in unpredictable growth, differentiation and metastatic properties (Hahn and Weinberg 2002, Balmain and Harris 2000). This raises issues of which of the two species will be dominant in contributing its characteristics to the chimeric tumours. Maybe tumours with a combination of the two characteristics will be formed. For example, in contrast to the invasive growth pattern of human cancer, mouse mammary tumours tend to be more expansile with pushing margins. Many mammary tumours that are known to metastasize in mice are regarded as histologically benign by most histopathologists (Cardiff 2001).

Unfortunately human breast cancer is one of the most difficult tumours to transplant into experimental animals. The reported success rate for human breast cancer xenografting is 7% - 20% (Mehta et al. 1993). In comparison the success

rates of pancreatic/colonic, renal cell, lung and urological carcinomas have been reported as 40-60%, 40%, 38-51% and 56% respectively (Cui et al. 2001, Angevin et al. 1999, Mourad and Vallieres 1995, Mattern et al. 1985, Okada and Yoshida 1984). Not until recent years (Hurst et al. in 1993) were mammary tumour xenograft lines which metastasized developed. Recently it has been reported that histomorphologically intact primary human breast lesions and cancers have been grown in athymic mice (Yang et al. 2000). Furthermore an experimental model system has been developed in which dissociated cells from surgical breast cancer specimens, after mixing with extracellular matrices, have been transplanted into nude mice (Yang et al. 2000). These transplanted cells undergo morphogenesis to reflect their original phenotype. This model presents a potential *in vivo* model for analysing specimens of primary human breast lesions and cancers. Traditionally established human tumour cell lines, when transplanted into immunodeficient mice, have proved to be relatively poor predictors of tumour response in humans. These xenograft tumours do not evolve *in situ* and lack the appropriate cellular interactions with the host microenvironment.

Until now there has been a distinct lack of a truly representative breast cancer model. The recent ability to immortalize fresh primary human mammary endothelial cells and fibroblasts by the insertion of the catalytic subunit of human telomerase (hTERT) and a mutant (U19A58) variant of simian virus 40 large T antigen has resulted in a readily available source of human mammary stromal cells. Therefore with the availability of appropriate stromal cells it has now become possible to improve xenograft models by incorporating more human components. The development of a complex 3 compartment heterologous xenograft model incorporating the relevant stromal elements would provide a

better alternative to currently available chimeric xenograft models. By xenografting human breast tumours in combination with human mammary fibroblasts and endothelial cells it is envisaged that the immortalized mammary stromal cells will provide a viable and much needed tumour microenvironment of human origin for tumour proliferation.

Models based on athymic nude mice have been playing an important role in evaluating many anti-cancer drugs. However, human tumours were transplanted subcutaneously and not at an orthotopically relevant organ site. The major problem of this model is that the transplanted tumours are located in an environment quite different from where most human tumours locate. Most subcutaneously transplanted tumours are surrounded by a pseudocapsule, and have little chance to invade and disseminate to the surrounding tissues and rarely metastasize, even when highly aggressive tumours have served as the source of the xenograft (Cui et al. 2001). However, human tumour cells implanted orthotopically in the corresponding organs of nude mice can increase the metastatic capability of human tumour cells in nude mice (Cui et al. 2001, Angevin et al. 1999, Mourad and Vallieres 1995, Mattern et al. 1985, Okada and Yoshida 1984).

The aim of this chapter was two-fold, firstly to investigate whether the immortalized stromal cells were tumourigenic in immunodeficient mice which would indicate that they have been transformed into a tumourigenic phenotype. Secondly, to determine the parameters of their survival and proliferation *in vivo* and also their ability to support and function as stroma to the co-engrafted human tumour cells when xenografted orthotopically. Success would allow us to develop a more representative model system incorporating more human

components for vital pre-clinical studies. In order to identify human cells in xenografts, cell type specific markers were selected.

6.2 Identification of human mammary epithelial and stromal cell markers

In order to identify the three different human cell types: breast epithelial cells, endothelial cells and fibroblasts in the context of fixed mixed cell populations both *in vitro* and *in vivo*, human cell specific antibody markers were chosen and staining protocols optimised as in Table 6.1 using both fixed cells *in vitro* (Figure 6.1) and tissue sections (Figure 6.2).

Antigen (specificity)	Antibody	Species	Sub-class	Dilution	Source
Human CD34	Q/BEND10	Mouse	IgG1	1:30 2 min PC	Dako
Human CD31	JC70A	Mouse	IgG1	1:30 2 min PC	Dako
Human Cytokeratin 5,6,8,17,19	MNF116	Mouse	IgG1	1:400 10 min CT	Dako
Human vimentin	V9	Mouse	IgG1	1:800 18 min MW	Santa Cruz
Mouse CD31	PECAM-1	Rat	IgG2a	1:20	Serotec
Vimentin *	RV202	Mouse	IgG1	1:400	BD Biosciences
Ki-67	MIB-1	Mouse	IgM	1:300 2 min PC	Dako
SV40 large T antigen	PAb 423	Mouse	IgG1	1:5	Dako

Table 6.1. Table showing all the antibodies used. Antigen retrieval methods were used for formaldehyde fixed paraffin embedded sections only; **2 min PC**- 2 minutes pressure cooking. **18 min MW** - 18 minutes microwaving, **10 min CT** - 10 minutes chymotrypsin enzyme digestion. Species specificity was confirmed by testing on either human or mouse tissues. (*) reacts with both human and mouse vimentin.

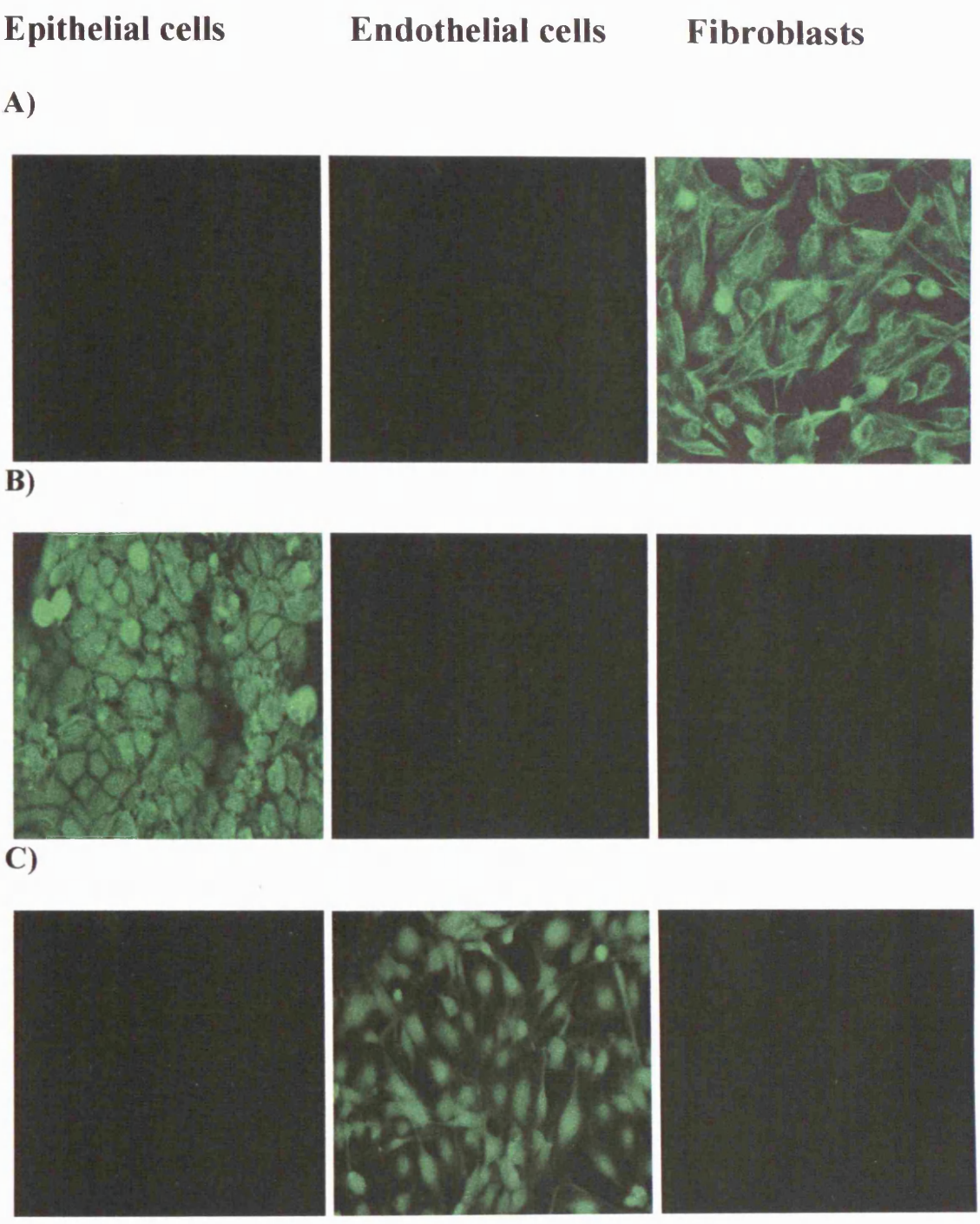


Figure 6.1. Cells grown on coverslips stained with (a) V9 anti-vimentin, (b) MNF116 anti-pan-cytokeratin antibody and (c) JC70A anti-CD31 antibody. The antibody against CD34 (QBEND/10) showed a similar staining pattern as (c). All primary antibodies were visualized using an anti-mouse FITC secondary antibody. Epithelial cells were MCF-7, endothelial cells HMME 7wt and fibroblasts HMF 3wt. Magnification at ×160.

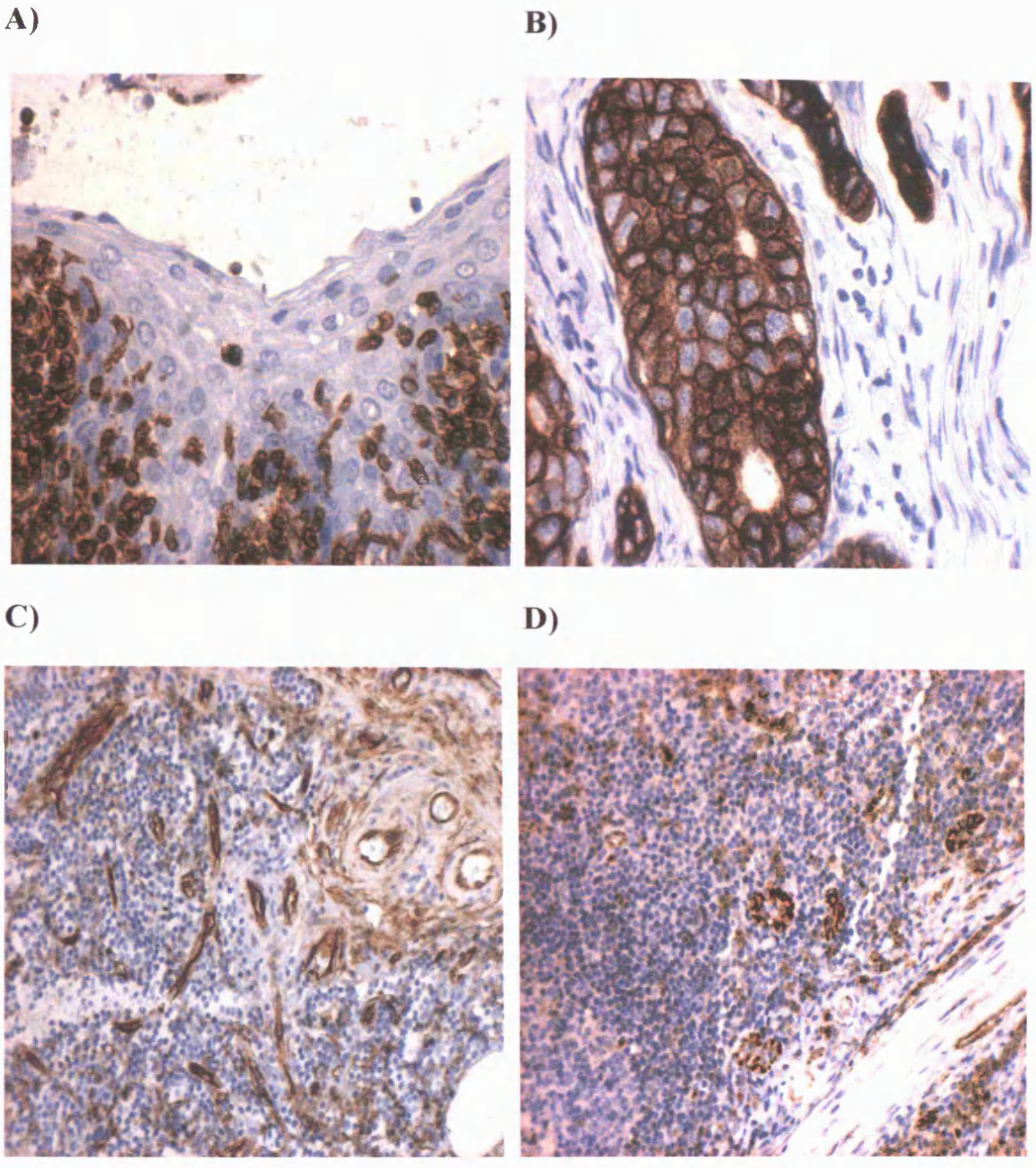


Figure 6.2. Staining of human tonsillar tissue as positive controls. Antibodies against (a) human vimentin, (b) human pan-cytokeratin (5,6,8,17,19), (c) human specific CD31 and (d) human specific CD34. Magnification a) & b) ×160, c) & d) ×80.

In addition, to identify host (murine) endothelial cells and blood vessels a mouse specific CD31 antibody was used. Murine fibroblasts were identified using non-species specific vimentin antibody that recognises the mouse antigen. The human

mammary stromal cell lines were further distinguished by SV40 Large T staining (Figure 6.3), and stains for the proliferation marker Ki67 using the antibody MIB-1 was also optimised for xenograft studies. As an additional means of differentiating mouse and human cells the nuclear stain Hoechst 33258 was used. This stains mouse nuclei with a characteristic punctuate pattern whereas human nuclei are stained homogenously (Figure 6.4).

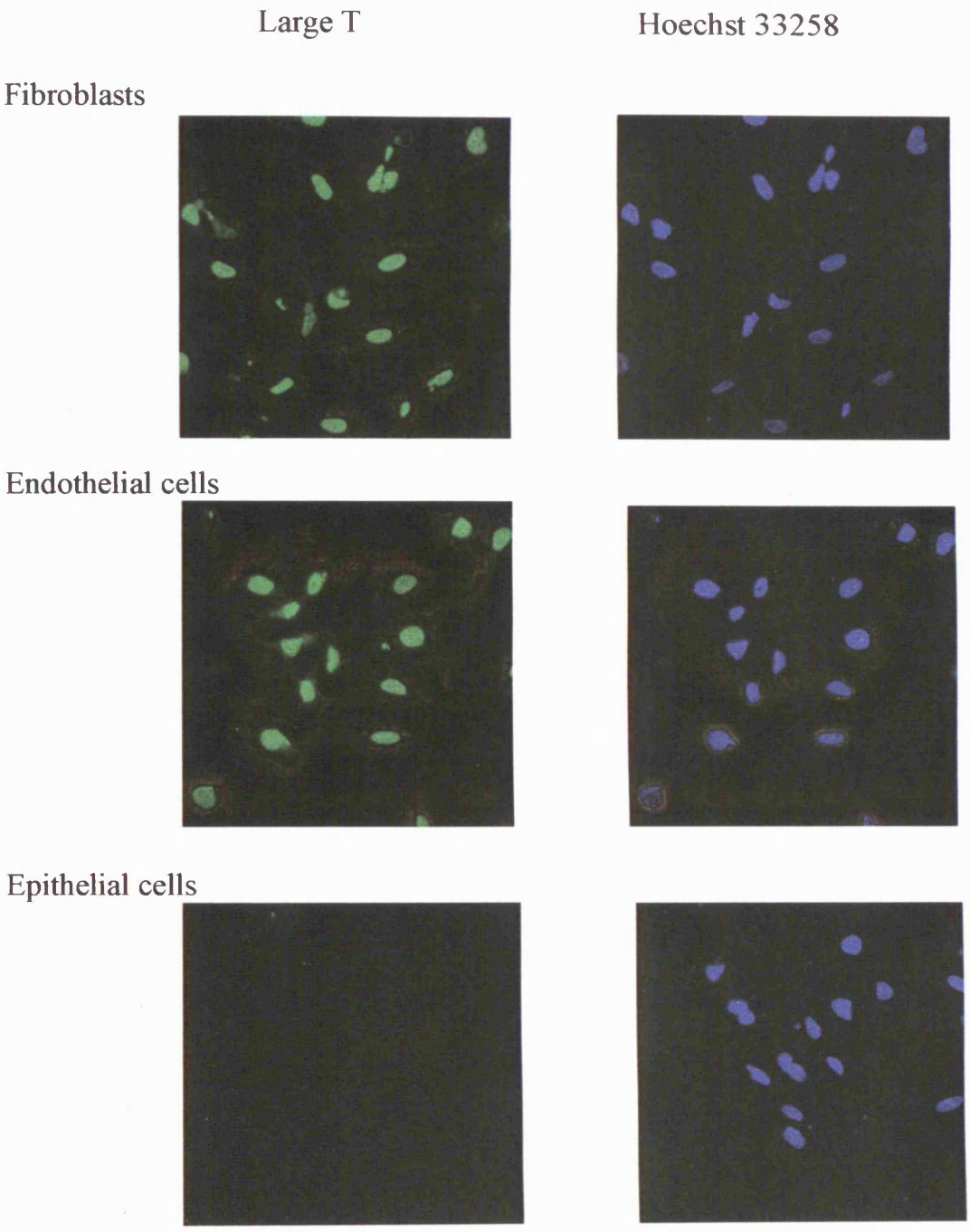


Figure 6.3. Human mammary fibroblasts (HMF 3wt), endothelial cells (HMME 7wt) and epithelial cells (MCF-7) cells stained with an anti-large T antibody and also with the nuclear stain Hoechst 33258. All primary antibodies were visualized using an anti-mouse FITC secondary antibody. Magnification ×160.

A) B)

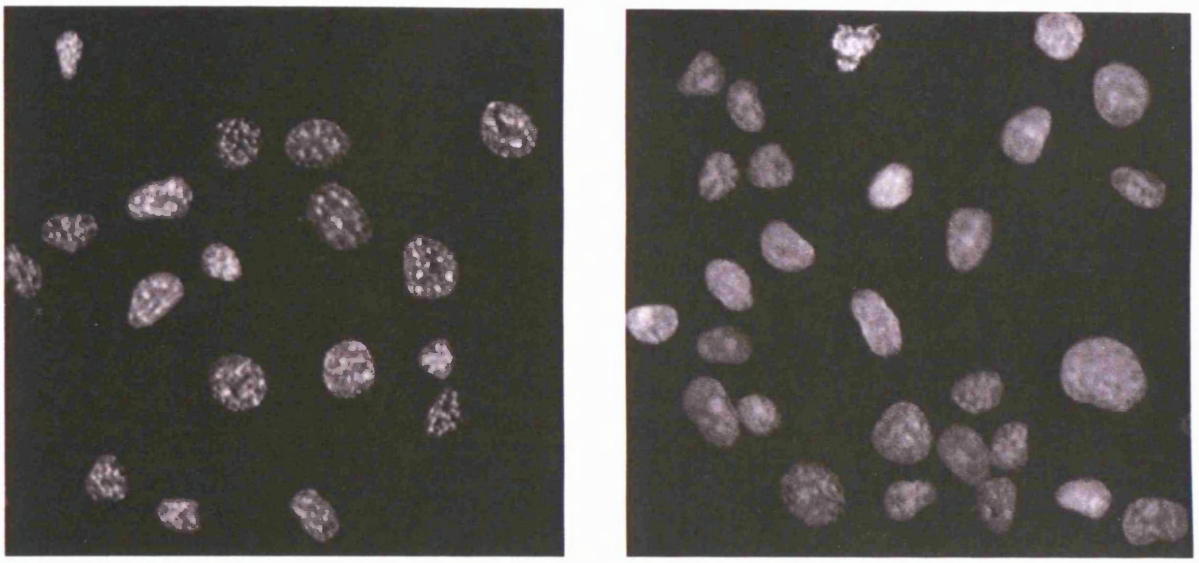


Figure 6.4. Hoechst 33258 staining of (a) mouse and (b) human endothelial cells. Note that mouse nuclei are stained with a punctuate pattern in contrast to a more homogenous pattern for human cells. Magnification ×320.

Results showed that the three human cell specific antibodies identified (see Table 6.1) were able to clearly distinguish between the three human cell types in fixed cells *in vitro* (Figure 6.1) and in *vivo* (Figure 6.2). In addition they were also confirmed as having human species specificity. The LT expressing immortalized stromal cell lines were also identified by a specific marker against LT (Figure 6.3). Human cells could also be differentiated from murine cells by the staining pattern produced by the nuclear stain Hoechst 33258 (Figure 6.4). Antibodies specific for mouse fibroblasts and endothelial cells were also identified and tested producing reliable reproducible results. Thus, a panel of reagents for confirming cell identity and origin in the context of xenografts was established.

6.3 Xenografts of immortalized human mammary stromal cells

All of the immortalized human mammary stromal cell lines including the temperature sensitive and wild LT type variants were xenografted, into 6 week old NCR-*nu* mice primed with slow release oestrogen pellets, to investigate whether any of them would give rise to tumours and whether surviving cells if any, could be detected immunohistochemically. The stromal cells (5×10^6) were xenografted either heterotopically (subcutaneously, SC) or orthotopically into the mammary fat pad (MFP). In addition, the cells were xenografted in either standard serum free media (SFM) or in the presence of a basement membrane preparation matrigel to potentially enhance take rates. In later experiments the immortalized fibroblasts and endothelial cell lines were combined before inoculation, including combinations of wild type LT variant cell lines (non temperature sensitive) with temperature sensitive lines. Xenograft harvests were carried out at 6 and 12 week time points post inoculation.

6.3.1 Xenografts of immortalized human mammary fibroblasts

All of the temperature sensitive and wild type fibroblast cell lines were individually xenografted into nude mice. The wild type LT variant of the human mammary fibroblast transfected subsequently with the mutant human activated *ras* oncogene Val^{12} (HMF 3wt *ras*) was also inoculated either subcutaneously or into the mammary fat pads of nude mice in Matrigel. The purpose of the HMF 3wt *ras* inoculations was to investigate the tumourigenicity of the immortalized fibroblasts following additional expression of the mutant *ras* oncogene, as it was

associated with many of the *in vitro* criteria of transformation (see Table 3.8). The results for all the fibroblast lines tested are summarized in Table 6.2.

Cell lines	Xenograft Take Rate SFM + SC	Xenograft Take Rate Matrigel + SC	Xenograft Take Rate SFM + MFP	Xenograft Take Rate Matrigel+MFP
HMF 3A	0/6, 0/6	0/6, 0/6	0/6, 0/6	0/6, 0/6
HMF 3B	0/6, 0/6	0/6, 0/6	0/6, 0/6	0/6, 0/6
HMF 3C	0/6, 0/6	0/6, 0/6	0/6, 0/6	0/6, 0/6
HMF 3D	0/6, 0/6	0/6, 0/6	0/6, 0/6	0/6, 0/6
HMF 3wt (wild type)	0/6, 0/6	0/6, 0/6	0/6, 0/6	0/6, 0/6
HMF 3wt *ras*	Not determined	0/8, 0/8	Not determined	0/16, 0/16

Table 6.2. Table summarizing the results of the immortalized fibroblast xenografts. Inoculations were performed in either serum free media (SFM) or extracellular matrix Matrigel, subcutaneously (SC) or into the mammary fat pad (MFP). $5x10^6$ cells of each cell type in 50μl of carrier were inoculated into each site. Xenografts were harvested and examined at 6 and 12 weeks points for which individual results are recorded. 6 sites were inoculated for each time point. The HMF 3wt *ras* line was only inoculated in Matrigel either subcutaneously or into the MFP; for the Matrigel + MFP group, 8 sites were inoculated on two separate occasions.

On gross inspection tumours were not visible in any of the fibroblast xenografts. Also, from the 8 subcutaneous and 16 paired mammary fat pad (8 on two separate occasions) injections of HMF 3wt *ras*, tumours did not develop in any of the mice (Table 6.2). Mammary fat pads and samples of subcutaneous tissues taken from the inoculation sites were harvested and either snap frozen for frozen sections or fixed in formaldehyde for paraffin sections. Immunohistochemical analysis of these sections using both immunoperoxidase and immunofluorescence, with an antibody specific for human vimentin (see Chapter 6.2), showed no evidence of surviving cells in any of the groups. 20 sections/inoculation site were stained. In addition, the sections were stained for the presence of the large T antigen, which is present in the immortalized fibroblasts. Using an anti-large T antibody all the sections tested were negative. Altogether a total of 2.4×10^8 cells were grafted for each different fibroblast cell line, including the HMF 3wt *ras* cell line, without apparent long term survival or tumour formation.

6.3.2 Xenografts of immortalized human mammary endothelial cells

All of the temperature sensitive and wild type endothelial cell lines were also xenografted in the same manner as the fibroblasts. The results are shown in Table 6.3.

Cell lines	Xenograft Take Rate SFM + SC	Xenograft Take Rate Matrigel + SC	Xenograft Take Rate SFM + MFP	Xenograft Take Rate Matrigel + MFP
HMME 2	0/6, 0/6	0/6, 0/6	0/6, 0/6	0/6, 0/6
HMME 7	0/6, 0/6	0/6, 0/6	0/6, 0/6	0/6, 0/6
HMME 7wt (wild type)	0/6, 0/6	0/6, 0/6	0/6, 0/6	0/6, 0/6

Table 6.3. Table summarizing the results of the immortalized endothelial cell xenografts. Inoculations were performed in either serum free media (SFM) or extracellular matrix Matrigel, subcutaneously (SC) or into the mammary fat pad (MFP). $5x10^6$ cells of each cell type in 50μl of carrier were inoculated into each site. Xenografts were harvested and examined at 6 and 12 weeks points for which individual results are recorded. 6 sites were inoculated for each time point.

The immortalized endothelial cell lines (wild type LT and temperature sensitive) did not give rise to tumours in any of the four group categories, after 6 or 12 weeks. As with the fibroblast lines, immunoperoxidase and immunofluoresence staining showed no evidence of long term survival of the grafted cells, when 10 sections/inoculation site were stained with a human specific anti-CD31 and 10 sections/inoculation site with a human specific anti-CD34 antibody. Staining for the large T antigen was also negative in all the sections tested. Again a total of 2.4×10^8 cells were grafted for each endothelial cell line.

6.3.3 Xenografts of combined immortalized human mammary endothelial cells and fibroblasts

In order to potentially improve survival and provide a more stringent test of tumourigenicity, xenograft combinations of endothelial cells and fibroblasts were performed (Table 6.4). All the combinations were inoculated, with the aid of Matrigel, either subcutaneously or into the MFP. 5 x 10^6 cells of each cell type were inoculated/combination.

Cell lines	Xenograft Take Rate Matrigel + SC	Xenograft Take Rate Matrigel + MFP
HMF 3A + HMME 2	0/6, 0/6	0/6, 0/6
HMF 3A + HMME 7	0/6, 0/6	0/6, 0/6
HMF 3B + HMME 2	0/6, 0/6	0/6, 0/6
HMF 3B + HMME 7	0/6, 0/6	0/6, 0/6
HMF 3C + HMME 2	0/6, 0/6	0/6, 0/6
HMF 3C + HMME 7	0/6, 0/6	0/6, 0/6
HMF 3D + HMME 2	0/6, 0/6	0/6, 0/6
HMF 3D + HMME 7	0/6, 0/6	0/6, 0/6
HMME 7wt (wild type) + HMF 3A	0/6, 0/6	0/6, 0/6
HMF 3wt (wild type) + HMME 7	0/6, 0/6	0/6, 0/6
HMME 7wt (wild type) + HMF 3wt (wild type)	0/6, 0/6	0/6, 0/6

Table 6.4. Table summarizing the results of the combined immortalized stromal cell xenografts. Inoculations were performed in Matrigel either subcutaneously (SC) or into the mammary fat pad (MFP). 5x10^6 cells of each cell type in 50µl of carrier (total) were inoculated into each site. Xenografts were harvested and examined at 6 and 12 weeks points for which individual results are recorded. 6 sites were inoculated for each time point.

Once again no tumours were evident on gross inspection at 6 and 12 weeks. The absence of surviving cells was confirmed by immunohistochemical staining using human cell specific antibodies and anti-large T antibodies (20 sections/inoculation site).

In summary, none of the stromal cell lines tested, including the wild type lines which might have been expected to survive better at mouse body temperature (~38°C), formed tumours. In addition, no surviving cells could be detected immunohistochemically. In total, over 5×10^8 cells of each cell line were inoculated, singly or in combination. The inability of the immortalized stromal cells to give rise to tumours when xenografted into mice could be due to many possible reasons. In addition to the temperature sensitive phenotype of some but not all off the lines tested, there are two possible other reasons for the failure to produce tumours. These are a residual innate immune response (e.g. natural killer cell activity) and the fact that the SV40 large T antigen only results in a partial transformation of the stromal cells resulting in a non-tumourigenic phenotype. The other possible reasons for their failure to survive could be due to either dispersion of cells at the graft site or the absence of other human cell types. The lack of support from cells normally present in the tumour or tissue environment (i.e. cells not in close proximity or contact) may be a crucial factor. Put simply, the stromal cells may need the close contact support of epithelial cells as well as other stromal components to survive and proliferate.

6.4 Xenografts of preformed *in vitro* multicellular spheroids

As described in Chapter 5, heterologous tumour spheroids were manufactured *in vitro* to provide a physiological 3-D environment in which the stromal cells were in close proximity both to each other and to the tumour cells. Multicellular tumour spheroids have a well organized spherical symmetry of morphological and physiological features including complex cell-cell and cell-matrix interactions resembling avascular tumour sites and or micrometastatic regions *in vivo* and thus are a very useful model in tumour biology. Heterologous two and three compartment tumour spheroids manufactured in the Rotary Cell Culture System (RCCS) as described and illustrated in Chapter 5, consisting of the 3 different cell types combined with Cytodex microcarrier beads were inoculated into the mammary fat pads of nude mice in Matrigel. The combinations of cells xenografted are shown in Tables 6.5 and 6.6 below. Due to the possible expected problems associated with the temperature sensitive lines, which will be close to their non permissive temperature (>39°C) in the mouse, only the wild type LT lines were used in these experiments.

6.4.1 Heterologous stromal spheroids

Heterologous stromal spheroids consisting of two different combinations of the immortalized fibroblasts and endothelial cells, manufactured in the RCCS, were inoculated into mammary fat pads of nude mice in Matrigel (Table 6.5). Xenograft harvests were carried out at 8 weeks post inoculation.

Cell lines	Xenograft Take Rate Matrigel + MFP (6 sites per group)
HMF 3wt + HMME 7 wt	0/6
HMF 3wt *ras* + HMME 7 wt	0/6

Table 6.5. Table summarizing 3-D heterologous stromal cell spheroid xenografts. Spheroids manufactured from the combination of two different fibroblast cell lines and endothelial cells were inoculated into the MFP with Matrigel. Cells were harvested at 8 weeks.

Both of the heterologous stromal spheroid combinations including the *ras* expressing cells failed to give rise to tumours after 8 weeks (Table 6.5). Immunohistochemical analysis of the all the inoculated mammary fat pads (20 sections/inoculation site), using antibodies against vimentin, CD31/CD34 and SV40 LT antigen, demonstrated no evidence of either stromal cell type survival.

6.4.2 Heterologous tumour spheroids

Tumour spheroids

Three different breast tumour cell lines were xenografted into the mammary fat pads of nude mice either as a cell suspension (5×10^6 cells) or as spheroids (Table 6.6). All the inoculations were performed in Matrigel. Tumours or mammary fat pads were harvested at 8 weeks.

Cell lines	Xenograft Take Rate Matrigel + MFP (6 sites per group)	
	Cell suspension	Spheroids
MCF-7	6/6	0/6
MCF-7 V12	5/6	0/6
T47D	6/6	0/6

Table 6.6. Table comparing the tumourigenicity of breast tumour cell lines when xenografted as spheroids and cell suspensions. Cell suspensions consisted of 5×10^6 tumour cells. Tumours or mammary fat pads were harvested at 8 weeks.

Two of the tumour cell lines (MCF-7 and T47D) produced 6 tumours from 6 inoculations (100%) with the other (MCF-7 V12) producing 5 out of a possible 6 inoculations (83%) when xenografted as cell suspensions. Immunohistochemistry using a human specific pan-cytokeratin marker confirmed that all the tumours produced were of human epithelial cell origin (10 sections/inoculation site). However when the same three tumour cell lines were xenografted as pre-formed multicellular spheroids none of the inoculations gave rise to tumours. The absence of the tumour cell survival in these inoculations was confirmed by immunohistochemistry using the same marker (10 sections/inoculation site).

2-compartment heterologous tumour spheroids

In an attempt to improve the success rate of establishment of the tumour cell only spheroids, xenograft experiments were performed using 2 compartment heterologous tumour spheroids with stromal elements already present in the pre-formed spheroids. The three breast tumour cell lines were combined with one of three different stromal cell lines before being inoculated into the mammary fat pads in Matrigel. The preparation and characterization of the spheroids has been described in Chapter 5.5. The different heterologous tumour-stromal combinations are shown in Table 6.7.

Cell lines	HMME 7wt	HMF 3wt	HMF 3wt *ras*
MCF-7	0/6	0/6	0/6
MCF-7 V12	0/6	0/6	Not determined
T47D	0/6	0/6	Not determined

Table 6.7. Table showing the xenografts of the 2-compartment heterologous tumour spheroids. All spheroid combinations were inoculated into the MFP in Matrigel (6 sites per group). Mammary fat pads were harvested at 8 weeks.

None of the 2 compartment heterologous tumour spheroid combinations gave rise to tumours after 8 weeks. Immunohistochemistry, using human specific antibodies described in Chapter 6.2 demonstrated that none of the human cells xenografted survived (10 sections/inoculation site).

3-compartment heterologous tumour spheroids

In a final attempt to provide a suitable cellular environment for establishing tumour spheroids, the three tumour cell lines were co-grafted as 3 compartment spheroids comprising tumour, fibroblast and endothelial cells (Table 6.8).

Cell lines	HMF 3wt + HMME 7wt	HMF 3wt *ras* + HMME 7wt
MCF-7	0/6	0/6
MCF-7 V12	0/6	Not determined
T47D	0/6	Not determined

Table 6.8. Table summarizing 3-compartment heterologous tumour-stromal cell spheroid xenografts. All spheroid combinations were inoculated into the MFP in Matrigel (6 sites per group). Mammary fat pads were harvested at 8 weeks.

Neither with HMF 3wt + HMME 7wt combinations or even HMF 3wt *ras* + HMME 7wt were successful in producing tumours. None of the heterologous 3-compartment inoculations gave rise to tumours. Again, analysis of all inoculation sites, by immunohistochemical analysis, to determine cell survival was negative (10 sections/inoculation site).

6.5 Establishment of heterologous tumour xenografts

6.5.1 Heterologous cell xenografts

The failure of the preformed heterologous tumour spheroids to form tumours did not provide a model of stromal-tumour interaction in the context of the immortalized stromal lines. One problem associated with the heterologous tumour spheroids is that they may not reach an adequate size and hence cell numbers necessary for *in vivo* establishment. To address the issue of whether the immortalized human mammary stromal cell lines could support tumour take and growth, experiments using cell suspensions, in which tumours cell lines and stromal cell lines were mixed at the point of inoculation, were performed. Furthermore, the cells numbers inoculated were carefully controlled. Recent research has shown that the human breast cell line MCF-7 produced significantly faster growing tumours when mixed with carcinoma associated fibroblasts than when mixed with normal fibroblasts (Shekhar et al. 2001).

Xenografts using wild type LT human mammary stromal cells

Cell numbers of breast tumour cell lines (1×10^5), generally insufficient to produce tumours when xenografted alone, were xenografted in combination with the immortalized human mammary stromal cells to determine whether the addition of these cells provided a tumour take advantage. It is generally observed that a minimum of 5×10^6 (threshold inoculum) of tumour cells/ inoculation are required to consistently produce tumours when xenografted in nude mice.

Tumour cells in the exponential growth phase were harvested and re-suspended in serum free medium, to give a of dose 1×10^5 cells/inoculation. The wild type LT mammary fibroblasts and endothelial cell lines were harvested in an identical manner. The stromal cells were then combined with the tumour cell

lines in either a 1:10 or 1:5 stromal cell to tumour cell ratio (Table 6.9). These ratios were chosen to provide a very approximate match to physiological tumour-stromal numbers *in vivo*. In addition to the MCF-7, the VEGF over-expressing tumour cell line MCF-7 V12 was also tested (Table 6.10). Controls consisted of tumour cell inoculations at the supra-threshold level of $5x10^6$cells/site. All inoculations were carried out in Matrigel into the mammary fat pads and harvested at 8 weeks. Tumours were permitted to reach a maximum size of 8×8mm before being harvested. Inoculation sites were inspected on a daily basis in order to identify the time at which the first sign of a palpable mass (a successful tumour take) was seen. Harvested tumours or mammary fat pads were first measured if tumours were present and then processed for immunohistochemical analysis.

A)

Cell lines	Xenograft Take Rate (8 sites per group)	Mean tumour volume (range) (mm^3)
MCF-7 (1×10^5)	1/8 (12.5%)	31.0
MCF-7 (1×10^5) + HMF 3wt	8/8 (100%)	134.6 (46.8 –183.3)
MCF-7 (1×10^5) + HMME 7wt	3/8 (37.5%)	75.2 (33.5 –102.1)
MCF-7 (1×10^5) + HMF 3wt + HMME 7wt	6/8 (75%)	192.8 (73.0 – 278.0)
MCF-7 Alone (5×10^6)	8/8 (100%)	66.7 (21.8 –104.8)

B)

Cell lines	Xenograft Take Rate (8 sites per group)	Mean tumour volume (range) (mm^3)
MCF-7 (1×10^5)	0/8 (0%)	N/A
MCF-7 (1×10^5) + HMF 3wt	8/8 (100%)	141.2 (65.4 – 187.3)
MCF-7 (1×10^5) + HMME 7wt	4/8 (50%)	74.5 (33.5 – 115.9)
MCF-7 (1×10^5) + HMF 3wt + HMME 7wt	7/8 (83.3%)	209.3 (86.7 – 294.0)
MCF-7 (5×10^6)	8/8 (100%)	70.3 (33.5 – 110.3)

Table 6.9. Heterologous cell xenografts using sub-threshold inocula of breast cancer cell line MCF-7 and stromal cells at a ratio of (a) 10:1 and (b) 5:1. Sub-threshold inoculum was defined as $1x10^5$ cells/ inoculation site with supra threshold level $5x10^6$ cells/inoculation site. All combinations were xenografted in Matrigel and harvested at 8 weeks. Tumour volume was calculated as follows: $V = (\pi/6)(d_1 \times d_2)^{3/2}$, where d_1 and d_2 are two perpendicular tumour diameters.

Cell lines	Xenograft Take Rate (8 sites per group)	Mean tumour volume (range) (mm^3)
MCF-7 V12 (1 x 10^5)	0/8 (0%)	N/A
MCF-7 V12 (1 x 10^5) + HMF 3wt	6/8 (75%)	187.8 (77.9 – 225.3)
MCF-7 V12 (1 x 10^5) + HMME 7wt	2/8 (25%)	72.2 (46.1–140.5)
MCF-7 V12 (1 x 10^5) + HMF 3wt + HMME 7wt *(10:1:1 ratio)*	4/8 (50%)	222.3 (94.4 – 283.4)
MCF-7 V12 (1 x 10^5) + HMF 3wt + HMME 7wt *(5:1:1 ratio)*	5/8 (62.5%)	264.7 (267.8 – 327.1)
MCF-7 V12 (5x 10^6)	6/8 (75%)	103.4 43.1–161.0

Table 6.10. Heterologous cell xenografts with sub-threshold inocula of breast cancer cell line MCF-7 V12 and stromal cells at a ratio of 10:1. Sub-threshold inoculum was defined as $1x10^5$ cells/ inoculation site. Stromal cells were xenografted in a ratio of 10:1 of each tumour cell to stromal cell type. All combinations were xenografted in Matrigel and harvested at 8 weeks. NB. tumours produced were in general larger and more hyperaemic than with MCF-7 cell with MCF-7 V12.

Results showed that the addition of stromal cells provided a dramatic growth advantage (Tables 6.9 and 6.10). These experiments demonstrate the effect of stromal cells on the promotion of tumourigenesis despite their failure to survive when grafted alone.

Unlike the heterologous spheroid xenograft experiments, the addition of the immortalized stromal cells produced a marked effect on the promotion of MCF-7 tumour cell growth when both were combined as cell suspensions. The heterologous cell xenografts described above differed significantly from tumour cell only xenografts both in the take rate (i.e. efficiency of tumour formation) and

the latency with which they formed palpable tumours, following orthotopic implantation in nude mice. Combining stromal cells at a ratio of 1:5 to tumour cells showed no significant difference in take rates compared to a more physiological ratio of 1:10 (Table 6.9).

Sub-threshold tumour only xenografts had a poor take rate with tumours arising in only 6.25% (1/16 sites) of injection sites. In comparison, the addition of fibroblasts produced a significantly enhanced take rate, resulting in a 100% take rate (16/16 sites). However, differences were noted in the take rates between fibroblasts and endothelial cells or both. In all cases the fibroblasts produced the highest overall take rates. While the addition of the endothelial cell lines did enhance tumour growth on their own (7/16 sites), this was significantly less than the fibroblasts (16/16 sites). The addition of both stromal cell types resulted in a take rate of 81.25% (13/16 sites), a take rate in between fibroblasts and endothelial cells.

Results using the MCF-7 V12 tumour cell line were broadly in agreement with the original MCF-7 results. Again fibroblasts were most effective (6/8 sites); endothelial cells (2/8 sites) least effective and the combination of both stromal cell types (4/8 sites) somewhere in between. However, there were two specific differences to the original MCF-7 cell line: 1) that overall take rates in this line were lower, including supra-threshold inoculum controls and 2), the tumours that were formed did so more rapidly on the basis of daily observations and were more hyperaemic when harvested. Six tumours grew so rapidly that the mice had to be sacrificed prematurely before the 8 weeks had elapsed, with three mice being sacrificed at 6 weeks.

In all cases heterologous tumours with added wild type LT fibroblasts not only grew more rapidly they also grew to significantly larger sizes, compared to

supra-threshold inoculum controls, within the same experiment period (see Table 6.11). The addition of fibroblasts reduced the latency period of tumour formation. The control groups (supra-threshold tumour cell inocula) displayed long latency periods (3-4 weeks) before tumours were visible on gross inspection (data not shown). Tumours were first visible at 2 weeks in all the fibroblast heterologous xenografts including those combined with endothelial cells. However, endothelial cells alone did not reduce the latency period when compared to the control group.

Cell lines	Take rate	Mean tumour volume (range) (mm^3)	Latency (weeks)
MCF-7 (5×10^6) **supra-threshold control**	8/8	66.7 21.8 –104.8	3-4
MCF-7 (1×10^5) + Fibroblasts **(10:1)**	8/8	134.6 46.8 –184.3	<2
MCF-7(1×10^5) + Fibroblasts **(5:1)**	8/8	141.2 65.4 –187.3	<2
MCF-7 V12 (5×10^6) **supra-threshold control**	6/8	103.4 43.1 – 161.0	3-4
MCF-7 V12 (1×10^5) + Fibroblasts **(10:1)**	6/8	187.8 77.9 – 225.3	<3

Table 6.11. Table comparing supra-threshold control xenografts with the tumour-fibroblast combination xenografts. Results taken from Tables 6.9 and 6.10.

Xenografts using temperature sensitive human mammary stromal cells

As a parallel experiment to the wild type LT heterologous cell xenografts, the conditionally immortalized temperature sensitive stromal cell lines were xenografted in combination with breast tumour cell lines to determine their effect on stromal-tumour interactions (see Table 6.12 for results). As previously explained in the introduction a temperature sensitive mutant of SV40 large T antigen (U19tsA58) was used to immortalize the stromal cell lines. HMME 2 and 7 endothelial cell lines were more temperature sensitive than the corresponding fibroblasts arresting completely at 36.5°C. The temperature sensitivities for growth has previously been characterised by O'Hare et al. (2001). Temperature sensitive LT function was essential for the continued proliferation of the stromal lines *in vitro* however a non-permissive temperature of 39.5°C halted growth of the cell lines. The body temperature of a mouse is generally around 38°C, but this fluctuates greatly with the animals' surroundings, and so a small increase in room temperature could result in loss of temperature sensitive LT functi

Cell lines	Xenograft Take Rate (8 sites per group)	Mean tumour volume (range) (mm^3)
MCF-7(1×10^5) Alone	0/8 (0%)	N/A
MCF-7 (1×10^5) + HMF 3A	8/8 (100%)	114.7 (63.5 –172.0)
MCF-7 (1×10^5) + HMME 7	4/8 (50%)	65.2 (31.0 – 116.0)
MCF-7 (1×10^5) + HMF 3A + HMME 7	5/8 (62.5%)	178.2 (56.1 – 203.6)
MCF-7 (5×10^6) Alone	8/8 (100%)	70.4 (33.5 – 110.3)

Table 6.12. Heterologous xenografts of sub-threshold inocula of breast cancer cell line MCF-7 and temperature sensitive stromal cells at a ratio of 10:1. Sub-threshold inoculum was defined as 1×10^5 cells/ inoculation site. Stromal cells were xenografted in a ratio of 1:10 of each stromal cell type to tumour. All combinations were xenografted in Matrigel and harvested at 8 weeks.

Results (Table 6.12) showed that the addition of the conditionally immortalized stromal cells to the tumour cells also resulted in not only a more successful tumour take rate compared with sub-threshold tumour only xenografts but they also produced quicker growing larger tumours compared with the supra-threshold tumour only controls. The addition of temperature sensitive fibroblasts produced 8/8 tumours, a 100% take rate; with 4/8 (50%) injection sites leading to tumours with the endothelial cells and 5/8 (62.5 %) in the combined stromal cell xenografts. It is clear from these results that non-proliferating stromal cells are as effective in assisting tumour take rate as the potentially proliferative non-temperature sensitive (wild type LT variants) equivalents (compare Tables 6.9A with 6.12). As the non-temperature sensitive cells could not be detected after 6

weeks when xenografted alone it is likely that their effects are confined to a short term influence on the tumour cells.

Summary

Thus to summarize, the addition of stromal cells supported sub-threshold tumour cell inoculates to produce tumours which would not generally have been produced. No significant differences in take rates between 1:5 and 1:10 stromal cell to tumour cell ratio xenografts were observed. The addition of fibroblasts produced better tumour take rates and provided shorter latency periods than the endothelial cells. The MCF-7 cell line was more efficient than the MCF-7 V12 line at producing tumours, however once the tumours had taken the MCF-7 V12 produced faster growing more hyperaemic tumours.

6.6 Histological and immunohistochemical analysis of the heterologous cell xenografts

Tumours generated in the mammary fat pads of nude mice by the mixed combinations of tumour cells, stromal cells and Matrigel as described above were fixed (frozen and formaldehyde) and processed for histology and immunohistochemistry after 8 weeks. 8 tumours from each group: tumour cell only, 2 and 3 compartment heterologous cell xenografts were harvested and analysed extensively. Positive and negative controls were performed on human tonsillar/appendix tissue and samples of mouse skin. In addition heterologous tumours using the VEGF over expressing tumour cell line MCF-7 V12 was also analysed. 20 sections/tumour were analysed using a variety of staining methods, these being haematoxylin and eosin, immunoperoxidase and, direct and indirect immunofluorescence for identification of specific human cell types. The

objective was to see if there were any differences in architecture, general histology and cellular composition between tumour cell only and heterologous xenografts.

6.6.1 Haematoxylin and eosin (H&E) staining

Tumour cell only xenografts (control)

Haematoxylin and eosin (H&E) staining of tumour cell (MCF-7) only xenografts with close examination of the centres of the tumours demonstrated, although occasional foci could be seen, a general lack of necrosis was observed indicating that the tumours were well vascularized (Figure 6.5). High power images demonstrated that they were poorly differentiated carcinomas equivalent to a high grade (Grade III) tumour in a patient. The tumours had large, pleomorphic nuclei, prominent nucleoli, and several mitotic figures per high powered field (Figure 6.6). The epithelial tumour cells were recognised as solid cords or tubules (Figure 6.7).

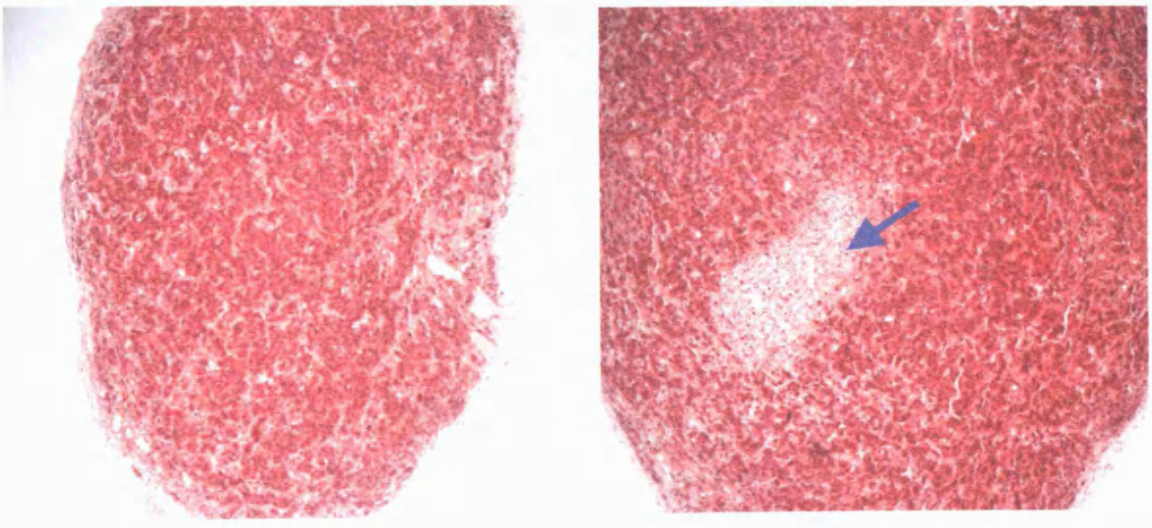


Figure 6.5. H&E sections of xenografts produced by MCF-7 inoculations. Tumours are comprised of densely packed MCF-7 epithelial cells with only occasional evidence of necrosis (arrow). Magnification ×80.

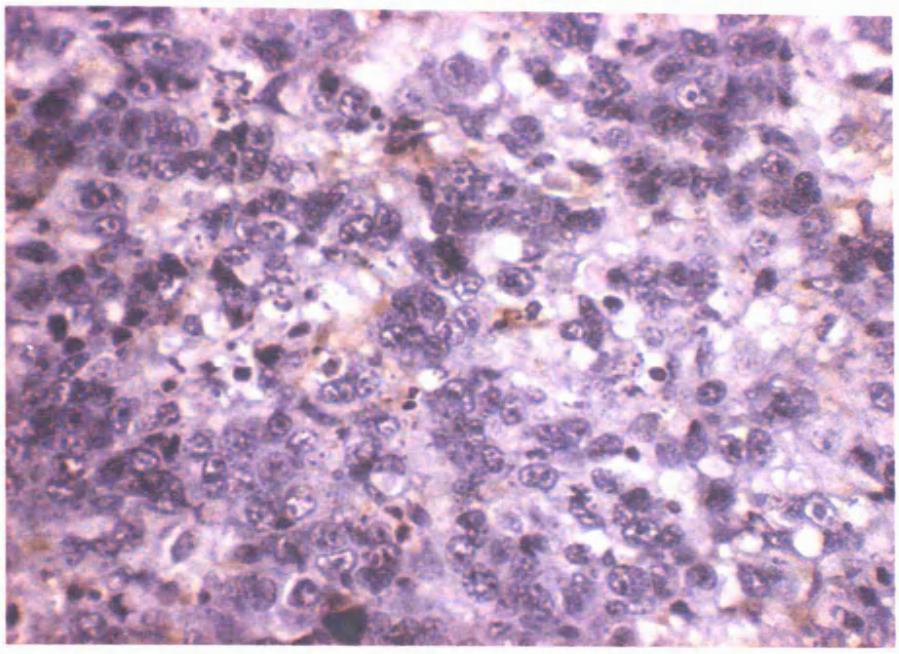


Figure 6.6. Haemotoxylin stained sections of xenograft tumours produced by MCF-7 inoculations. Xenografts were poorly differentiated cells with large pleomorphic nuclei and prominent nucleoli visible. Magnification ×320.

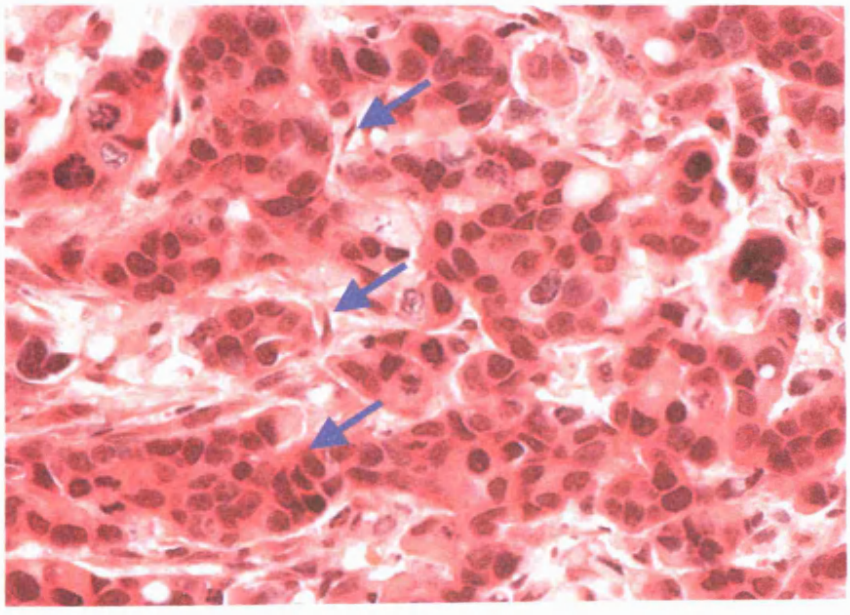


Figure 6.7. H&E sections of xenografts produced by MCF-7 inoculations. MCF-7 epithelial cells are easily recognisable as cords (arrows). Magnification ×320.

The tumours that arose from mammary fat pad injections demonstrated invasive characteristics, as they infiltrated through several types of adjacent tissue. They infiltrated throughout the gland, spreading through adipocyte layers and migrating around ducts (Figure 6.8a). They also spread through the thin layers of skeletal muscle and the adipose tissue just beneath the skin surrounding the mammary fat pad (Figure 6.8b). One mammary gland tumour also showed infiltration into the skeletal muscle of the abdominal wall. There was no evidence of the equivalent of a desmoplastic response (i.e. extensive tumour-induced proliferation) by the host fibroblasts which were relatively few in number and present as isolated cells.

No signs of metastatic spread were observed, on gross inspection, in any of the tumour bearing mice. H&E staining of sections of the lungs, livers, spleens, and kidneys of MCF-7 tumour bearing mice showed no evidence of distant metastases (S Clinton, personal communication).

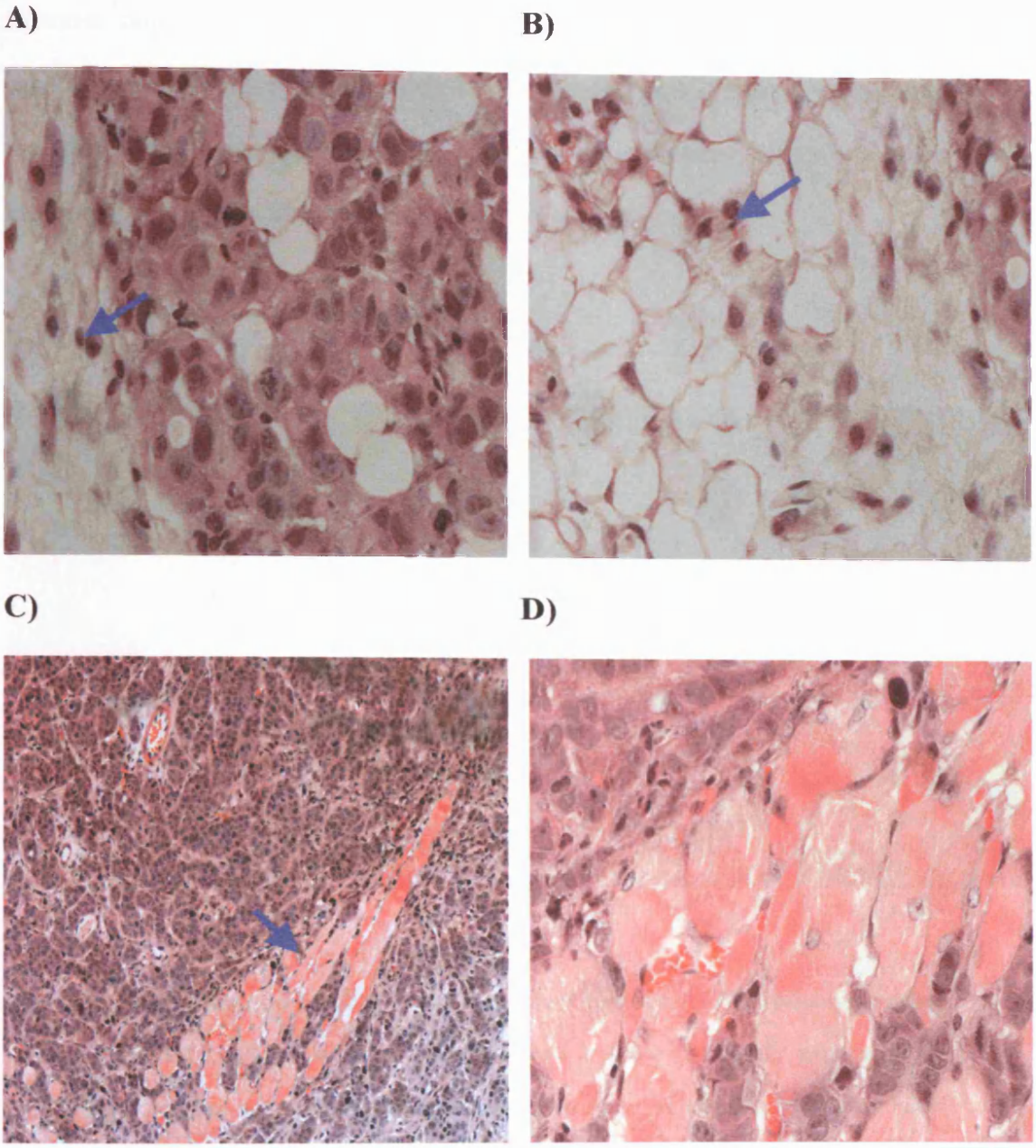


Figure 6.8. H&E sections of MCF-7 xenograft tumours demonstrating invasive growth. Tumours (arrow) invaded into adipose tissue (a) with tumour epithelial cells (arrow) clearly visible within the adipose tissue (b). They also invaded into local muscle (c) beneath the mammary fat pad with striated muscle visible at high power (d). Magnification a), b) & d) ×320, c) ×80.

However tumours produced by the MCF-7 V12 cell line produced were in general noticeably more vascular with large vascular lakes (Figure 6.9). As with the MCF-7 cell line there was no evidence of metastases either on gross inspection or in histological sections of brain, liver and lungs.

A) B)

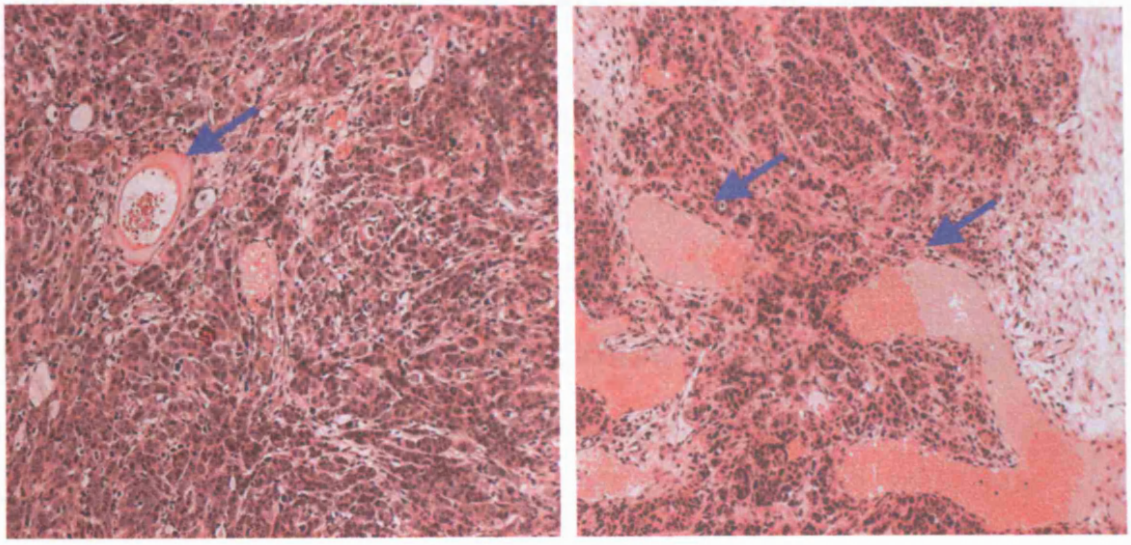


Figure 6.9. H&E sections of MCF-7 V12 xenograft tumours (VEGF overexpressing variant of MCF-7). MCF-7 V12 containing xenografts were noticeably more vascular than MCF-7 containing tumours and in some parts showed large (b) vascular lakes (arrows). Magnification ×80.

Heterologous cell xenografts

Analysis of 2 and 3 compartment heterologous cell xenografts, by H&E sections, showed no significant differences in architecture compared to the tumour cell only xenografts (both MCF-7 and MCF-7 V12 containing xenografts) with again the MCF-7 V12 tumour appearing more vascular.

6.6.2 Immunoperoxidase staining

Formaldehyde fixed paraffin embedded sections of all tumours from each group (10 sections/antibody), were labelled using a standard immunoperoxidase labelling technique following individual antigen retrieval procedures (see Chapter 2.8). As described previously in the methods, antibodies specific for human fibroblasts (vimentin), endothelial cells (CD31/CD34) and breast epithelial cells (pan-cytokeratin) were used (Table 6.13).

Antigen	Antibody	Species	Sub-class	Dilution	Sources
CD34	Q/BEND10	Mouse	IgG1	1:30 2 min PC	Dako
CD31	JC70A	Mouse	IgG1	1:30 2 min PC	Dako
Pan-cytokeratin	MNF116	Mouse	IgG1	1:400 10 min CT	Dako
Vimentin	V9	Mouse	IgG1	1:800 18 min MW	Santa Cruz

Table 6.13. Table showing the human cell specific antibodies and antigen retrieval techniques used. PC (pressure cooker), CT (chymotrypsin), MW (microwave).

Staining of 3 compartment heterologous xenografts for human endothelial cells with anti-CD31/CD34 antibodies, fibroblasts with anti-vimentin and tumour epithelial cells with a pan-cytokeratin are shown in Figures 6.10, 6.11 and 6.12.

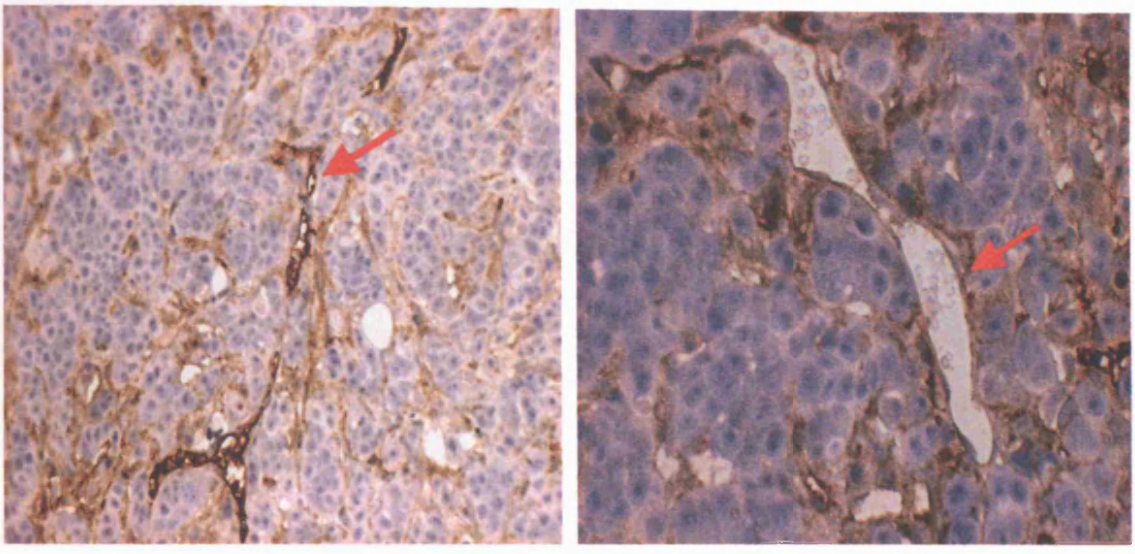


Figure 6.10. Immunoperoxidase staining of 3-compartment heterologous tumours with a human specific antibody against CD31. Sections demonstrated apparent stromal staining in some areas (arrows). Anti-CD34 (not shown) and CD31 produced similar staining patterns. Magnification ×80 and ×320.

The pattern of staining produced by labelling of endothelial cells and fibroblasts using human specific antibodies against CD31/CD34 and vimentin respectively, demonstrated that the human stromal cells apparently not only survived but they even proliferated to form complex structures (Figure 6.10 and 6.11). Endothelial cells lining blood vessels present in the tumours, were clearly labelled by positive staining with both human specific anti-CD31 and CD34 markers, suggesting that the xenografted human endothelial cells had formed complex structures in the form of blood vessels (Figure 6.10). Staining for human specific

vimentin was also positive with abundant intermixing visible throughout the tumour indicating the fibroblasts had also proliferated *in vivo* (Figure 6.11).

A) **B)**

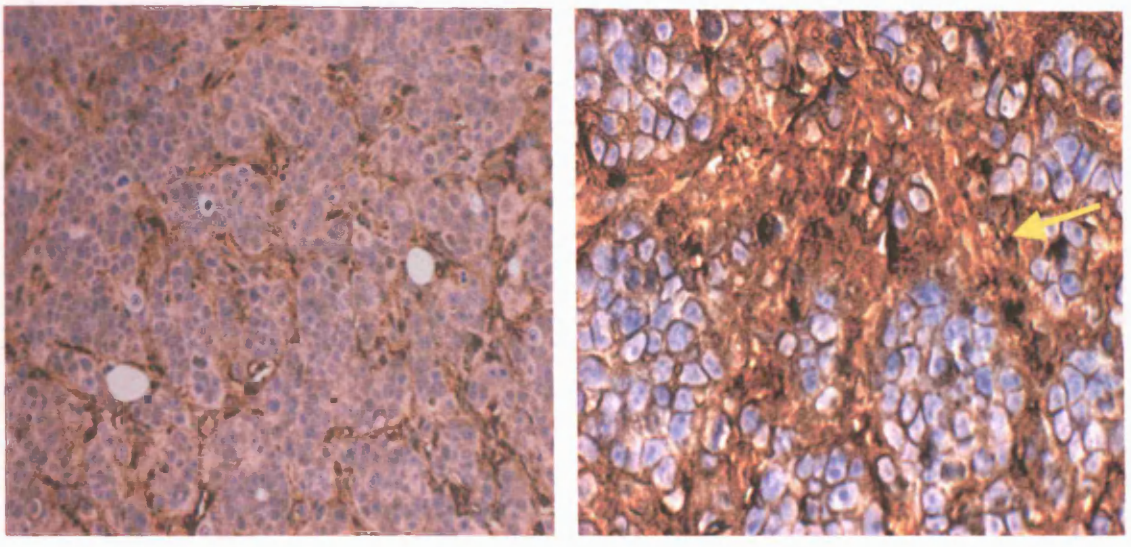


Figure 6.11. Immunoperoxidase staining of a 3-compartment heterologous tumour xenograft with a human specific antibody against vimentin. Note the apparent specific staining in (a) but reactivity of epithelial membranes (arrow) in other regions of the same section (b) similar to that seen with the pan-cytokeratin staining in Figure 6.12. At ×80 and ×160 magnification.

Immunoperoxidase staining of all tumour xenografts using a human specific pan-cytokeratin (MNF116) antibody with antigen retrieval techniques for the identification of human epithelial cells showed strong staining throughout the tumour, validating the epithelial nature of the tumour (Figure 6.12).

A) B)

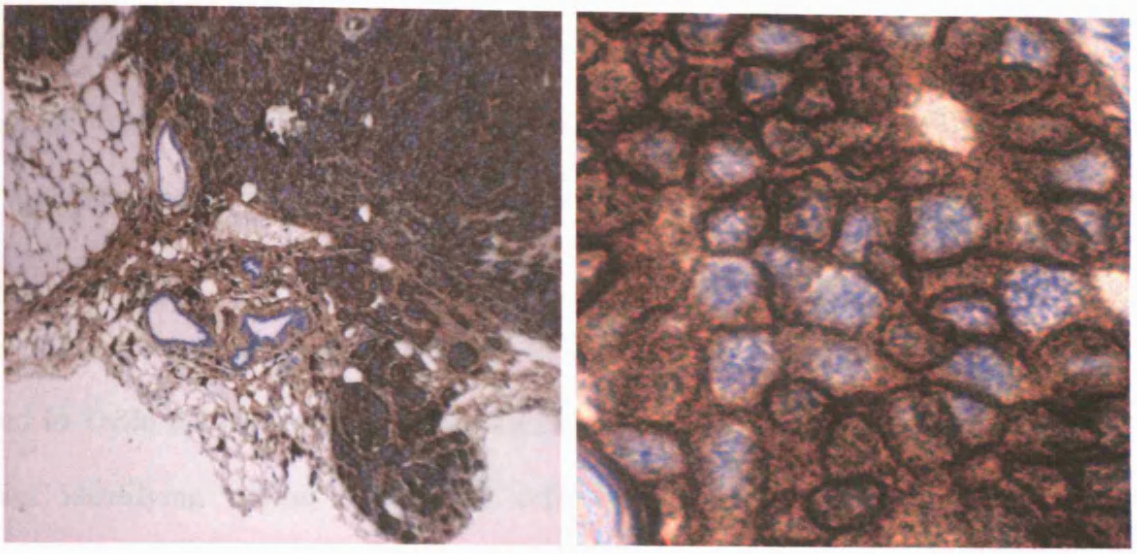


Figure 6.12. Immunoperoxidase staining of 3-compartment heterologous tumours with a human specific antibody against pan-cytokeratin. At high power (b) many individual tumour cells are stained just at their edges. Magnification at ×80 and ×320.

Some of the sections shown in the figures were selected as they appeared to represent true staining (i.e. cell type specific). However, there were several signs of indiscriminate staining in the xenografts, contrasting with the generally clean staining of the human tissue controls. Firstly, a significant amount of non-specific background staining was present in a large proportion (70%) of the sections tested some of it typical 'edge effect' but also patches of apparently non-specific reaction deeper in the xenograft. In several sections tested the staining patterns produced by antibodies to vimentin and CD31 were nearly identical, indicating that maybe the staining pattern was not cell type specific but rather indiscriminate (non-specific). More specifically, high magnification visualization of vimentin staining revealed anomalous staining of epithelial cell membranes in some sections (Figure 6.11b).

6.6.3 Immunofluorescence staining

Due to the variable patterns of staining observed with immunoperoxidase labelling, on formaldehyde fixed paraffin tumour sections with antigen retrieval techniques, direct and indirect immunofluorescence staining of frozen sections of the same tumours were performed to either confirm or refute the results. Frozen sections did not require antigen retrieval techniques. The same antibodies identified in Table 6.12 were used. In addition all sections were labelled with antibodies identifying mouse endothelial cells and a non species specific fibroblast marker.

The human mammary stromal cells in the xenografts were labelled using either direct (Zenon Alexa fluor dyes) immunofluorescence or indirect immunofluorescence using fluorophore conjugated secondary antibodies (TRITC or FITC). With the indirect immunofluorescence technique significant background fluorescence was encountered using the anti-mouse IgG reacting secondary antibodies. For example, while cytokeratin in MCF-7 cells was strongly stained and clearly visible, more specific weaker background staining was seen in the associated stroma. When sections were stained by the indirect method using antibodies against fibroblasts and endothelial cells the only reactivity seen was a weak stromal localisation no more intense than when an anti-epithelial antibody was used. It is unclear whether or not this represented specific staining or reaction of secondary antibodies with mouse IgG in the blood and tissue fluids. No differences were seen between vimentin and CD34/31 staining with this method again suggesting that there were no specifically stained human stromal cells present.

To overcome the problem of non-specific background staining caused by secondary antibodies raised against mouse staining mouse tissues in the xenograft experiments, the Zenon (Molecular Probes) direct immunofluorescence system was, therefore, adopted. This system works as follows: an unlabelled IgG is incubated with the Zenon labelling reagent, which contains a fluorophore-labeled Fab fragment (A). The labeled Fab fragment binds to the Fc portion of the IgG antibody (B), and excess Fab fragment is neutralized by the addition of a non-specific IgG (C). The addition of non-specific IgG prevents cross-labeling of the Fab fragment in experiments where multiple primary antibodies of the same type are present (Figure 2.7 in Chapter 2.8.5). Thus, this system should in theory overcome the problem of background staining observed in the sections labelled using indirect immunofluorescence techniques.

Controls sections of a primary human breast cancer were used to validate the results and check that the system used was able to label the primary antibodies chosen for the study (Figure 6.13).

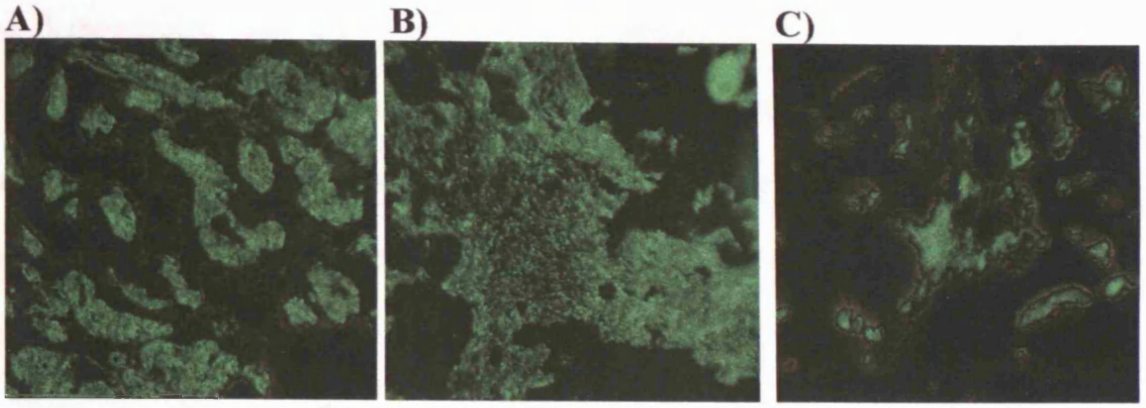


Figure 6.13. Direct immunofluorescent labelling of controls, consisting of a primary human breast cancer, using human specific (a) anti-pan-cytokeratin antibody, (b) human specific anti-vimentin and (c) an anti-CD31 antibody. Note that human tumour cells are not so tightly packed as the MCF-7 cell xenografts. Magnification ×80.

Following successful labelling of a primary human breast cancer control (Figure 6.13) with the Zenon Alexa fluor direct labelling kit, direct immunfluorescence labelling of 3-compartment xenografts was now performed. The sections were labelled with antibodies specific for human vimentin, CD31/CD34 and a pan-cytokeratin. Results are shown in Figure 6.14.

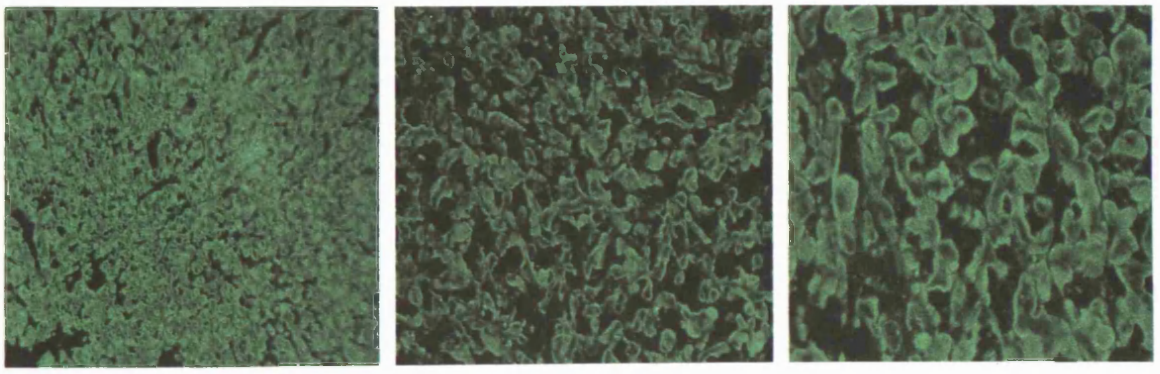


Figure 6.14. Direct immunofluorescence labelling of 3-compartment heterologous xenografts using a human specific pan-cytokeratin marker. At ×80, ×160 and ×320 magnifications.

Staining demonstrated that the xenografts were predominantly MCF-7 cells surrounded by non cellular connective tissue. Staining for vimentin and CD34 and or CD31 was negative in all sections tested. These pictures were representative of the staining pattern seen in both tumour only and heterologous cell xenografts.

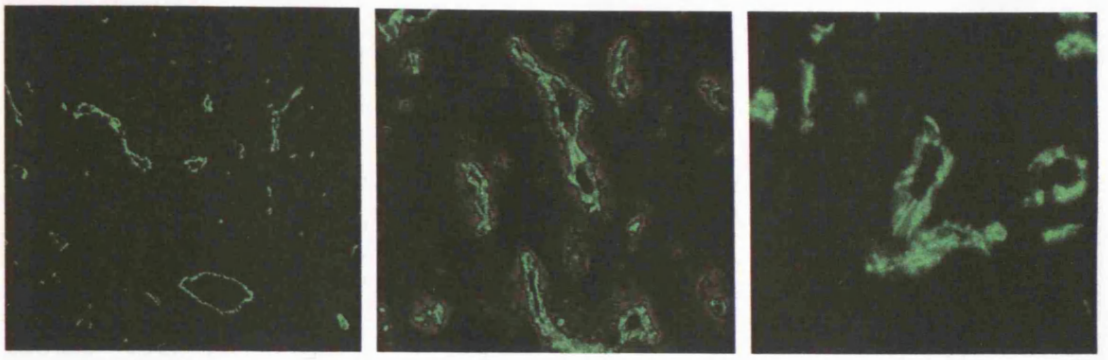
There was almost no background staining seen in the xenograft sections labelled using the Zenon system. The intensity of specific staining (e.g. cytokeratin in MCF-7 cells) was very similar for that seen using indirect immunofluorescence methods. This made the assessment of, in particular, endothelial cells and fibroblasts markers more robust. In both cases no cells of this type were seen despite an extensive immunohistochemical analysis of 20 sections/tumour harvested at 60 days. The results from direct immunofluorescence are summarised in Table 6.14.

Antibodies tested				
Tumour composition	**Pan-cytokeratin**	**Vimentin**	**CD34**	**CD31**
MCF-7	**+ve**	**–ve**	**–ve**	**–ve**
MCF-7 +HMME 7 (temperature sensitive)	**+ve**	**–ve**	**–ve**	**–ve**
MCF-7 + HMF 3A (temperature sensitive)	**+ve**	**–ve**	**–ve**	**–ve**
MCF-7 + HMF 3A + HMME 7 (temperature sensitive)	**+ve**	**–ve**	**–ve**	**–ve**
MCF-7 + HMME 7wt	**+ve**	**–ve**	**–ve**	**–ve**
MCF-7 + HMF 3wt	**+ve**	**–ve**	**–ve**	**–ve**
MCF-7 + HMF 3wt + HMME 7wt	**+ve**	**–ve**	**–ve**	**–ve**
MCF-7 V12	**+ve**	**–ve**	**–ve**	**–ve**
MCF-7 V12 + HMME 7wt	**+ve**	**–ve**	**–ve**	**–ve**
MCF-7 V12 + HMF 3wt	**+ve**	**–ve**	**–ve**	**–ve**
MCF-7 V12 + HMF 3wt + HMME 7wt	**+ve**	**–ve**	**–ve**	**–ve**

Table 6.14. Table summarizing the combinations of cells xenografted to produce tumours and their staining pattern using direct immunofluorescence. 20 sections per xenograft combination were labelled using the antibodies shown above.

To control for the possibility that the human stromal cells were present but had, for example, down-regulated the expression of cell type specific antigens, sections were stained for LT antigen, present in the nuclei of all the cell lines used in the study. Negative staining using an antibody against SV40 LT antigen confirmed the absence of the human cell lines. Also, no evidence of host (mouse) fibroblasts was seen in any of the sections studied using the non-species specific vimentin antibody. This was validated by using positive controls consisting of human tonsillar tissue and mouse skin tissue in which fibroblast staining was seen. However, all blood vessels present in the tumours stained positive using an anti-CD31 antibody specific to mouse, with blood vessels being significantly more abundant near the periphery of the tumours (Figure 6.15).

A)



B)

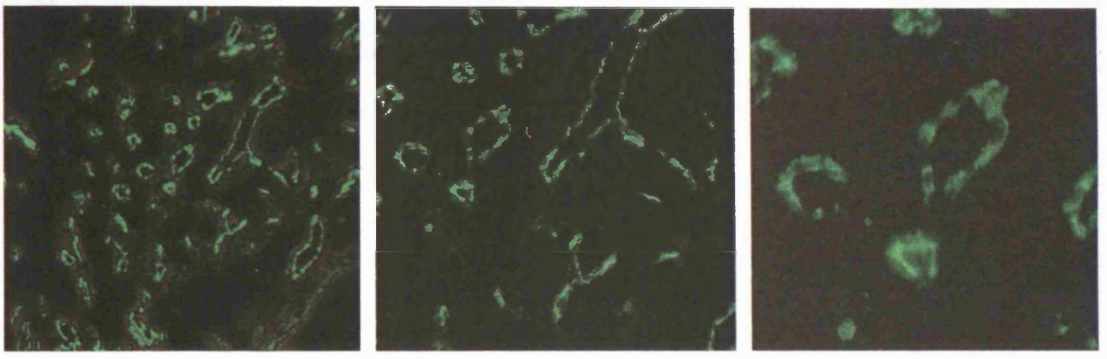


Figure 6.15. Fluorescent labelling of mouse endothelial cells in (a) MCF-7 containing heterologous xenografts and (b) MCF-7 V12 containing heterologous xenografts using an anti-CD31 rat antibody specific to mouse. Tumours stained negative for human anti-CD31 antibody. MCF-7 V12 containing xenografts were noticeably more hyperaemic and vascular compared to the MCF-7 tumours. Blood vessels were significantly more abundant near the periphery of the tumours. Taken at ×80, 160 and ×320 magnifications.

Therefore the staining pattern resulting from direct immunofluorescence did not correlate with that seen with the immunoperoxidase staining. Direct immunofluorescence staining showed that the xenografts were predominantly human tumour epithelial cells (MCF-7 or MCF-7 V12 cells) admixed with host connective tissue (ECM) vascularized by murine blood vessels. A summary of the positive labellings is demonstrated in Figure 6.16.

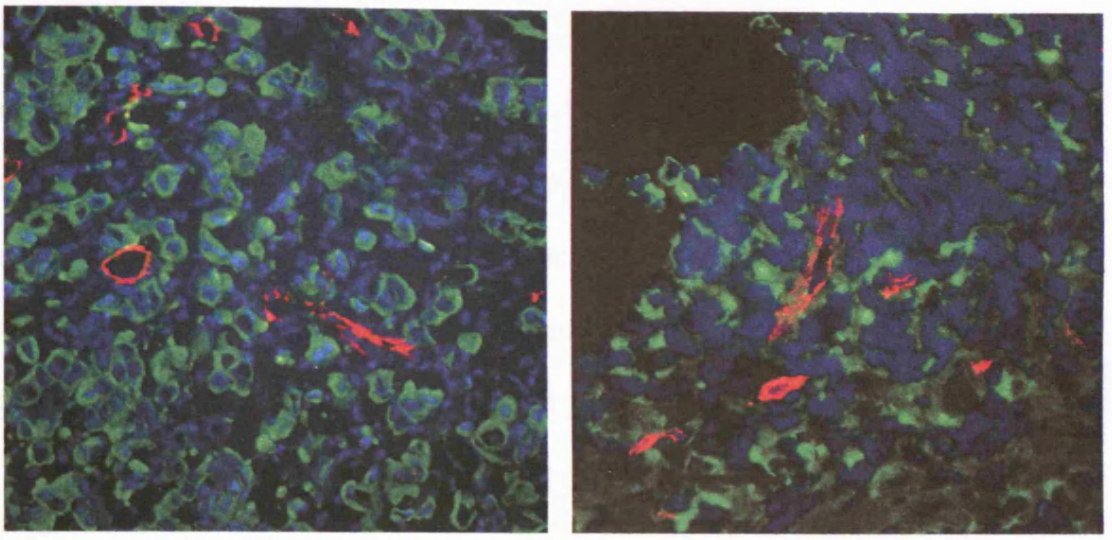


Figure 6.16. Triple immunofluorescence staining of 3-compartment heterologous tumours containing MCF-7, HMF 3wt and HMME 7wt cell lines. Alexa fluor dye labelling of MCF-7 cells (green) with mouse anti-CD31 labelling of blood vessels (red) and the nuclear stain Hoechst 33258 (blue).

6.6.4 Summary and discussion of immunohistochemistry

In summary, immunoperoxidase staining appeared to show, in some sections tested, that xenografted immortalized human mammary fibroblasts and endothelial cells not only survived but proliferated within the tumours, whereas direct and indirect immunofluorescence showed that the human stromal cells did not survive beyond 8 weeks and that such stromal cells that were present in the xenografts were predominantly mouse blood vessels.

It is generally held that immunoperoxidase staining is a more sensitive labelling technique than immunofluorescence. Immunofluorescence labelling works by completely saturating the antigens with primary antibodies and then saturating the primary antibodies with fluorophore conjugated secondary antibodies. Once both reactions are completed to saturation, no further increase

in fluorescence intensity can occur however long the sections are incubated. In comparison, immunoperoxidase labelling is not dependent on antigen saturation for intensity of labelling as the colour product is the result of an enzymatic reaction which only terminates when the chromogen (DAB) is washed off or removed. Therefore, this method is should be better at detecting even low levels of antigen, which perhaps immunofluorescence may not make evident.

Unlike immunofluorescence labelling immunoperoxidase staining presented some very contrasting and variable staining patterns. Although apparent specific staining could be seen in certain regions of xenograft sections, the rest of the same sections could demonstrate non-specific staining. In some sections, CD31/CD34 and vimentin staining were positive even though one or the other of these two cell types was not even inoculated. Also the pattern of staining resulting from vimentin and CD31/CD34 markers were remarkably similar in the many of the sections tested. Furthermore, these same patterns were visible in sections of MCF-7 tumour cell only xenografts although fibroblasts and endothelial cells were not inoculated. Vimentin staining of fibroblast containing xenografts appeared to be specific to stroma at low magnification; however at higher magnification it was clearly not restricted to fibroblasts alone. Overall, the percentage of non-specific staining areas were much greater than the apparently specifically stained regions

There are several other possible reasons for the discrepancy in results produced by the two different immunohistochemical staining methods. Firstly, the severe conditions required for some antigen retrieval methods may have produced false positives due to cellular damage and therefore indiscriminate unmasking of antigens. This may explain how appropriate specific staining in one region was replaced by non-specific staining in another region in the same

histological section. However, positive (controls) staining on human tonsillar tissue and negative controls consisting of mouse skin samples were not subject to these problems even with the same antigen retrieval procedures. Thus the problems of interpreting staining with immunoperoxidase seemed confined to the xenografts. Furthermore, the Zenon direct immunofluorescence labelling system, which bypasses the problems of antigen-antibody species reactivity showed no evidence of human stromal cell presence. Thus, two methods which do not suffer from the problems of antigen-antibody species reactivity; immunoperoxidase controls on human tissue and direct antibody labelling of the xenografts, produced the same pattern of staining.

Another possible reason for the discrepancy in results could be that, although the human lines were present, they were no longer expressing these normally characteristic cell markers. If this was the case, then LT antigen staining should have been positive. However it was negative on extensive immunohistochemical analysis, again showing that there was no long term survival of the grafted human stromal lines despite their clear influence on take rates and latency of grafted human breast tumour cell lines (Chapter 6.5.1)

With these results in mind, experiments were next performed to investigate how long the stromal cells survived *in vivo* as part of the heterologous xenografts using the direct immunofluorescence method to detect the cells.

6.7 Heterologous tumour harvests at different time points - investigation of stromal cell survival

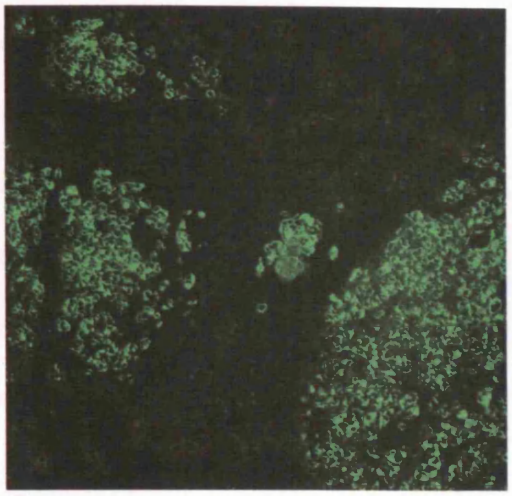
Experiments were conducted in which the 3-compartment heterologous tumours (consisting of MCF-7 tumour cells, wild type LT human mammary fibroblasts

HMF 3wt and endothelial cell line HMME 7wt inoculated orthotopically as mixed cell suspensions) were harvested at 2, 4 and 6 week time points for immunohistochemical analysis. Two weeks was chosen as the first time point because this was the earliest time at which palpable tumours could be detected and harvested. Six mammary fat pads were inoculated for each time point with 20 frozen section samples taken from different levels of all the tumours. These were stained for each of the three cell types using the Zenon direct immunofluorescence staining method conjugated with human cell type specific antibodies. Results of the direct immunofluorescence labelling are shown in Figure 6.17.

Figure 6.17. Direct immunofluorescence staining of 3-compartment heterologous xenografts containing MCF-7, HMF 3wt and HMME 7wt cell lines at 2, 4 and 6 weeks post inoculation. At 2 weeks xenografts stained positive for (a) human specific pan-cytokeratin (b) and vimentin. At 4 weeks a similar pattern was seen (c & d) as 2 weeks. However at 6 weeks xenografts stained positive for pan-cytokeratin but no vimentin staining was observed. Staining for human specific CD31 and CD34 was negative at all time points; images are therefore not shown.

(See next page for figure)

A)



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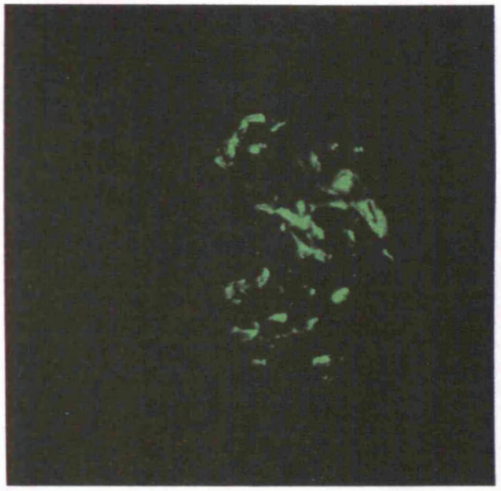
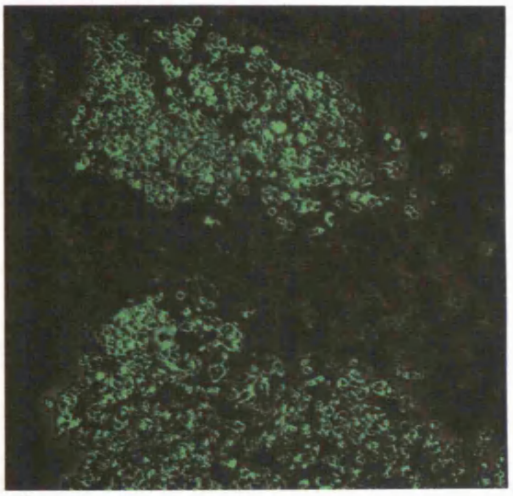
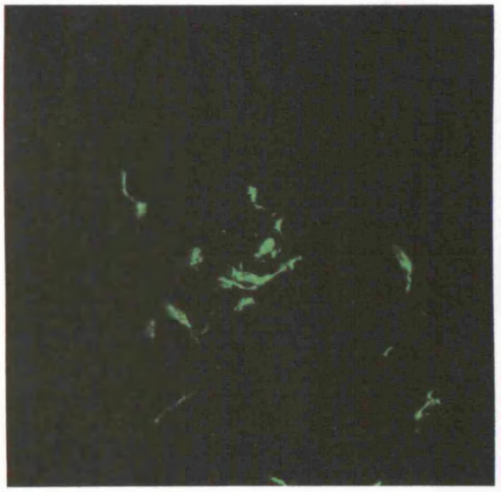


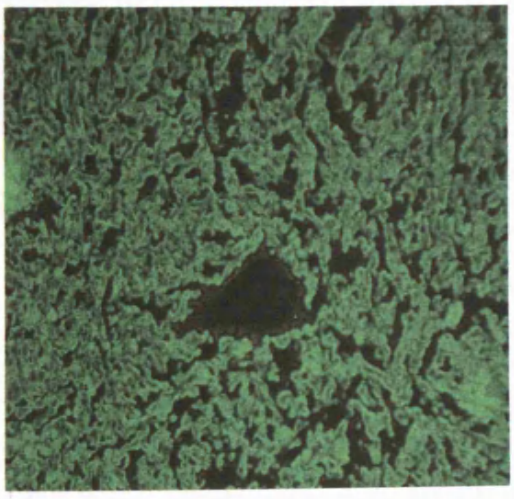
C)



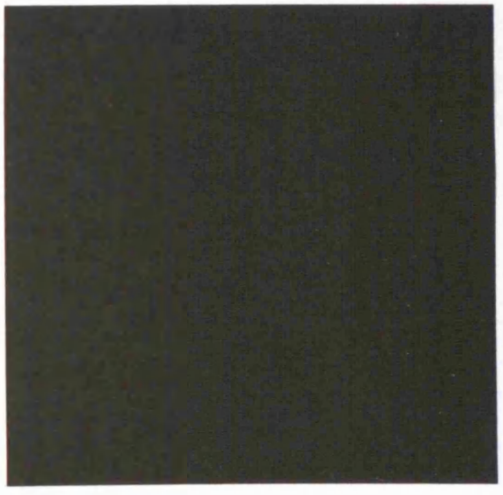
D)



E)



F)



Immunofluorescence staining for the three different cell types at 2 weeks consistently revealed tumours comprising of clusters of proliferating MCF-7 breast tumour cells with a sparse scattering of the immortalized human mammary fibroblasts as visualized by vimentin staining (Figure 6.17). A similar density and pattern of vimentin staining was seen in all the 2 week tumour sections tested (20 sections/tumour). There was no obvious intermingling of tumour and fibroblasts (i.e. the clusters of solid tumour did not contain human fibroblasts which were confined to tumour free areas), as demonstrated by staining on consecutive sections. Furthermore there did not seem to be an obvious or intimate relationship with the distribution of the MCF-7 cell clusters (i.e. fibroblasts did not cluster around the tumour cells). Additional staining for vimentin using a non-species specific antibody produced a near identical staining pattern as the human specific vimentin marker with few if any additional cells reacting indicating very little evidence of host (mouse) fibroblast infiltration (i.e. it appeared to detect only human cells) (Figure 6.18). Staining was negative for human endothelial cells using the anti-CD31/34 antibodies on all sections tested demonstrating that endothelial cells had not survived even to two weeks in detectable numbers.

A) B)

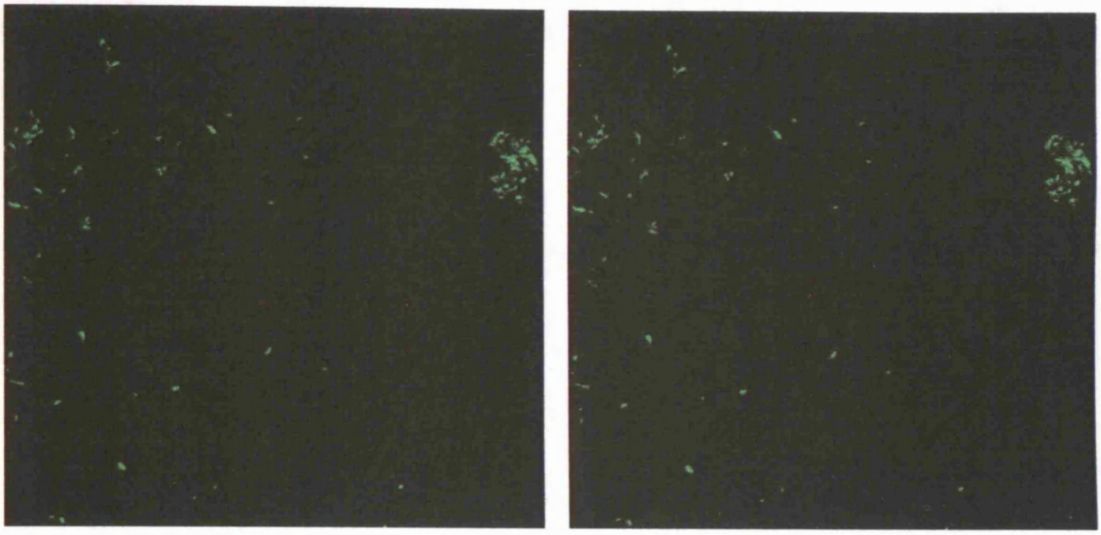


Figure 6.18. Direct immunofluorescence labelling of the 3-compartment heterologous cell xenografts (containing MCF-7, HMF 3wt and HMME 7wt cell lines) at 2 weeks with (a) human specific anti-vimentin and (b) non species specific vimentin on another tissue section. Staining patterns is identical demonstrating that there is vitually no mouse fibroblast presence.

At the 4 week time point, the tumours had a higher density of MCF-7 cells with much less intermingling connective tissue visible (Figure 6.17d). Vimentin staining was also much less obvious with much sparser isolated areas of positive staining. Again staining for human endothelial cells with anti-CD31 and anti-CD34 markers was negative. Finally at 6 weeks (Figure 6.17a), tumours had a similar appearance and structure as the xenografts left to grow for 8 weeks. The tumours were predominantly composed of tightly packed MCF-7 cells with murine vessels and extracellular matrix (as previously demonstrated).

To summarize, human fibroblast lines were clearly more robust than the corresponding endothelial cell lines, with signs of survival up to 4 weeks albeit in

relatively small numbers compared with the human tumour cells, unlike endothelial cells which showed no evidence of survival even at 2 weeks despite their effect on tumour take rates.

6.8 Primary human breast tumour cell xenografts

Successful growth of primary human breast cancer cells in immunodeficient mice is reported to be very low (Mehta et al. 1993), quoted to be in the region of 7-20%. The effect of the addition of stromal cell support was investigated because results from the heterologous cell xenograft experiments, presented earlier in this chapter, clearly demonstrate that these cells did support and promote establishment as xenografts of established cell lines such as MCF-7. To test whether that these stromal cells could also support the establishment of freshly isolated primary human breast cancer cells in immunodeficient mice the following xenografts were carried out.

6.8.1 Heterologous cell xenografts using primary human breast tumour cells

Metastatic breast tumour cells (PE1050) isolated from a large fresh pleural effusion of a patient with an aggressive invasive ductal breast carcinoma had been previously harvested and frozen for storage. Five attempts at culturing the metastatic tumour cells from this patient *in vitro*, from 5 consecutive pleural harvests, had been carried out without success. Cells cultured in the standard (DMEM/Ham's F12 & 10% v/v FCS) medium remained viable in culture for periods of up to 2-3 months but with little evidence of proliferation (MJ O'Hare, personal communication). The take rates *in vivo* and *in vitro* of such freshly

isolated primary breast tumour cells are well recognised as being very low. However with the support provided by relevant human mammary stromal cells; fibroblasts and endothelial cells, it was hoped that the primary cells would survive and form tumours *in vivo* as xenografts, since despite their failure to survive in the longterm the immortalized stromal cell lines had a very significant effect on the establishment of MCF-7 tumour cell line.

PE1050 cells were thawed quickly by warming in a water bath for 1-2 hours and then incubated in L15 medium with 5% (v/v) FCS prior to xenografting. The cells were then spun in a centrifuge to obtain a pellet and resuspended in 1:1 SFM and matrigel solution for grafting into the mammary fat pad. The PE1050 cells (5×10^6 cells) were xenografted alone, with HMF 3wt cells, with HMME 7wt cells and with a combination of HMF 3wt and HMME 7wt cells in each case at a ratio of 10 tumour cells to 1 stromal cell. Six mammary fat pads were inoculated per group and harvested at 8 weeks. A total in excess of 1.2×10^8 of the primary human tumour cells were inoculated into mice.

Xenografts of the primary human tumour cells PE1050/stromal cell combinations gave rise to one tumour out of a possible 24 sites (Table 6.15).

Cells and cell lines	**Xenograft Take Rate (6 sites per group) MFP +Matrigel**
PE1050 Alone	**0/6**
PE1050+ HMF 3wt	**1/6 (16.7%)**
PE1050 + HMME 7wt	**0/6**
PE1050+ HMF 3wt + HMME 7wt	**0/6**

Table 6.15. Primary breast tumour cell xenografts.

The tumour was first noticed at 35 days post inoculation. Following the long latency period the tumour proliferated rapidly to exceed the permitted 8mm in diameter over a period of one week and so was harvested. The single tumour was simultaneously processed for immunohistochemistry, *in vitro* cell culture and serial re-engrafting. The contra-lateral negative mammary fat pad together with all the other inoculation sites were left to complete the full 8 week experiment period. Samples of all the negative mammary fat pads were dissected and processed for immunohistochemistry.

6.8.2 *In vitro* culture and serial xenografts of the primary tumour

The single tumour produced was carefully dissected, washed in PBS and processed for serial xenografting *in vivo* and for *in vitro* cell culture. Post mortem analysis of the tumour bearing mice after 8 weeks showed no evidence of local or distant metastases. Gross inspection of the brain, liver, lungs and peritoneal cavity was unremarkable.

2 × 2 mm pieces of the original tumour produced were re-grafted back into the mammary fat pads of nude mice. This resulted in a take rate of 75% (3/4 sites) is shown in Table 6.16. Intact tumour pieces were serially re-passaged by surgical xenotransplantation a further two times with 100% take rates (Table 6.16).

Xenograft (Passage number)	Xenograft Take Rate (4 sites per group) MFP
Tumour pieces (p1)	3/4
Tumour pieces (p2)	4/4
Tumour pieces (p3)	4/4

Table 6.16. Table summarising the serial re-xenotransplantation of the original xenograft produced by the combined injection of primary human breast cancer cells and human mammary fibroblasts.

Separation and culture of the tumourigenic cells.

A portion of the original tumour was rinsed in 30ml of HBSS, containing 100U/ml penicillin and 100μg/ml streptomycin. The tumour was minced into small pieces (1mm^2 squares) in a 60mm tissue culture dish using sterilized razor blades, and then enzymatically digested in 20ml of 1ml/mg collagenase A (Sigma) in L15 medium and 1mg/ml of bovine trypsin at 37°C for 45 minutes with occasional agitation.

Following digestion, DMEM with 10% (v/v) FCS was added and centrifuged for 10 minutes at approximately 300×g (1500 rpm) to form a pellet. The pellet was then resuspended in 5ml of DMEM with 10% (v/v) FCS and then the cellular digest was filtered sequentially through sterile 40μm and 31μm nylon meshes to remove clumps of cells, centrifuged at 400 ×*g* for 10 minutes to pellet cells, and washed twice in DMEM medium with 10% (v/v) FCS. The cell pellet was then resuspended in 3ml of DMEM with 10% (v/v) FCS.

The resulting cells were cultured in medium consisting of DMEM, 2mM glutamine, 10% (v/v) heat-inactivated FCS, 50units/ml penicillin, and 50μg/ml streptomycin at either 37°C or 33.5°C in a humidified incubator in an atmosphere

of 5% (v/v) CO_2 + 95% air. They attached to the tissue culture flasks quickly and grew rapidly in culture (Figure 6.19).

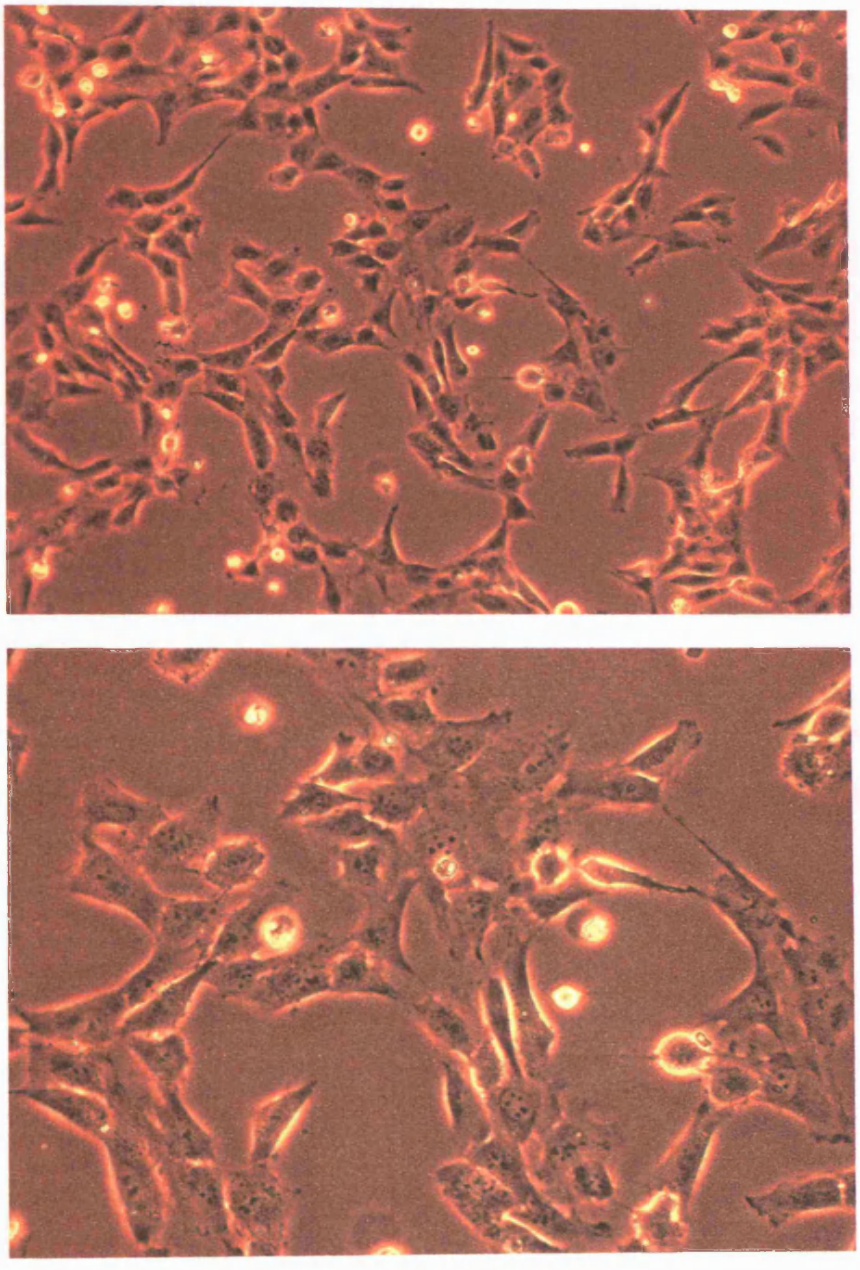


Figure 6.19. *In vitro* culture of cells isolated from the primary heterologous tumour. At ×80 and ×160 magnifications.

Cell numbers were then bulked up and re-xenografted, as a cell suspension, back into the mammary fat pads of nude mice to assess its ability to form tumours (Table 6.17). 5×10^6 cells were inoculated into each mammary fat pad in serum free medium. Results showed that, at 3 different passage numbers, these cells produced a take rate of 100% (Table 6.17).

Xenograft (Passage number)	**Xenograft Take Rate (4 sites per group) MFP**
Cultured cells (p1)	4/4
Cultured cells (p3)	4/4
Cultured cells (p5)	4/4

Table 6.17. Table summarising the serial re-grafting of the original xenograft produced by the combined injection of primary human breast cancer cells and human mammary fibroblasts as a cell suspension.

This experiment had therefore resulted in a single xenograft tumour that subsequently had very high take rates and growth rates when re-grafted directly or after culture. This behaviour was so different to that of the original primary human breast tumour cell PE1050 cells that further investigation to determine the identity of the tumourigenic cells was carried out including histology and immunohistochemical examination.

6.8.3 Histological and immunochemical analysis of xenograft tumour

Tumour analysis with H&E

H&E staining of the single tumour produced was carried out and results are shown in Figure 6.20.

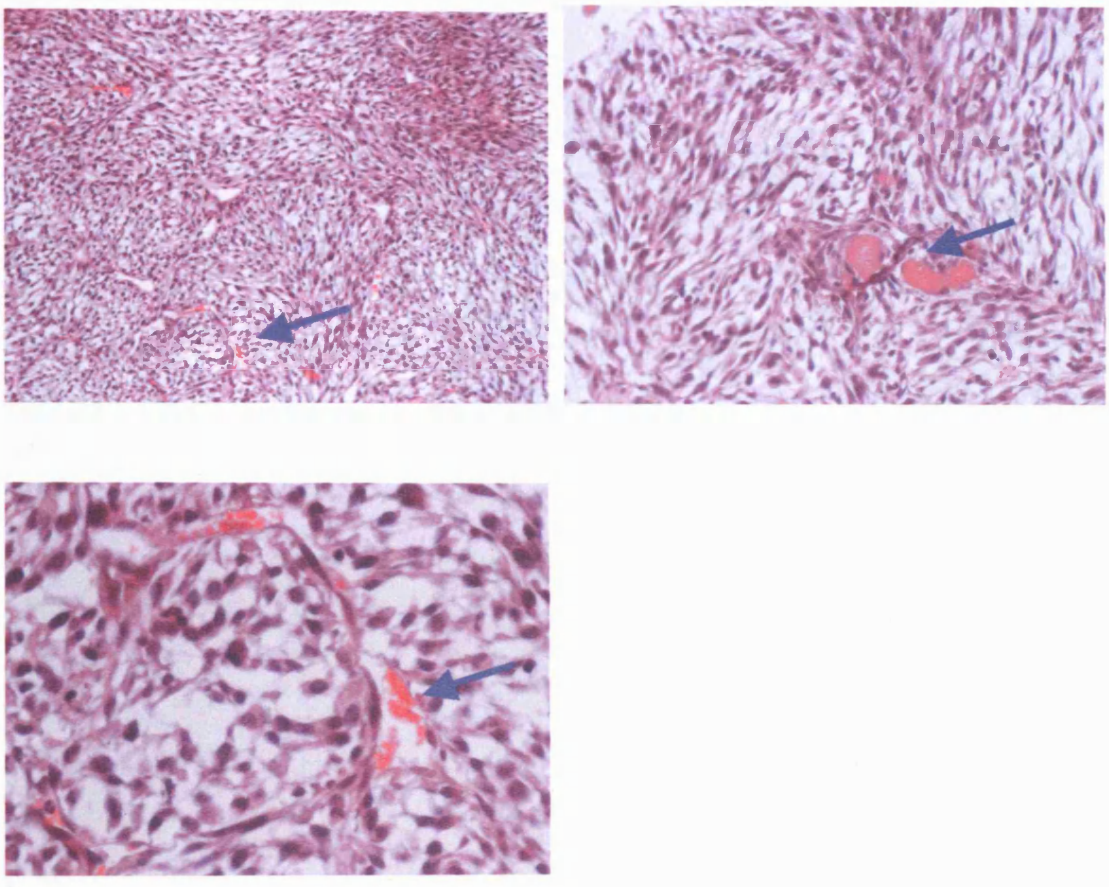


Figure 6.20. H&E sections of the original xenograft tumour formed by the combined injection of primary human breast cancer cells and immortalized fibroblasts. Blood vessels with blood are clearly visible (arrows) at ×80, ×160 and ×320 magnifications.

Staining showed long elongated structures more typical of sarcomas than epithelial tumour cells. There were no signs of lumina, cords or tubular structures

as seen with the MCF-7 xenografts (Figure 6.21). Vasculature including red blood cells was visible in the tumour sections, but less than in the MCF-7 tumours. There were no signs of local invasion.

Ultra-thin section

Ultra-thin sections of both the original PE1050 cells and the resulting tumour were compared in Figure 6.21.

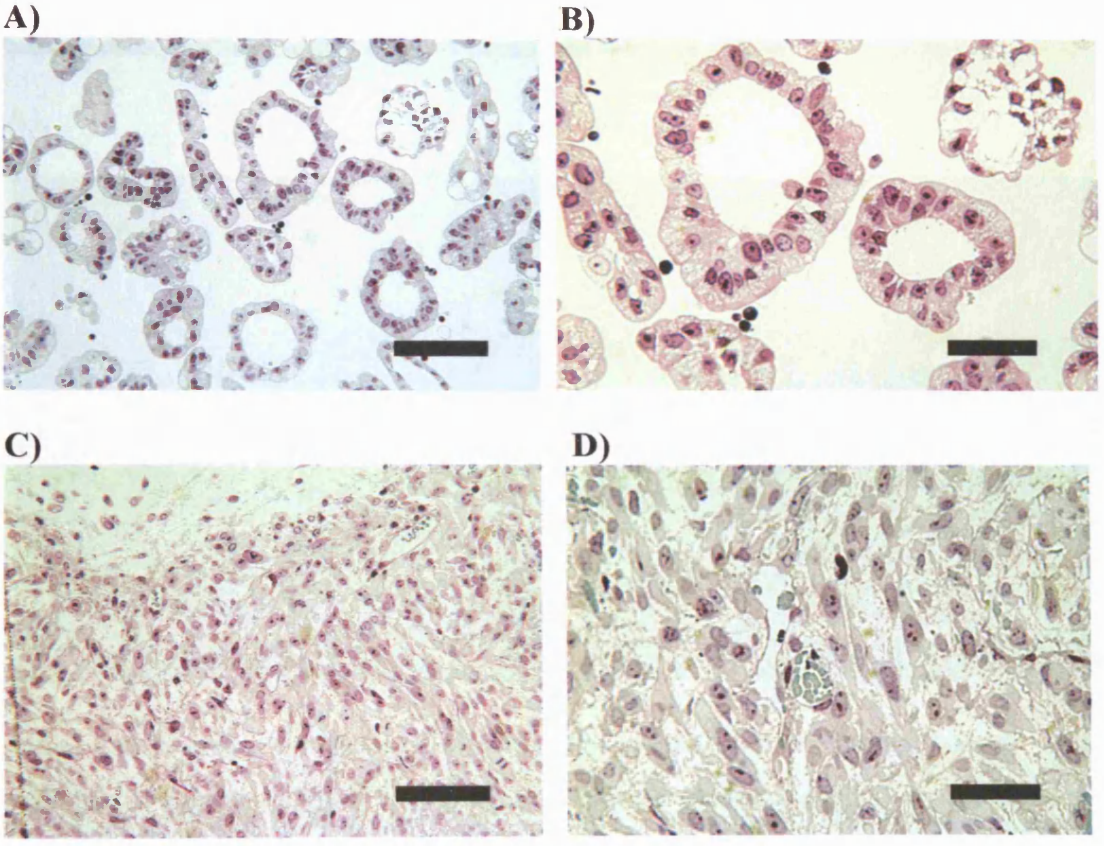


Figure 6.21. Ultra-thin sections of the (a & b) original primary human cancer cell PE1050 and (c & d) its resulting xenograft tumour. Note the hollow cluster or organoid morphology of the original PE1050 cells compared to the 'undifferentiated' appearance of the xenograft tumour stained with toluidine blue. Bar: a) 20μm, b) 5μm, c) 80μm and d) 40μm.

Immunofluorescence

Immunohistochemical analysis, using direct immunofluorescence, of all the sections of the tumour produced, demonstrated positive staining for human specific vimentin (Figure 6.22). Staining was negative for human pan-cytokeratin MNF116 and a commonly used epithelial membrane antigen (BrEP4). These results suggest that the tumour produced was in fact a sarcoma derived from fibroblasts HMF 3wt originally inoculated with the PE1050 primary human tumour cells. Further studies *in vitro* confirmed this conclusion.

A) **B)**

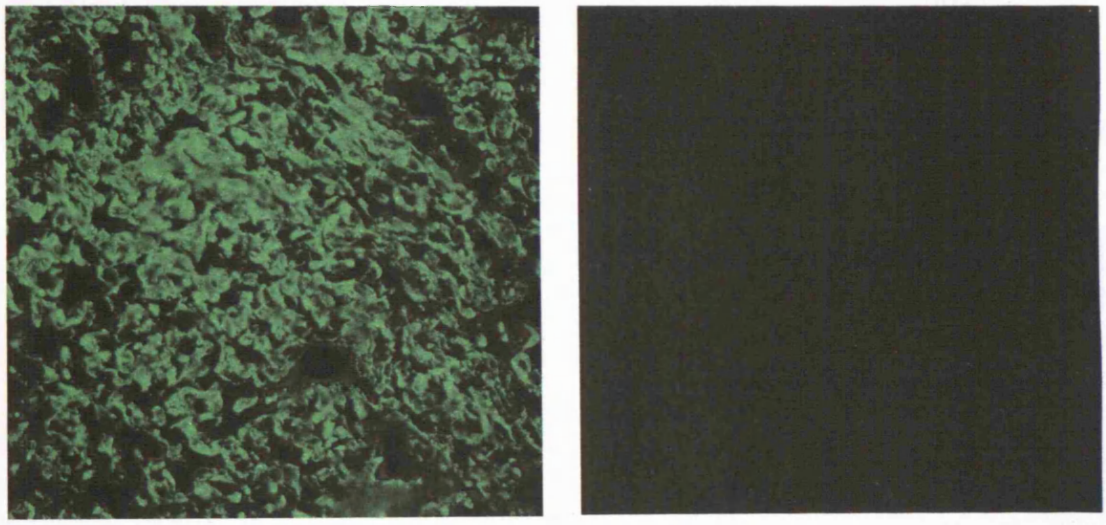


Figure 6.22. Immunohistochemical staining of the original primary human tumour xenograft (PE1050/HMF 3wt) for (a) human specific vimentin and (b) a human specific pan-cytokeratin. All sections were negative for the human pan-cytokeratin and epithelial membrane antigen (BrEP4). Magnification ×320.

Analysis of live cells on coverslips using immunofluorescence.

Cells isolated from the original tumour for *in vitro* culture were plated on coverslips in 24 well plates for direct immunofluorescence (Figure 6.23).

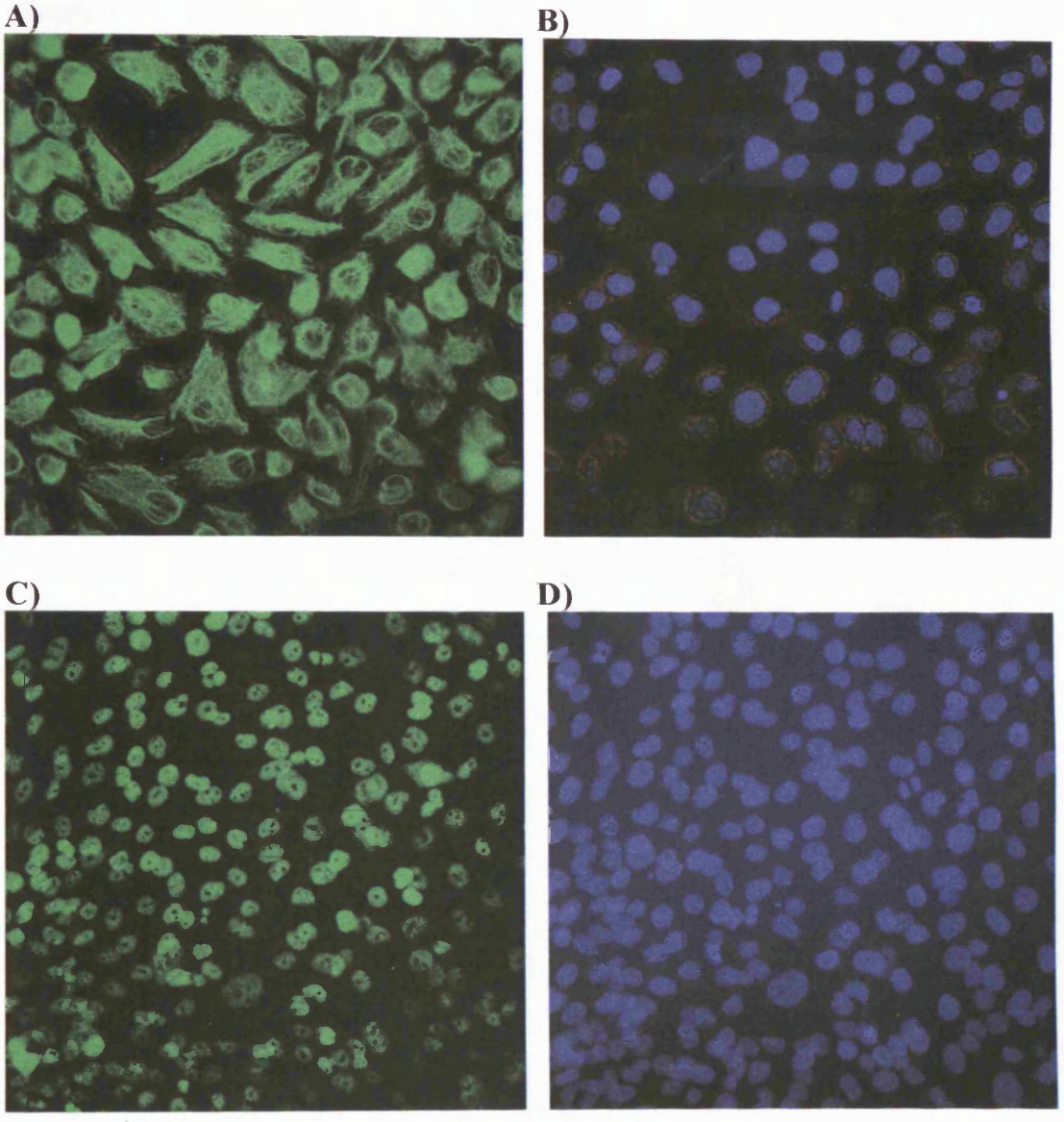


Figure 6.23. Immunofluorescence labelling of cultured cells isolated from the primary xenograft tumour. Staining for (a) human specific vimentin and (b) with Hoechst 33258 stain are shown as well as (c) SV40 large T staining of the same cells with its corresponding (d) Hoechst 33258 staining. All cells were stained on coverslip. Note the lack of punctuate staining with Hoechst 33258 (b and d) indicating their non-mouse origin.

In correlation with results produced from staining of the original tumour, the cells stained positive for human specific vimentin and SV40 large T antibody (Figure 6.24). Positive labelling for the large T antigen confirmed that the fibroblasts forming the tumour must be a transformed variant of the immortalized human mammary fibroblast cell line, HMF 3wt that was originally grafted with the primary human tumour cells.

Human origin was further confirmed by the homogenous staining pattern observed with Hoechst 33528. Again the cells stained negative for human pan-cytokeratin MNF116 and the epithelial membrane antigen (BrEP4). These results confirmed that the tumour was a sarcoma derived from a fully transformed variant of the immortalized human mammary fibroblast cell line HMF 3wt.

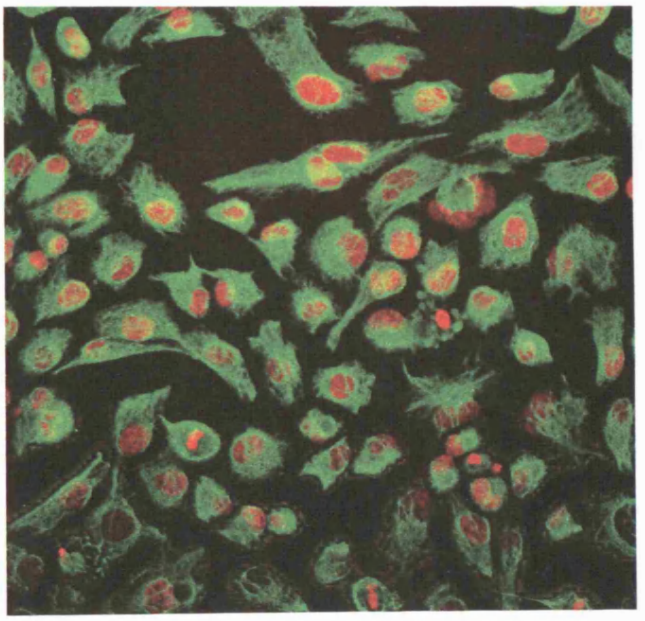


Figure 6.24. Cells isolated from the primary tumour stained for a human species specific vimentin and SV40 ×large T antigen. Double immunofluorescence staining with vimentin (green) and SV40 LT antigen (red). Magnification ×320.

Results from all the previous xenograft experiments have clearly shown that the immortalized fibroblast cell line HMF 3wt was not tumourigenic in nude mice when inoculated on its own, combined with endothelial cells or in 3-D heterologous spheroids. Also the HMF 3wt cell line failed to survive in significant numbers when xenografted with a well established human mammary tumour cell line MCF-7. However, the results in this section demonstrate that the HMF 3wt cell line, in this case, has been transformed into a tumourigenic line (HMF 3wtX), a very rare event (>1 in 3×10^8). The capacity of these cells to form xenografts persisted for up to five passages in culture, indicating that something had fundamentally changed resulting in a stable tumourigenic phenotype.

6.9 Discussion and conclusions

To recapitulate, the aim of this chapter was three-fold. Firstly, to investigate whether the immortalized stromal cells would produce tumours when xenografted into immunodeficient mice, suggesting that they had been transformed into a completely tumourigenic phenotype, as a result of LT antigen expression. Secondly, if the stromal cells did not form tumour as such, to determine their parameters of survival and proliferation *in vivo*. The third objective was to examine their ability to support and function as stroma to co-engrafted human tumour cells when xenografted orthotopically. Success in the last objective would allow us to develop a more representative model system incorporating more human components for vital pre-clinical studies.

The transformation of a cell line has frequently been assessed by its ability to induce tumours when transplanted *in vivo* into immunodeficient mice, such as nudes and SCIDs. Production of tumours would signify complete

transformation into a fully tumourigenic phenotype. However, it is important to recognise that, in this case, if the stromal cell lines had indeed been transformed to become neoplastic by the immortalization process, the tumours produced would be sarcomas or angiomas. Thus, they would not be representative of the stromal cells found in association with human mammary epithelial carcinomas. The ideal situation would be the proliferation and differentiation of these non-tumourigenic stromal cell lines *in vivo* when supported by the appropriate stimuli i.e. mammary epithelial cell tumours.

6.9.1 Xenografts of immortalized human mammary stromal cell lines

Results showed that none of the stromal cell lines, singly or in combination, formed tumours (with a single exception) or survived in the longer term (≥6 weeks) when xenografted into the mammary fat pads of nude mice. Furthermore immunohistochemical analysis with cell-type specific antibodies showed no evidence of survival at 8 weeks. A total in excess of 2.4×10^8 cells of each stromal cell line was inoculated *in vivo*. Cell number inoculations of this quantity should have been sufficient to give rise to many tumours if the cells were intrinsically tumourigenic.

To enhance the chances of survival and establishment the stromal cell lines were inoculated into the mammary fat pads with the aid of an extracellular support matrix (Matrigel). The two main reasons behind orthotopic inoculations were to keep a) the tumours in a physiological an environment as possible and b) overcome the problem of inadequate or inappropriate stromal microenvironment in subcutaneous sites for tumour formation. It was also hoped that extracellular matrix would support the establishment of the cell lines within the mice, and so

increase the likelihood of tumour formation when they were grafted with tumour cells. Previous studies have reported that altering the tumour microenvironment with Matrigel, a complex mixture of extracellular matrix proteins derived from the Englebreth-Holm-Swarm sarcoma (Kleinman et al. 1986), or stromal fibroblasts (normal and tumour associated) enhances the efficiency of tumour formation and decreases the tumour latency in other human breast cancer cell lines (Noel et al. 1993). In a final attempt to aid establishment the two stromal cell lines were combined in Matrigel in order to provide cell-cell and cell-ECM interaction.

However, even with these various attempts of enhancing stromal cell survival the stromal cells failed to survive. All pertinent factors were precisely defined to avoid technical reasons for failure. For example, the number of cells/inoculation, total quantity of cells inoculated, use of adjuncts such as Matrigel, experimental period and method of inoculations were consistent with similar studies by others (Hahn et al. 1999, Elenbass et al. 2001).

There are several possible reasons why the stromal cells did not survive when engrafted into nude mice. Firstly, the immortalized stromal cell lines may simply not possess the necessary phenotype. Characterization of the immortalized stromal cell lines (Chapter 3) demonstrated with the exception of the *ras* variant (see Chapter 3.6) that these cells were only partially transformed according to the *in vitro* criteria. Therefore their inability to form overt tumours *in vivo* is to be expected or even predicted, but that does not explain their failure to survive.

There may be a more general explanation for the failure to survive and establish, particularly in the temperature sensitive stromal cell lines. It is possible that the body temperature of the nude mice approached non-permissive

temperatures (negative effects on growth *in vitro* become apparent above 38.5°C, disrupting temperature sensitive SV40 large T antigen function). Although the body temperature of a mouse is generally around 38°C this fluctuates greatly with the animals surroundings (Sage 1981). A temperature of 38°C would effectively prevent the conditionally immortalized temperature sensitive lines from proliferating and slow down the wild type LT lines' growth rate. At this point the cells could become more susceptible to NK cell activity or self induced apoptosis. However, as the wild type LT sensitive stromal cell lines (non-temperature) also did not survive this does not seem to provide a robust explanation.

Another reason may be that even orthotopic inoculations (mammary fat pad) are too hostile an environment for exogenous stromal cell growth with inadequate support in the form of cell-cell interaction (tumour-stromal cell interaction) and or growth factors. The majority of the mouse gland is composed of adipocytes with fibroblasts prominent only in narrow sheaths around the ducts. In addition the mouse stroma itself provides a foreign microenvironment for the xenografted human stromal cells to survive. In the human gland, the ducts are embedded in large tracts of connective tissue (composed predominantly of fibroblasts) that is adjacent to deeper layers of adipose tissue (Hovey et al. 1999). Epithelial cells are never found in the adipose tracts. Human mammary stromal cells may therefore require and in turn thrive in the specialized stromal microenvironment of the human gland rather than the largely adipose tissue microenvironment of the mouse mammary gland. In order to enhance human tumour cell xenograft take rates a required number and/or presence of associated stromal cells is needed to be present in microenvironment. Lack of sufficient stromal cell presence/component can lead to low take rates and long latency

periods due to the time taken to recruit the necessary host stromal cells to commence proliferation (Shekhar et al. 2001). In addition to the inhospitality of the mammary fat pad a factor further compounding failure of cell survival could be dispersion of cells at the graft site providing inadequate cell–cell interaction despite the presence of Matrigel ECM support.

A residual innate immune response provided by functional murine natural killer cells (NK) and B lymphocytes, which is known to be present in nude mice, may also be a cause of death in the xenografted human stromal cells (Lin et al. 1997). The recessive *nu* gene, which is responsible for the lack of a thymus, when homozygous in mice, produces the nude mouse (*nu/nu*). Because it lacks a thymus, nude mice cannot generate mature T lymphocytes essential to the immune system. Therefore they are unable to mount most types of immune responses, including: antibody formation that requires $CD4^+$ helper T cells, cell-mediated immune responses, which require $CD4^+$ and/or $CD8^+$ T cells: delayed-type hypersensitivity responses (require $CD4^+$ T cells) killing of virus-infected or malignant cells (requires $CD8^+$ cytotoxic T cells) graft rejection (requires both $CD4^+$ and $CD8^+$ T cells). The absence of functioning T cells prevents nude mice from rejecting not only allografts but also xenografts which are grafts of tissue from another species. However NK cell activities in nude mice are not suppressed which may be a possible explanation for the destruction of the xenografted stromal cells. In fact, nude mice have near normal numbers of B lymphocytes and normal to elevated numbers of NK cells (Dandekar and Perlman 2002, Benveniste et al. 1990, Kennedy et al. 1992).

NK cells are a type of lymphocyte representing a small fraction (~2%) of the lymphocytes circulating in the blood. They are neither T cells nor B cells and so form a component of the non-specific immune defence. They are called NK

cells because they are already specialized to kill certain types of target cells, especially host cells that have become infected with virus and host cells that have become cancerous (Okumura et al. 1982, Trinchieri 1989, Biron 1997, Scott and Trinchieri 1995). Therefore they are able to respond rapidly, thus providing another arm of innate immunity. The immortalized stromal cell lines used in the present study were generated in such a way that they express both bacterial and viral proteins due to their puromycin resistance gene derived from *E. coli* and expression of SV 40 LT antigen. These factors may enhance their susceptibility to NK cells. In addition to killing target cells, NK cells secrete cytokines such as the anti-viral cytokine IFN-γ and the inflammatory cytokine TNF-α. The host response to a xenograft is characterized by a large number of infiltrates by monocytes and NK cells suggesting an important role of the innate immunity in the process of its rejection (Rossini et al. 1999). This response is reported to occur within hours and be complete by 12-24 hours. For example in a model system by Kim et al. (2002), the abrogation of the ability of nude mice to reject xenografted metastatic carcinoma cell lines, by injecting anti-mouse NK cell antibodies, underscores the role of these cells in tumour rejection.

An argument against the NK cell hypothesis is that the co-xenografted MCF-7 tumour cells are not destroyed. However, this applies only to supra-threshold inoculations ($>5\times10^6$) because at sub-threshold inoculations tumours generally do not form. It is conceivable that at lower cell numbers (sub-threshold inoculation) the cells are largely killed shortly after inoculation by NK cells. It is perhaps the remaining tumour cells that have survived NK cells activity that give rise to tumours. Opposing this argument is the fact the stromal cell lines were also inoculated in numbers equivalent to supra-threshold MCF-7 cells ($>5\times10^6$) but even this did not result in their long term survival. Furthermore, the results

from the primary human cell tumour xenograft experiments have demonstrated that the co-engrafted HMF 3wt cell line survived long enough to generate the tumourigenic variant (HMF 3wtX) and form tumours in several consecutive xenografts at high frequency. The fact that the HMF 3wtX cells can survive and give rise to tumours following xenografting indicates that these immortalized stromal cell lines may be able in rare instances, to overcome this residual innate immune response and act in the same manner as tumourigenic breast cell lines. However, as mentioned in Chapter 6.8.3, this is a very rare event occurring in less than 1 in 3×10^8 cells inoculated.

The issue of immunodeficiency and a residual immune response could be further investigated experimentally. Firstly, the level of NK cell activity could be determined, by measuring their numbers in nude mice shortly after xenograft inoculations to establish whether there is increased activity at this time. The numbers of NK cells present could be determined by using an antibody specific for mouse NK cells (Sentman et al. 1989, Newman et al. 1983). Comparison of levels between xenografted and non-xenografted mice would provide an indication of increased NK activity. In a different approach, irradiation of nude mice can suppress NK cell activity and could be a possible simple means of testing whether NK cell activity is at the root of the failure of the immortalized stromal cells to survive (Feuer et al. 1995). Alternatively, an animal species with an overall greater degree of immunodeficiency, including lower NK cell activity, could be used. Possible alternative mouse strains are the severe combined immunodeficient (SCID) and non-obese diabetic (NOD)/SCID mice both of which are more immunodeficient than the nude.

Tumours from other species (rat, cow, human) are more easily transplanted into SCID mice and will grow without being rejected (Greiner et al.

1998). For certain specific tumours (e.g. thyroid), SCID mice showed improved transplantability (take rates) over nude mice (Kawai et al. 1998). SCID mice have greater levels of immunosuppression compared to nude mice as they lack both B and T cell immunity due to a lack of the catalytic subunit of a DNA-dependent protein kinase (Bosma et al. 1983).

The NOD/SCID mouse, generated by crossing the SCID mutation onto the NOD background, has multiple defects in innate immunity in addition to the lack of B and T cell immunity (Shultz et al. 1995). As these mice have defects in NK cell activity (Kataoka et al. 1983), as well as defective macrophage (Serreze et al. 1993) and complement function (Baxter and Cooke 1993) on a severe T- and B-lymphoid–immunodeficient background they may represent an even better animal carrier than SCIDs. They have recently been shown to be more successful for xenografting 'difficult' cells such as progenitor and stem cell transplantations (Rice et al. 2000, van der Loo et al. 1998, van Hennik et al. 1999). This is probably because the levels of NK cells and macrophages in NOD/SCIDs were lower than in SCID mice (Rajan et al. 1994, Greiner et al. 1998).

Another possibility is the beige (*bg*) mouse which has a recessive mutation of chromosome 13 resulting in impaired NK cell activity, specifically severe impairment of NK cell cytolysis with decreased chemotaxis of cytotoxic T-cells, macrophages, and polymorphonuclear leukocytes macrophages (Talmadge et al. 1980). Therefore crossing SCID with beige mice (Mosier et al. 1993) may provide a model satisfying the criteria of a reduced innate immune response on a background of severe B and T cell immunodeficiency.

Nude mice were used as the initial model, in the present study, as they have been shown repeatedly to successfully xenograft human breast cancers in particular the MCF-7 cell line. Also some studies have shown that SCIDs and

beige mice are not universally superior to nudes in that breast cancer do not grow and disseminate more readily compared to nude mice (Welch 1997). In fact some solid tumours, such as breast cancers (cell lines), metastasize more readily in nude mice for reasons not known (Welch 1997).

6.9.2 Xenografts of *ras* transfected immortalized fibroblasts

The purpose of this experiment was to investigate the potential tumourigenicity of the immortalized fibroblasts following expression of the activated *ras* oncogene. The wild type LT variant of the immortalized fibroblast cell line HMF 3wt, already expressing LT and hTERT, was further transformed by H-*ras* V12 to produce the cell line HMF 3wt *ras*. This cell line was described in Chapter 3 to have a 'fully' transformed phenotype *in vitro* as shown by its anchorage independent growth in soft agar and loss of contact inhibition of movement and growth (MJ O'Hare, personal communication).

Results showed that out of a possible 8×SC and 16×MFP injections (> 2 $\times 10^8$ total cells), tumours failed to develop in any of the mice (Table 6.2). In addition, there was again no evidence of survival, on immunohistochemical analysis, at 8 weeks. This suggests that the further expression of the *ras* oncogene in this system was insufficient to produce a fully tumourigenic line *in vivo* and that the acquisition of neoplastic growth requires the progressive loss cell death signals or gain of other functions, including the loss of further senescence and suppressor functions.

The activated *ras* gene (H-*ras*V12), which is mutated in many human cancers although rarely mutated in breast cancers (~5% of cases) (Clark and Der 1995), is an oncogene which has been used successfully to transform certain

immortalized human cells (fibroblasts and hepatocytes) (Hahn et al. 1999, Elenbaas et al. 2001). The most convincing evidence comes from Hahn et al. (1999) who have shown that the ectopic expression of the telomerase catalytic subunit (hTERT) in combination with two oncogenes (the SV40 LT oncoprotein and an oncogenic allele of H-*ras*) resulted in the direct tumourigenic conversion of normal human fibroblast and hepatocytes. The ability of these different cells to form colonies in soft agar (a simple, *in vitro* test for whether cells need to be anchored to a solid surface for growth-normal fibroblasts and epithelial cells do, where as transformed cells do not) as well as form tumours in nude mice clearly showed that only the combination of all three genetic elements was sufficient to cause efficient tumour growth. They concluded that the mutations causing telomerase to become active can cooperate with other mutations in oncogenes to induce full transformation in nude mice.

When the results of the present study were compared with those of Hahn et al. (1999) and other similar studies of Elenbaas et al. (2001) and Morales et al. (1999) it was noted that the numbers of cells inoculated (10^6), site of inoculation (SC) and host animal used (nude mice) were consistent throughout the studies. Therefore other reasons must exist to explain the discrepancy.

Hahn et al. (1999) showed that the insertion of the two oncogenes and hTERT, in the order SV40 LT followed by hTERT and finally the *ras* V12 into normal human diploid BJ foreskin fibroblasts, and embryonic hepatocytes, resulted in fully tumourigenic cell lines both *in vitro* and *in vivo*. Elenbaas et al. (2001) produced similar results to Hahn et al. (1999) when they inserted the same two oncogenes and hTERT in the same order into normal mammary fibroblasts. In contrast to these two studies, the HMF 3wt *ras* cell line, in the present study, was generated by inserting hTERT first followed by the two oncogenes LT and

then *ras*. This suggests that the order of insertion of the LT oncogene with respect to hTERT may be important. Indeed, it has been reported that immortalized human mammary endothelial and fibroblast cell lines that received hTERT first followed by LT were more chromosomally stable with less structural aberration events than the reverse order of gene transduction (Fauth et al. 2004). Also, in a not too dissimilar study, Morales et al. (1999), reported that they could not transform human fibroblasts using hTERT and *ras* combined with the human papillomavirus-16 E6/E7 viral oncoproteins (which also inactivates the retinoblastoma protein and p53). Again hTERT was inserted before the E6/E7 viral oncoprotein. Therefore, with the well known fact that the inactivation of p53/retinoblastoma (Rb) functions increases genomic instability (leading to mutations), whereas switching on hTERT does not, this study further underlines the fact that the large T protein and or the inactivation of p53/Rb may perturb additional cellular targets making the order in which the mutations occur important.

To test the hypothesis that the order of LT and hTERT insertion, in the generation of cell lines, affects their tumourigenic properties the wild type HMF 3B fibroblast line which was generated by the insertion of LT followed by hTERT could be transduced with the *ras* oncogene and then its tumourigenicity determined. If this line is tumourigenic and gives rise to tumours in immunodeficient mice the issue of order of oncogene expression could be clarified.

Another possible reason for the discrepancy in the present study may be that the expression of the potent oncogene H-*ras* V12 was insufficient to induce a fully malignant phenotype in the HMF 3wt *ras* cell line. In the study by Elenbaas et al. (2001) normal foreskin fibroblasts and human kidney hepatocytes

had 10-fold and 60-fold over-expressions of the H-*ras*V12 oncogenes respectively compared with endogenously expressed wild-type H-Ras protein. The study also showed that expression of H-*ras*V12 above a critical threshold level (approximately >12.5 fold over-expression) is required for tumourigenic transformation of cells in the presence of the co-expressed LT and hTERT genes. It is clear that the level of *ras* expression is a critical determinant of tumourigenicity (Hahn et al. 1999). However the level of mutant *ras* in the HMF 3wt *ras* cell line was high (~10 fold) compared with endogenous *ras* levels when examined by immunoblotting (K Hardy, personnel communication). Thus the level of *ras* expression seems a less likely explanation of why the HMF 3wt *ras* cell line did not give rise to tumours, although it cannot be ruled out.

6.9.3 Xenografts of heterologous stromal and tumour spheroids

The purpose of the spheroid xenograft experiments was to enhance establishment of the immortalized stromal cells *in vivo*, by overcoming several obstacles encountered in the cell suspension xenografts as described in Chapter 6.9.1. It was postulated that a possible cause for the failure of stromal cells to survive *in vivo* could be either dispersion of cells at the graft site and/or the absence of other human cell types. The lack of support from cells normally present in the tumour or tissue environment (i.e. cells not in close proximity or contact) may be a crucial factor. The stromal cells may require the close contact support of epithelial cells as well as other stromal components to survive and proliferate. Therefore, the stromal lines were inoculated as pre-formed spheroids manufactured *in vitro* containing a combination of stromal-stromal and tumour-

stromal cells so as to ensure a high concentration of cells at the graft site in a form unlikely to disperse.

However, contrary to expectation, spheroid xenografts using two different breast tumour cell lines (MCF-7 and T47D), failed to produce tumours. In contrast, the suspensions consistently resulted in tumour formation of both cell lines (Table 6.6). The inability of the tumour spheroids to give rise to tumours could be due to the architectural nature of the spheroids which may not allow host vascularization and therefore the influx of host stromal cells. In an attempt to overcome this problem, heterologous tumour-stromal spheroids containing human stromal cells were manufactured and xenografted. However, these heterologous spheroids did not give rise to any tumours. These results were surprising as these spheroids when grown *in vitro* in extracellular matrix demonstrated good viability demonstrating proliferation by growth in size as well as promotion of endothelial cell differentiation (see Figure 5.5.2).

A possible reason why the heterologous tumour spheroids did not produce tumours may be in the fact that they were manufactured in the RCCS. One of the key advantages of the RCCS is the promotion of differentiation of epithelial cell types such as prostate (O'Connor 1999) and colon (Jessup et al. 1997) as well as other cell types cartilage (Vunjak-Novakovic et al. 1999, Unsworth and Lelkes 1998). This could lead to a relatively more differentiated phenotype causing the tumour lines to become less aggressive with regards to tumourigenicity with reduced or absent tumourigenic properties. The spheroids formed in the RCCS were shown to possess organised morphologies mimicking the structural arrangement of well differentiated *in vivo* tissue (Chapter 5).

In addition, it was unlikely that the required number of living tumourigenic cells (supra-threshold level of $5x10^6$cells/site) were present in the

spheroids to give rise to successful xenograft establishment. Even if the required number of cells had been physically present in the spheroids, due to the very nature of the spheroid architecture only the cells in the peripheries of the tumour may have been effective as they would be most amenable to host stromal and vascular support for survival. In other words the cells at the centre of the spheroids may be dormant, less viable or in the process of dying due to inadequate host vascularization.

6.9.4 Heterologous 3-compartment cell xenografts

Due to the failure of establishment of the heterologous tumour-stromal spheroids, an adequate model of tumour-stromal interaction could not be manufactured. Thus, heterologous cell suspension xenografts with strictly controlled cell number inoculations were performed. The main purpose of this experiment was to determine whether the immortalized human mammary stromal cell lines, as a cell suspension, could support tumour establishment and proliferation. By providing a more stromal cell-concentrated environment, which is a requirement of human tumours, it was hoped that human tumour cell proliferation could be further promoted and accelerated. The proliferating tumour cells may then reciprocate by providing the necessary support for stromal cell survival and growth.

The results showed that the addition of the immortalized stromal cells (both the wild type LT and temperature sensitive lines) to sub-threshold tumour cell inoculates (MCF-7 and MCF-7 V12 lines) provided a significant growth advantage resulting in a consistent take rate (Table 6.9). Not only did these combinations produce better take rates, but the fibroblast cell lines in

particular promoted shorter latency periods (~2 weeks) compared with the controls (i.e. supra-threshold tumour cell only inoculates), which took 3-4 weeks to become palpable. Surprisingly, there was no difference in effect between the wild type LT and temperature sensitive stromal cell lines in this respect suggesting that the mouse body temperature was not high enough to inhibit the cells. Results also showed that a ratio low as 1:10 stromal cell to tumour cells was sufficient to greatly enhance tumour cell establishment. The VEGF over-expressing MCF-7 V12 breast tumour cell produced noticeably more vascular tumours than the original MCF-7 tumour cell line presumably by stimulating growth and differentiation of host endothelial cells. However this stimulation seemed to only affect the mouse and not the co-xenografted human endothelial cells because the latter could not be detected immunohistochemically.

These results concur with the now well recognised fact that human tumour cell xenografts, in particular breast, are more successful in terms of take rate and latency when extra stromal cells are added (Brouty-Boye et al. 1993, Horgan et al. 1987). Also the fact that although only one cell type was injected, the tumours (breast and prostate) were histologically complex with a significant proportion of the cells being normal stromal cells, including fibroblasts, epithelial cells, mast cells and macrophages underlines the requirement for stromal cells for support (Tuxhorn et al. 2001, Ronnov-Jessen et al. 1996). This suggests that a lack of a sufficient stromal cell presence/component will lead to poor success and long latency periods due to the time taken to recruit the necessary host stromal cells. As the majority of the mouse mammary gland is composed of adipocytes, with fibroblasts prominent only in narrow sheaths around the ducts, the addition of exogenous stromal components may be required to improve or accelerate human tumour xenograft establishment. It is noteworthy

that there was a virtual absence of host fibroblasts within these heterologous xenografts (see Figure 6.18). In the human gland, the ducts are embedded in large tracts of connective tissue (composed predominantly of fibroblasts) that are adjacent to deeper layers of adipose tissue (Hovey et al. 1999). This may explain why, in the present study, the supra-threshold tumour cell-only inoculates had long latency periods in comparison to the heterologous inoculates. The question remains as to how the added stromal cells produced this enhancement, given that that they clearly do not survive very long *in vivo*.

The precise mechanism of stromal cell stimulation of tumour epithelial cell growth is not clear at present, although it may be associated with extracellular matrix (ECM) factors. Normal human fibroblasts may enhance tumourigenicity by a similar mechanism to Matrigel, which is derived from a mouse sarcoma that secretes ECM factors (Kleinman et al. 1986) similar to those that are deposited by primary human fibroblasts. Fibroblasts have previously been shown to regulate mammary epithelial function through interaction with ECM constituents (Haslam 1986, Haslam 1991). However, other studies have shown that the latency of tumour formation by MCF-7 xenografts was markedly shorter with admixed stromal fibroblasts than with admixed Matrigel (Noel et al. 1993, Noel and Foidart 1998). This suggests that normal human fibroblasts may either secrete different ECM factors to Matrigel or that this effect is mediated through additional or different mechanisms. It is likely that the human fibroblasts produce human specific ECM. This may explain how the fibroblasts promote tumour growth more efficiently than Matrigel, whose role may be limited to enhancing the initial establishment of implanted tumour cells in the host tissue. The results from the present study support this hypothesis in that while the fibroblasts did not survive beyond 2 weeks, probably having a similar duration of

action as Matrigel, their effect on epithelial cell growth was dramatic. Furthermore the temperature sensitive lines, which would have tended to take on a non-proliferative senescent phenotype at the high temperatures in the mice, produced the same effects as the wild type stromal cell lines. This fact and the results from Chapter 6.7 suggest that it is the initial short term influence that is all important. Therefore, it opens the possibility that an alternative mechanism of action other than ECM factors may be the cause.

One such alternative mechanism could be mediated by soluble paracrine factors such as cytokines and or growth factors. With stromal cells now well recognised as having major roles in epithelial growth and differentiation in adult tissues (Cunha et al. 1985), it has been reasoned that stromal cells closest to the epithelial cells would be the most likely to be involved in emitting regulatory signals in a paracrine manner (Wu et al. 2003). For example fibroblast growth factor (Giri et al. 1999, Foster et al. 1999), transforming growth factors α and ß (Dumont and Arteaga 2000, Akhurst and Balmain 1999) insulin like growth factor (IGF) (Singer et al. 1995) and hepatocyte growth factor (Kasai et al. 1996) have all been shown to promote epithelial tumour cell progression in both *in vitro* and *in vivo* model systems. Although potentially any of the growth factors derived from breast fibroblasts may function as an important regulator of mammary tumour growth, IGFs, and IGF-II in particular which is also produced by human fibroblasts, may play a key role in mediating breast tumour growth because IGF-II expression has been reported in the stroma of invasive breast cancers, but not in normal breast (Singer et al. 1995).

The hypothesis of a paracrine mediated stimulation of tumour cells by the immortalized human mammary stromal cell lines provides a reasonable explanation for the dramatic influence on tumour cell growth. Considering this

hypothesis in conjunction with the issue of NK cell activity it is possible that the immortalized stromal cell lines have released growth stimulatory paracrine factors (cytokine or growth factors) prior to being selectively destroyed by the NK cells in favour of the tumour cells as they are more susceptible (as they contain viral and bacterial transgenes). The initial focus of NK cell activity on the more susceptible immortalized fibroblasts may divert activity away from the tumour cells allowing their proliferation to such an extent which could permit the tumour cells to effectively out proliferate NK cell activity. This temporary diversion or reprieve from NK cell activity together with the influence of growth promoting factors may provide an additional explanation for their dramatic growth rates in comparison to tumour cell only inoculates.

6.9.5 Primary human tumour cell xenografts

The results from the present study demonstrated that a combined inoculation of primary human breast tumour cells and the immortalized fibroblast cell line HMF 3wt cells resulted in the transformation of the HMF 3wt cell line so that it not only survived but also proliferated to form tumours *in vivo*. This is surprising as both the HMF 3wt fibroblast cell line and the *ras* transfected variant HMF 3wt *ras* alone and in combination with established breast tumour cell lines repeatedly failed to be tumourigenic in other *in vivo* xenograft experiments. The new tumourigenic fibroblast cell line HMF 3wtX produced was more aggressive than the MCF-7 or MCF-7 V12 breast tumour cell lines in terms of *in vivo* take rate and latency rates.

The most likely explanation of this rather rare event is the spontaneous mutation in one or a few of the HMF 3wt cells possibly during their routine

culture or after their inoculation into the nude mice. However the co-inoculated primary breast tumour cells, PE1050, could have played a role. There are emerging studies which support the concept of neoplastic transformation of tumour associated stromal cells in breast and colon tumours by tumour-epithelial interactions (Kurose et al. 2001, Wernert et al. 2001, Moinfar et al. 2000). Wernert et al. (2001), for example, demonstrated that frequent genetic alterations in non-hereditary invasive human colon and breast cancers (loss of heterozygosity (LOH) and TP53 mutations) occur not only in the neoplastic epithelial cells, but also in the adjacent fibroblastic stroma and that both components can share clonal features. Similarly Kurose et al. (2001) reported that out of 41 sporadic invasive adenocarcinomas of the breast examined, the great majority of markers (13 chromosomal regions) showed a higher frequency of LOH in the neoplastic epithelial compartment than in the stroma, suggesting that LOH in neoplastic epithelial cells precedes LOH in surrounding stromal cells.

As all stages in this stromal-tumour progression (i.e. primary fibroblasts, transformed with hTERT only, transformed with hTERT and LT, transformed with hTERT, LT and *ras* and the tumourigenic HMF 3wtX cells) exists they can be compared so as to define what changes have taken place at these five stages. One way this could be achieved is using global microarray analysis which will define changes in levels of gene expression. Also specific mutations in known proto-oncogenes could be studied.

Chapter 7

Investigating the role of circulating endothelial progenitor cells

7.1 Introduction

Vasculogenesis, the *in situ* differentiation of primitive endothelial progenitors known as angioblasts, into endothelial cells that aggregate into a primary capillary plexus, is responsible for the development of the vascular system during embryogenesis (Peichev et al. 2000). In contrast, angiogenesis, defined as the formation of new blood vessels by a process of sprouting from pre-existing vessels, occurs both during embryonic development and in postnatal life (Peichev et al. 2000, Watt et al. 1995, Reyes et al. 2002). Until recently, it was believed that the formation of the foetal capillary networks from migrating endothelial precursor cells was restricted to early embryonic development with blood vessel formation in postnatal life being mediated by sprouting of endothelial cells from existing vessels (i.e. derived from endothelial cells located *in situ*). However, recent evidence suggests that endothelial stem cells may persist into adult life, playing an important role in postnatal endothelialization, whereby they contribute to the formation of new blood vessels (Nishikawa et al. 1998, Gehling et al. 2000, Rafii et al. 1994, Asahara et al. 1997). This in turn suggests that, as during development, neo-angiogenesis in the adult may depend at least in part on a process of vasculogenesis. Precursors of endothelial cells have been isolated from bone marrow and peripheral blood (Peichev et al. 2000, Watt et al. 1995). The ontogeny of these endothelial progenitors is unknown.

Identification of circulating endothelial progenitor cells

A search of unique endothelial markers to facilitate the isolation and characterization of circulating endothelial progenitor cells (CEPs), has shown that CD133 is expressed on a subset of CEPs but not on mature differentiated endothelial cells. CD133, also known as AC133, is a novel 120-kd glycosylated polypeptide that contains 5-transmembrane domains with an extracellular N-terminus and a cytoplasmic C-terminus (Miraglia et al. 1997, Yin et al. 1997). The function of CD133, which does not share homology with any previously described haematopoietic cell surface antigen, is not known. However, isolation of a sub-population of $CD34^+$ cells using a monoclonal antibody to human CD133 has identified functional $CD34^+$ haematopoietic stem cells that can differentiate into mature endothelial cells (Peichev et al. 2000, Reyes et al. 2002). Incubation of these non-adherent $CD34^+CD133^+$ cells with VEGF, fibroblast growth factor (FGF-2) on a substratum of fibronectin or collagen results in their proliferation and differentiation into adherent $CD133^+VEGFR\text{-}2^+$ mature endothelial cells. These results suggest that circulating $CD34^+$ cells expressing VEGFR-2 and CD133 comprise a functional population of CEPs cells that may play a role in postnatal angiogenesis or vasculogenesis.

One study has demonstrated that human derived $CD133^+$ cells can repopulate sheep bone marrow (Yin et al. 1997) and, therefore, can be considered pluripotent haematopoietic stem cells. Expression of CD133 is rapidly down regulated in haematopoietic progenitors and stem cells as they differentiate into more mature post-mitotic cells (Peichev et al. 2000, Gehling et al. 2000). In fact, virtually all mature haematopoietic cells, including mature myeloid, megakaryocytes, erythroid, and lymphoid cells and terminally differentiated

haematopoietic cells, fail to express CD133 (Miraglia et al. 1997, Yin et al. 1997).

The emerging potential of circulating endothelial progenitor cells

$CD34^+$ haematopoietic stem cells can be detected at low numbers in bone marrow, foetal liver, and umbilical cord blood. The fact that CD133 is expressed on immature haematopoietic stem and progenitor cells, and is not found on mature blood cells provides a valuable method of isolation and tracking.

CEPs can be isolated from adult bone marrow, foetal liver, umbilical-cord blood and cytokine-mobilized peripheral blood and have been reported to give rise to mature endothelial cells when cultured *in vitro* (see Table 7.1). Freshly isolated CEPs are a reported to be highly motile, non-adherent population of cells that express characteristic cell surface markers. Several markers are expressed by both CEPs and mature endothelial cells, including VEGFR2 (Gill et al. 2001), CD34, PECAM and CXCR4 (Peichev et al. 2000). With this information now available it is now feasible to isolate, characterise and study the potential of CEPs to differentiate *in vitro* and *in vivo*.

CD133-expressing cells in different haematopoietic tissues (1)		
	% $CD34^+$	**% $CD133^+$**
Normal bone marrow (2)	1.47 ± 0.23 (36.3 ± 2.2)	0.52 ± 0.11
Cord blood (3)	0.37 ± 0.06 (51.0 ± 1.6)	0.16 ± 0.03
Apheresis (4)	1.75 ± 0.31(75.3 ± 0.8)	1.37 ± 0.27

Table 7.1. Table summarizing the percentage of $CD34^+$ and $CD133^+$ cells present in different haemopoietic sources. Figures in brackets represent % of $CD34^+$ cells also expressing $CD133^+$. References 1) de Wynter et al. 1998, 2) Horn et al. 1999, 3) Green et al. 2000, 4) Verfaillie 2002.

Circulating endothelial progenitor cells and their role in cancer

CEPs as detected by CD133 have been detected at increased frequency in the circulation of cancer patients and lymphoma-bearing mice (Mancuso et al. 2001, Monestiroli et al. 2001). In addition tumour volume and tumour production of VEGF were found to be correlated with CEPs mobilization in the animal model system. There is increasing evidence that these cells actually contribute to the formation of tumour associated blood vessels. Several studies have shown that CEPs when engrafted into immunocompromised mice, incorporate into the vasculature of xenotransplanted tumours (Lyden et al. 2001, Reyes et al. 2002, Moore 2002, Asahara et al. 1999, Gehling et al. 2000). For example, human $CD133^{+}/VEGFR2^{+}$ cells were isolated from human umbilical cord blood, injected *in vivo* into immunocompromised mice and allowed 2 weeks to engraft. Mice were then inoculated with human tumour cells, and the relative contributions of donor-derived and host-derived bone marrow cells in the tumour vasculature was determined on the basis of immunohistochemical staining for human antigens (VEGFR2, CD34 and PECAM). On average, 5–8% of cells in the newly formed blood vessels within the tumour tissue were found to be of human donor origin (Rafii et al. 2002), indicating that the transplanted donor cells contribute to the newly formed vasculature. In another relevant *in vivo* model, it was shown that the neo-intimal surface of left ventricular assist devices ranging from 28 days to 6 months old was also colonized with large numbers of $CD133^{+}VEGFR\text{-}2^{+}$ cells determined by dual-colour flow cytometry (Rafii et al. 1995, Peichev et al. 2000).

The purpose of this chapter was three-fold. The first objective was to isolate and characterize CEPS according to methods established in the literature. The second objective was to demonstrate that these isolated cells could

proliferate and differentiate as mature endothelial cells when exposed to the appropriate stimuli. The third objective was to determine if such CEPs preparations could participate in the vascularization of human tumour cell xenografts by incorporating them in multi-compartment xenograft models, because of the failure of the differentiated endothelial cells to survive or participate in xenograft formation (Chapter 6).

7.2 Isolation, culture, characterization and differentiation of circulating endothelial progenitor cells

7.2.1 Isolation of circulating endothelial progenitor cells

Low-density mononuclear cells (LD-MNCs) were extracted from two different sources. These were fresh bone marrow aspirates (~50ml) collected from healthy donors for allogeneic bone marrow transplantation and cytokine mobilized 'buffy coat residues', blood samples rich in mononuclear cells obtained from the National Blood Transfusion Centre.

LD-MNCs were isolated from fresh bone marrow aspirates and cytokine mobilized 'buffy coat residues' by layering on a Ficoll-Hypaque gradient. Following isolation of LD-MNCs the haemopoietic stem/progenitor cells were isolated using positive selection of $CD133^+$ expressing cells using the AC133 Cell Isolation Kit (Miltenyi Biotec) and the MS Magnet (MiniMACS™, Miltenyi Biotec). Briefly LD-MNCs were added to $CD133^+$ monoclonal antibody conjugated super paramagnetic microbeads (AC133 Cell Isolation Kit), washed and processed through a MACS magnetic separation column to obtain a pure population of $CD133^+$ cells as shown in materials and methods (Chapter 2.3.2).

A 500ml bag of 'buffy coat residue' provided a yield of approximately 10^8 LD-MNCs cells. Isolation of $CD133^+$ cells from this sample resulted in a yield of ≤1% producing approximately 10^6 $CD133^+$ cells. A typical 50ml marrow aspirate produced a smaller yield of between 10^6-10^7 LD-MNCs cells. This provided a $CD133^+$ cell population of 10^4-10^5 $CD133^+$ cells. The isolation procedure was carried out on over 10 samples of each source, producing similar results each time in terms of yields. These results were consistent with previous reports on the isolation of CEPs (Table 7.1).

7.2.2 Culture of circulating endothelial progenitor cells

To induce differentiation into endothelial cells, freshly isolated $CD133^+$ (CEPs) cells were plated at 1 x 10^4 to 2 x 10^4 cells in $25cm^2$ fibronectin coated tissue culture vessels, in a mix of 60% (v/v) low-glucose DMEM and 40% (v/v) MCDB-201 , supplemented with 1x insulin-transferrin-selenium, 1x linoleic acid–BSA, 10^{-8} M dexamethasone, 10^{-4} M ascorbic acid 2-phosphate, 100U/ml penicillin and 100μg/ml streptomycin, 10ng/ml VEGF, bFGF-2 1ng/ml, 2ng/ml IGF-1, and 10% (v/v) FBS as described by Reyes et al. (2002). Cultures were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2 with medium changes every 4–5 days.

After 14 days of culture, between 50-60% of the initially non-attached cells, had formed isolated colonies each containing an average of 60 cells (Figures 7.1a and b), their morphology was characterized by a round central body with some cytoplasmic projections. These cells were then cultured for a further 2 weeks by which time they had proliferated to confluency (Figure 7.1c and d). The confluent cultures consisted of a monolayer of 'spindle-shaped' cells,

sometimes forming a focal area of cell aggregates with typical 'cobblestone' morphology similar to primary mammary endothelial cells. In some instances, cells were sub-cultured using trypsin after day 9 at a 1:4 dilution under the same culture conditions. The sub-cultured cells which could be passaged < 5 times completed at least 20 population doublings before they ceased to grow and eventually died.

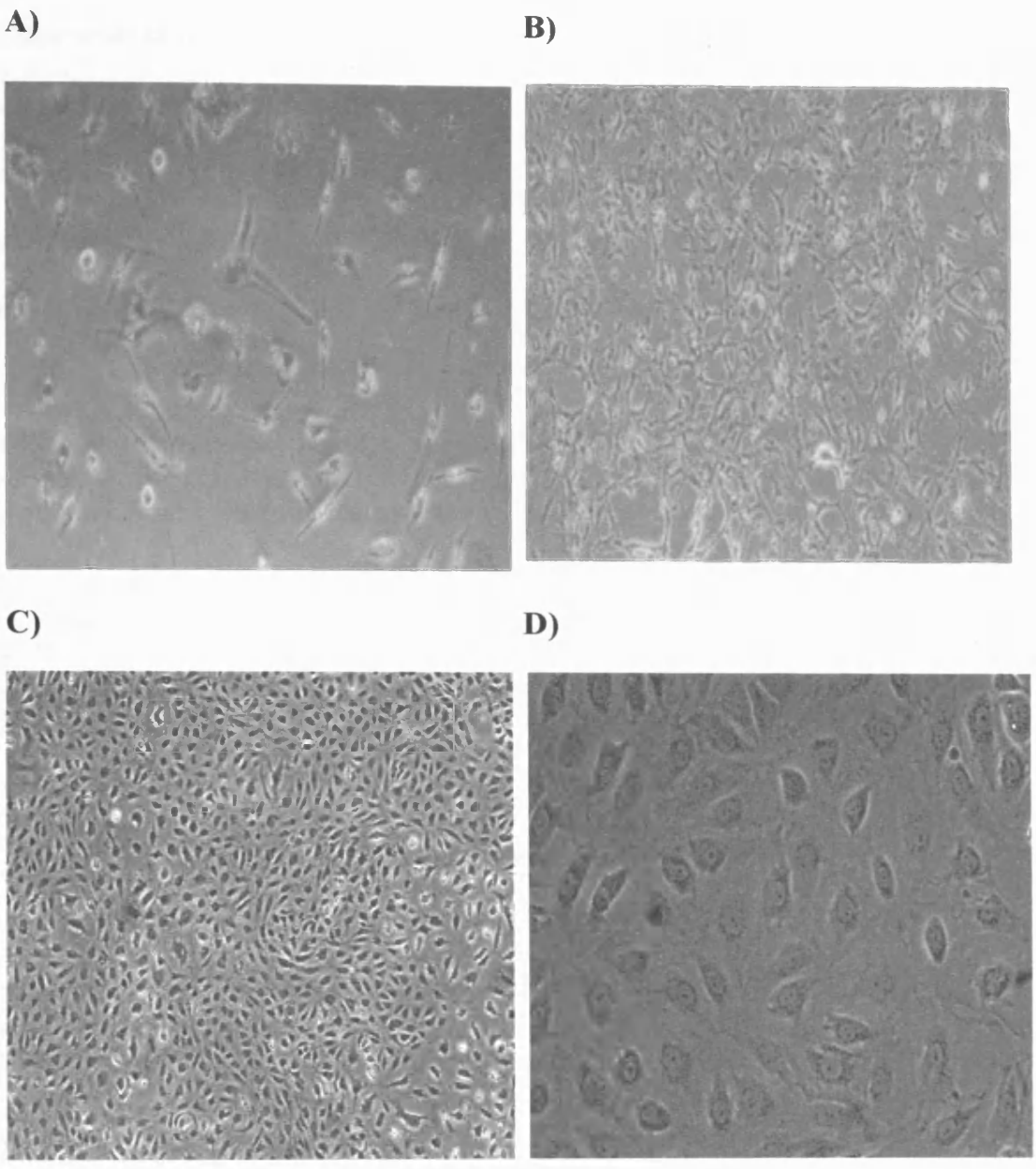


Figure 7.1. Phase contrast micrographs of circulating endothelial progenitor cells ($CD34^+$ $CD133^+$) cultured on fibronectin coated tissue culture flasks at (a) 2 days, (b) 8 days and 2 weeks (c & d). Figure (d) represents a high power image of (c). a) & c) taken at ×80 with c) & d) at ×320 magnification.

7.2.3 Characterization of circulating endothelial progenitor cells

Expression of endothelial cell markers

The endothelial cell markers PECAM-1/CD31, CD34 and endothelial progenitor cell marker CD133 were used to characterize CEPs that had been in culture for 2 and 5 days. Live cells were plated on coverslips in 24-well plates, allowed to attach before being fixed in methanol (Figure 7.2). The cells were stained using indirect immunofluorescence. In three separate experiments using independent cell preparations from both bone marrow and cytokine mobilized blood samples, all of the samples taken at 2 days were $CD133^+$ with the majority of cells (>80%) staining with the mature endothelial cell marker CD34. However staining for CD31 was negative. Preparations from experiments in which cells were cultured for longer (5 days) were negative for CD133, with weak $CD34^+$ (5-37%) staining visible. However the cells stained positive for CD31 at 5 days. Therefore CD133 expression present at 2 days had disappeared after 5 days, CD34 staining had reduced and CD31 had appeared indicating progressive maturation towards an endothelial phenotype.

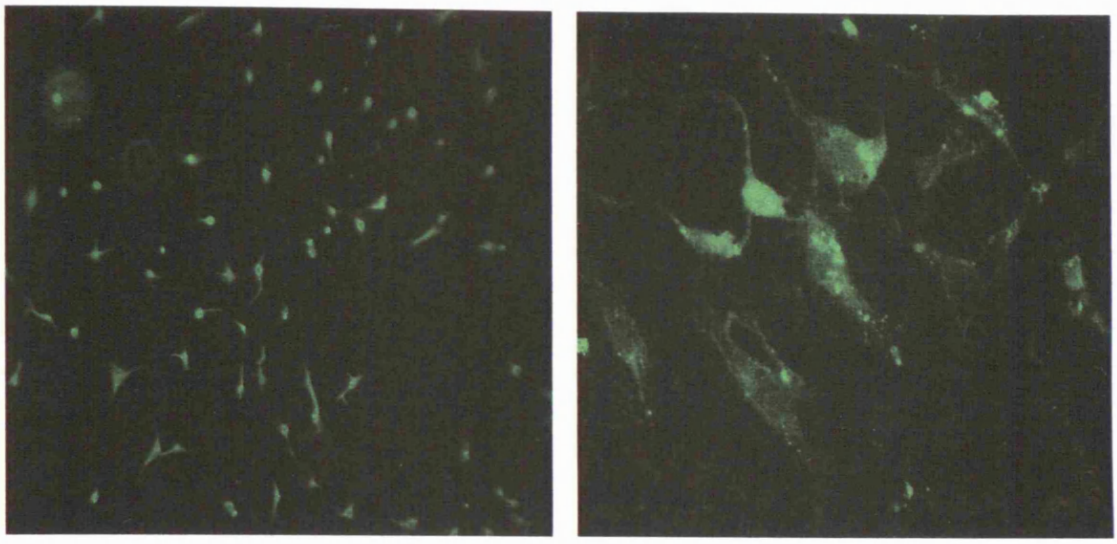


Figure 7.2. Immunofluorescence staining of CEPs after 5 days of culture using an anti-CD31 antibody. CEPs isolated from both bone marrow and cytokine mobilized blood were cultured on fibronectin coated tissue culture flasks before being labelled. Magnification x80 and x320.

7.2.4 Differentiation analysis of circulating endothelial progenitor cells

The ability of mature human microvascular endothelial cells to form a network of capillary like structures when cultured on Matrigel is a sign of normal endothelial cell differentiation. Therefore the ability of $CD133^+$ cells (CEPs), which had been cultured *in vitro* as described above, to differentiate on Matrigel to form microcapillaries was examined. $CD133^+$ cells that had been in culture for 5-7 days following isolation were harvested using trypsin. 1×10^3 cells were counted and plated on 200µl of pre-prepared Matrigel in 96-well plates (and also 500µl on 24-well plates) containing medium and observed for differentiation into microcapillary networks (Figure 7.3). Similar to mature human endothelial cells

the $CD133^+$ were seen at first to move to aggregate before forming recognisable capillary like networks after 3-4 hours.

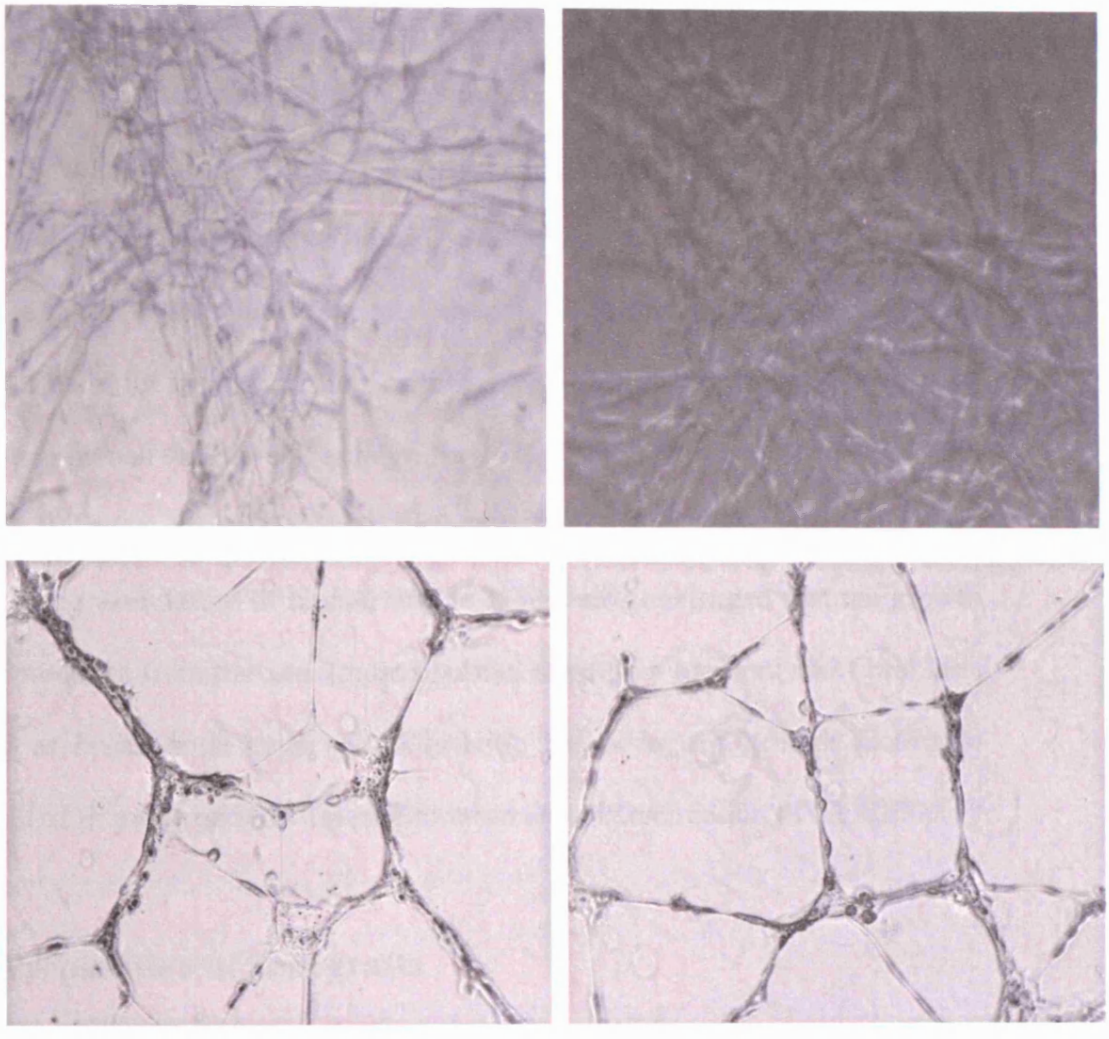


Figure 7.3. Phase contrast micrographs of primitive microcapillary like networks formed from $CD133^+$ (CEPs) that had been in culture for 7 days. $CD133^+$ cells were isolated from a bone marrow aspirate. Cells isolated from cytokine mobilized blood produced similar structures. Images were taken after 12 hours of plating on Matrigel. Magnification x80 and x320.

7.3 Xenotransplantation of CEPs

The purpose of this experiment was to investigate the role of $CD133^+$ cells (CEPs) in tumour neo-vascularization by incorporating them as part of the heterologous 3-compartment xenograft model. Chapter 6 demonstrated that the heterologous tumour xenografts involving the use of immortalized human mammary endothelial cells to form human endothelial cell vascularized tumours were unsuccessful. With the knowledge that human embryonic stem cells have the potential to differentiate into various cell types and, thus, are useful as a source of cells for transplantation or tissue engineering, it was postulated that inoculating human tumour cell xenografts with endothelial precursor cells, which have the ability to differentiate into endothelial cells, would improve the chance of producing vasculature of human origin. It was also envisaged that the growth factors produced from the xenografted human mammary tumours and fibroblasts as well as host/murine cells would provide the necessary growth factors to activate and promote endothelial proliferation and differentiation of the CEPs.

7.3.1 Preparation of xenografts

To determine the capacity of CEPs to contribute to tumour angiogenesis and or neo-vascularization, $CD133^+$ cells (CEPs) were xenografted in combination with the VEGF over-expressing mammary tumour cell line MCF-7 V12 and/or human mammary fibroblasts together into the mammary fat pads in matrigel. Both freshly isolated CEPs and adherent cells that had been cultured for <48 hours were tested.

Due to the limited number of CEPs available from single isolates, experiments utilized cells from both bone marrow and cytokine mobilized blood.

In the first experiment three 500ml 'buffy coat residue' bags were processed to yield a $CD133^+$ cell count of approximately of 3×10^6 cells. These cells were cultured *in vitro* for 2 days before being harvested for xenotransplantation. Six mammary fat pads of 3 mice were inoculated with MCF-7 V12 at a supra-threshold number (5×10^6) and $CD133^+$ cells at a ratio of 10:1. This experiment was repeated on a separate occasion but this time with 5×10^5 cells of the immortalized fibroblast line HMF 3wt was added as well as the CEPs.

In the second series of experiments, three 50ml bone marrow samples were also processed to yield a $CD133^+$ cell count of approximately of 2×10^6 cells which were cultured *in vitro* for 2 days before being harvested and inoculated into the mammary fat pads as before. 4 mammary fat pads were inoculated with 5×10^5 cells of $CD133^+$ cells and 5×10^6 of MCF-7 V12 cells together with 5×10^5 cells of HMF 3wt cells. In addition, 1×10^6 CEPs from the same preparation were xenografted into two mammary fat pads (5×10^5 CEPs cells/MFP) with MCF-7 V12 and HMF 3wt cells in the same ratios as above but this time as freshly isolated cells without prior *in vitro* culture. Results for all the CEPs inoculations are shown in Table 7.2.

Cells and cell lines	Xenograft Take Rate MFP + Matrigel
MCF-7 V12 + CEPs (2 days in culture)	5/6 (blood)
MCF-7 V12 + HMF 3wt + CEPs (2 days in culture)	6/6 (blood)
MCF-7 V12 + HMF 3wt + CEPs *(freshly isolated)*	2/2 (bone marrow)
MCF-7 V12 + HMF 3wt + CEPs (2 days in culture)	4/4 (bone marrow)

Table 7.2. Table summarizing the results of circulating endothelial progenitor cell xenografts. MCF-7 V12 cells were inoculated in supra-threshold numbers/site (5×10^6). CEPs and fibroblasts (HMF 3wt) were inoculated at a ratio of 1:10 to tumour cells. All inoculations were carried out in Matrigel into the MFP. Results are a summary of several experiments.

7.3.2 Analysis of xenografts

Xenografts were harvested between 4-6 weeks when a substantially palpable tumour was present. In keeping with previous experiments of MCF-7 V12, tumours were visibly hyperaemic. The inoculations gave rise to 17 tumours out of a possible 18 inoculations. Extensive immunohistochemical analysis (20 sections/tumour), using direct immunofluorescence, of the xenografts with specific markers for mature human endothelial cells (CD31/CD34) was carried out. Results showed very few scattered and isolated fluorescent dots within the tumours. However there was no evidence of any significant vascular structure staining in the tumours with either the human specific anti-CD31 or CD34 antibodies. This was consistent throughout sections taken from 4 week and 6 week old tumours. All blood vessels within the tumours tested stained positive using a mouse specific CD31 antibody.

7.4 Discussion and conclusion

The close similarities in the development of haemopoietic and endothelial cells during embryonic life have led to the hypothesis that the two lineages may derive from a common precursor called the haemangioblast (Muller et al. 1994, Garcia-Porrero et al. 1995, Smith and Glomski 1982). Indeed, recent studies have shown circulating $CD34^+$ cells expressing VEGFR-2 and CD133 comprise a functional population of circulating endothelial progenitor cells (CEPs) that may play a role in postnatal angiogenesis or vasculogenesis.

The expression of CD133 on immature haematopoietic stem and progenitor cells but not on mature blood cells provided a valuable method of isolation and tracking CEPs. The stimuli provided by fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), fibronectin and/or collagen, have been shown by the present study to provide CEPs with the necessary nutrients to differentiate into adherent anchorage-dependent endothelial cells in accordance with other studies (Bagley et al. 2003, Quirici et al 2001, Kalka et al. 2000, Peichev et al. 2000). As reported in the literature, the expression of CD34 and CD133 diminished with maturation and differentiation (Miraglia et al. 1997, Yin et al. 1997, Delia et al. 1993). Therefore, subsets of $CD34^+$ cells that express CD133 are truly a phenotypic and functional marker of an immature population of haematopoietic stem and endothelial progenitor cells.

In summary, bone marrow and cytokine mobilized blood derived $CD133^+$ cells can be isolated with a single step of immunomagnetic separation and cultured in appropriate conditions, to give rise to an almost pure population of endothelial cells, probably derived from 'putative' haemangioblasts.

Role of CEPs in tumour xenograft vascularization

Studies have shown that multi-potential progenitor/stem cells when xenografted into immunocompromised mice incorporate into the vasculature of xenotransplanted tumours (Lyden et al. 2001, Reyes et al. 2002, Moore 2002, Asahara et al. 1999, Gehling et al. 2000). In one particular study, human multi-potential adult progenitors (MAPC) (bone marrow mononuclear cells depleted of $CD45^+$ and glycophorin A^+ cells using micromagnetic beads) which had been induced to differentiate into endothelial cells *in vitro*, were injected into immunocompromised mice that carried mouse Lewis lung carcinomas. After 5 days, 30% of the newly formed tumour associated vessels were derived from human MAPC-derived endothelial cells. MAPC-derived human endothelial cells also contributed to the neovasculature of spontaneously formed lymphomas that commonly develop in ageing immunocompromised mice (Reyes et al. 2002). Similarly Asahara et al. (1999) engrafted tumour bearing mice with transgenic mouse bone marrow cells that were engineered to express a cell marker lacZ under the transcriptional regulation of the VEGFR2 and TIE2 endothelial-specific promoters. Cells that expressed this marker were detected throughout the implanted mouse syngeneic colon tumours, indicating that bone marrow-derived cells, such as CEPs, can home to the tumour vasculature and differentiate into endothelial cells. None of these studies, however, could prove that recruitment of bone marrow-derived cells was absolutely required for tumour growth.

In the present study it was hypothesized that injecting CEPs ($CD133^+$ cells) directly into the mammary fat pads of nude mice, with the relevant tumour cells and fibroblasts already present, would provide the simplest and most direct method for CEPs establishment and differentiation. However, results showed that differentiation and incorporation of CEPs as mature endothelial cells into the

multi-compartment xenograft models was unsuccessful. This was surprising as similar type studies in the past indicated that they would incorporate into the neo-vasculature as summarized as above. Furthermore, the present study introduced several novel attempts at optimising the environmental conditions as well as enhancing the establishment and growth of the CEPs. For example, the VEGF over-expressing cell line MCF-7 V12 was used to promote maturation and differentiation into mature endothelial cells whilst the addition of fibroblasts and Matrigel was intended to provide additional growth factor and nutritional support.

In order to identify the possible causes for the failure of CEPs to establish, differences between the present study and other similar type studies were examined. There were several possible factors which could affect whether the CEPs successfully established or not. These were: origin of progenitor/stem cells, cell numbers inoculated, route and site of engraftment, host (animal) type, tumour site and type, criteria for identification and duration of experimental period. These pertinent features were identified in several similar type studies and are shown in Table 7.3.

Reference	Origin & Cell numbers	Origin of CEPs Cultured /fresh	Host animal	Injection method/site	Tumour type & site	Criteria for identification	Duration of study
Reyes et al 2002	Bone Marrow 2.5 x 10^5	Multipotent adult progenitor cells cultured + fresh	NOD / SCID	Tail vein injection	Lewis lung carcinoma / SC	CD31, CD34, CD36, CD44, HLA class I, HLA class II, ß2-microglobulin, VCAM, ICAM-1, VE-cadherin, H1P12, ZO-1, MUC18, $\alpha_v\beta_3$, $\alpha_v\beta_5$, ß-catenin, γ-catenin	14 days & 12 weeks
Gehling et al. 2000	Blood 1×10^6	$CD133^+$ LD-MNCs Cultured	SCID	SC	Human lung cancer cell line A549/ SC	CD34, CD41a, CD41b,vWF, VE-Cadherin, KDR, P1H12, EN4	5 weeks
Asahara et al. 1999	Bone marrow 2 x10^6	LD-MNCs Fresh	Transgenic mice expressing ß-galactosidase	Intravenous	Mouse colon cancer cells (MCA38)/ SC	lacZ-expressing cells	1 or 3 weeks
Lyden et al 2001	Bone Marrow 1 x10^6	LD-MNCs	Id1-Id3 protein double knockout mice	Tail vein injection	B6RV2 lymphoma Intra-dermal	vWF, VEGFR1, MOMA-1, CD11b, VE-Cadherin	14 days
Moore et al 2004	Umbilical cord blood 1.0 × 10^6 per mouse	Human $CD34^+$ LD-MNCs Cultured	SCID	Tail vein injection	Mouse glioma Brain	Cells pre-labelled with a fluorophore	7 & 14 days

Table 7.3. Table summarizing various xenografts studies of endothelial progenitor cells.

The number of CEPs/inoculate and the source of CEPs was ruled out as a possible cause of failure as similar numbers of CEPs from both cytokine mobilized blood and bone marrow aspirates have been xenografted previously in other studies resulting in successful CEPs establishment (see Table 7.3). Both sources were used in the present study.

There were, however, some noticeable differences between the present study and those reported in the literature. Unlike the present study, SCID and NOD/SCID mice were predominantly used as the host with CEPs generally being inoculated haematologically, rather than directly into the tumour microenvironment. NOD/SCID mice in particular have been shown to provide better conditions for stem cell engraftment (Rice et al. 2000, van der Loo et al. 1998, van Hennik et al. 1999). However, in the present study the hypothesis that the residual innate immune responses (e.g. NK cells, macrophages and complements) are the cause for the failure to survive is unlikely. This is because CEPs are primary cells and not genetically engineered and so do not express bacterial and viral proteins like the engineered immortalized endothelial cells. Thus, they should be less susceptible to NK cell activity if this was indeed a significant factor.

The site of inoculation may be very important in providing the initial support required for survival and differentiation. Endothelial precursor cells need to adhere to the endothelium to proliferate and so, like many of the studies listed in Table 7.3, a haematological implantation method may be more advantageous in the establishment of CEPs. The majority of the successful engraftment studies injected the CEPs into the vascular system following establishment of tumour xenografts and then examined tumour associated blood vessels for CEPs differentiated endothelial cells (Reyes et al. 2002, Asahara et al. 1999). The

recruitment of CEPs to tumour angiogenesis probably represents a multistep process, including (a) active arrest and homing of the circulating cells within the angiogenic microvasculature; (b) trans-endothelial extravasation into the interstitial space; (c) extravascular formation of cellular clusters; (d) creation of vascular sprouts and cellular networks; and (e) incorporation into a functional microvasculature.

In addition, adhesion of CEPs to the tumour vessel endothelium of xenografts may be further facilitated by the tumour-specific micro-haemodynamics, which are characterized by a much lower blood cell velocity (~350 μm/s) and wall shear stress (~4.0 dyne/cm^2) when compared with physiological conditions in post-capillary venules of non-angiogenic vasculature (Vajkoczy et al. 2002, Vajkoczy et al. 2003). The observation that after permanent adhesion, CEPs extravasate into the interstitium further indicates similar mechanisms underlying CEPs and leukocyte recruitment and migration through the endothelial lining. This is in contrast to a non-angiogenic microvasculature, where CEPs are arrested by passive plugging of small calibre capillaries and remain intravascular (Vajkoczy et al. 2001, Butcher 1991, Menger et al. 1996).

In the studies summarized in Table 7.3, CEPs were generally inoculated after tumours xenografted into the mice had had sufficient time to establish and proliferate. However, in present study as CEPs were inoculated at the same time as the tumour cell lines. Consequently, there would be a lag period in which it took the tumour cell line to establish and commence vascularization. Thus, the attraction of CEPs by angiogenic signals in tumour bearing mice may provide a key role in promoting the differentiation and establishment of CEPs. However, the molecular and cellular mechanisms underlying CEPs recruitment and

differentiation are not yet understood, and remain as one of the central issues in stem cell biology (Blau et al. 2001). For instance, it is unclear how CEPs specifically home to angiogenic sites, which critical steps underlie their incorporation into new blood vessels, and how effectively these processes compare with the activation of local, pre-existing endothelial cells (Risau 1997). Furthermore, little is also known about which tumour types are most susceptible and dependent on CEPs and haematopoietic cells for their growth. The present study used breast cancer cells, something not attempted in the other reported studies. Based on available evidence, mobilization and functional incorporation of CEPs into the tumour vasculature is essential for the growth of some tumours; lymphomas, Lewis lung carcinoma and colon cancers (Rafii et al. 2002). This was concluded because a significant proportion of the tumour vasculature was consistently derived from CEPs derived endothelial cells.

Another important difference between the present study and those in Table 7.3 is that the experiment period of those studies was generally much shorter, with the search for CEPs incorporation beginning much earlier. This perhaps may be a crucial factor as a longer time period may have resulted in little or no evidence of CEPs presence as they may have changed their expression phenotypes or even been replaced by other cells and new vessels (i.e. rapid cell turnover in angiogenic vasculatures leading to short cell life). Based on mouse tumour models, it seems that these cells are most likely to contribute to the early stages of tumour growth, but are only involved in the angiogenic phase.

To conclude therefore, as the hypothesis that the CEPs, too, are being destroyed by a remaining immune response is unlikely the most probable alternative reason maybe due to inadequate support for endothelial cell growth, in terms of nutrition and endothelial cell specific growth factors. Comparison

with other similar xenograft studies demonstrated that the type of animal (usually SCID), site of inoculation (IV) and duration of experiment maybe be crucial factors in providing the required nutritional support and growth factors. Having said this, the combination of factors released by the co-engrafted tumour cells, fibroblasts and growth factor rich Matrigel should have provided sufficient growth support.

To test the above hypothesis, freshly isolated CEPs could be injected intravenously into SCID or NOD/SCID mice once the MCF-7 cells had established in the mammary fat pads. The injected CEPs could then be tracked by pre-labelling or looking for specific human endothelial markers at regular earlier time points (< 2 weeks). To further improve success, repeated injections of greater cell numbers into the vasculature of human breast tumour bearing SCID mice could be performed to overcome the problem of cell arrest when trapped in small calibre vessels.

Chapter 8

Final discussion and future prospects

8.1 Aims of the thesis

The overall aim of this thesis was to develop a more authentic xenograft model of human breast cancer by incorporating the relevant and essential constituents of a tumour microenvironment: human stromal cells and extracellular matrix. Ideally this would be an organotypic, 3-compartment xenograft model of human breast cancer in mice comprising solely of human cells. Until now, the great hurdle impeding multi-compartment xenograft model development was due to a lack of readily available human stromal cells. With the recent availability of a series of immortalized human mammary stromal cell lines (O'Hare et al. 2001) it has now been possible to commence development of a multi-compartment *in vivo* breast tumour model.

The development of a complex multi-compartment heterologous xenograft model incorporating the relevant stromal elements would provide a realistic alternative to currently available chimeric xenograft models. If successful this model could be utilized as an accurate and credible pre-clinical model for the development and testing of new therapeutic strategies, such as those that target breast cancer stroma itself.

8.2 General summary of results

The results of this thesis fall into two broad areas that can be summarised as follows:

In vitro

The main purpose of the first results Chapter 3 was to determine whether the immortalized human mammary stromal cell lines had retained the basic characteristics of normal cellular growth or whether the immortalization procedure had transformed the cells. This was performed to assess the suitability of the available stromal cell lines for use in the development of tumour-stromal xenograft models, and more specifically, to assess the effects and the extent to which the SV40 LT antigen had on cell transformation during the immortalization process. Characterization of the immortalized stromal cell lines demonstrated that the stromal cell lines displayed some of the hallmarks of *in vitro* transformation. However they also retained many of the characteristics of normal cells. Therefore, although they could not be strictly regarded as normal cells they were also by no means fully transformed. In fact they were much closer phenotypically to normal cells than fully transformed cells. The results also demonstrated that the each of immortalized human mammary stromal cell lines tested was unique, with each line possessing slightly different phenotypic properties. The cause of variability between the cell lines (discussed in Chapter 6) was attributed to the order and timing of hTERT and SV40 LT gene transduction (Fauth et al. 2004). However it was also clear from the results that the characteristics of the cell lines were not just a manifestation of LT antigen expression alone as they had gained other additional properties which made then individual.

Thus it seemed that a useful series of robust and stable human mammary stromal cell lines have been generated. Although these cells that possess an immortal growth potential, they function essentially like normal cells without expressing a typical fully transformed malignant phenotype (Table 3.3). As a result they represent a readily available source of essentially normal human mammary stromal cells, potentially able to support a tumour microenvironment when xenografted *in vivo*. However, with the available *in vitro* results it was not possible to select a particular cell line as being better suited or more superior for the *in vivo* studies. Their individual characteristics meant that each had to be tested separately.

Following characterisation of the available human mammary stromal cell lines, the ability of human and host (mouse) endothelial cells to interact to form vasculature was investigated (Chapter 4). *In vitro* differentiation assays incorporating the immortalized human mammary microvascular endothelial cells and the normal mouse microvascular endothelial cells showed that the two cells, from the different species, interacted to form primitive micro-capillary networks. These results were very promising in that they indicated that the immortalized human mammary endothelial cell line, when xenografted, would interact with host endothelial cells to integrate into host vasculature. It, therefore, provided a potentially effective means of vascularizing the human tumour cell xenografts with human vessels.

The next goal was to bring together the tumour and stromal cells to manufacture a 3-D *in vitro* tumour-stromal co-culture. The rationale was to transplant these preformed tumour-stromal units directly into the mammary fat pad in order to optimise tumour develop and spread. The availability of the immortalized stromal cells, together with increasingly sophisticated 3-D cell

tissue culturing techniques meant that complex 3-D tumour-stromal models could now be created without the limitations associated with monolayer cultures and single cell type spheroids (Chapter 5). The 3-compartment heterologous tumour-stromal spheroids manufactured in the Rotary Cell Culture System demonstrated that more complex organotypic tissues can be manufactured *in vitro* realistically mimicking the 3-D structure and environment of *in vivo* cancers. These 3-D heterologous tumour models incorporating human endothelial cells were shown to be viable tissue units capable of growth and differentiation in an appropriate environment such as Matrigel. They also provided a novel way of promoting tumour-stromal interactions prior to xenotransplantation.

As an alternative source of endothelial cells the potential of human circulating endothelial progenitor cells (Chapter 7) for vascularizing human tumour xenografts with species-specific blood vessels was investigated. CEPs could be isolated from bone marrow aspirates and cytokine mobilized blood using a simple magnetic bead isolation protocol, with an antibody for CD133, which is a marker specific for CEPs. CEPs, with the appropriate stimuli, differentiated into cells that had the same phenotype and characteristics as mature endothelial cells. Differentiated CEPs formed primitive micro-capillary like networks on Matrigel. These results were promising for the engraftability of CEPs in an *in vivo* setting. If in the future CEPs are found to be a significant contributor of tumour vascularization then they may provide a new target for chemotherapy.

In vivo

Results showed that none of the stromal cell lines, singly, in combination or as spheroids formed survived or tumours, in the long term (≥6 weeks) when xenografted into the mammary fat pads of nude mice (Chapter 6). Even when the stromal cell lines were inoculated as a part of a 3-compartment xenograft, immunohistochemical analysis showed no evidence of survival 8 weeks post inoculation. Overall a total in excess of 2.4×10^8 cells of each stromal cell line was inoculated singly or as a mixture *in vivo*. Generally, cell number inoculations of this quantity should have been sufficient to give rise to tumours if the cells were intrinsically tumourigenic. Therefore to clarify the survival characteristics of the stromal cells 3-compartment tumours were harvested at earlier time points (2, 4 and 6 weeks). This showed that the fibroblasts survived up to 4 weeks albeit in very small numbers. However, the endothelial cells were much more sensitive in that they were no evidence of survival after just 2 weeks. Surprisingly the 3-compartment heterologous co-culture spheroids previously manufactured in the Rotary Cell Culture System also failed to form tumours.

However, the co-engraftment of the immortalized human mammary fibroblasts and or endothelial cell lines, with human breast cancer cell lines, as mixed cell suspensions had a dramatic effect on the promotion of tumour cell take rates including reduction of latency periods. The addition of the stromal cell lines to sub-threshold tumour cell inoculates consistently gave rise to tumours, which would not ordinarily have formed in the absence of the stromal cells. The fibroblasts had the greatest effect with combined inoculations of the fibroblast and endothelial cells not producing an additional cumulative effect. Although the immortalized stromal cells had a dramatic effect on established breast cancer cell

lines they did not support the establishment of primary human breast cancer cells (PE1050). In addition, the xenografts of the CEPs were unsuccessful in that they also did not survive when inoculated as a combination with tumour and or the human mammary fibroblast cell lines.

A surprising positive outcome of the xenograft studies was the full transformation of the wild type HMF 3wt fibroblast cell line, on one occasion, into a tumourigenic cell line (HMF 3wtX). This resulted when the HMF 3wt fibroblast cell line was engrafted with primary human breast cancer cells (PE1050). Tumours produced from the HMF 3wtX and PE1050 mixture were confirmed as being fibroblastic of human origin expressing the LT antigen, originally inserted into derivative immortalized cell line. Not only was this line tumourigenic *in vivo*, it had a near 100% serial re-transplantation rate. Now that we have the complete series of fibroblasts ranging from the primary fibroblasts, immortalized, immortalized *ras* oncogene expressing and now the fully tumourigenic HMF 3wtX line we can start to investigate the crucial step by step changes that have occurred in the original unaltered cells to confer a fully transformed malignant phenotype.

8.3 Potential improvements to current models

The failure to establish a fully human humanized xenograft model, in nude mice, by using SV40 LT immortalized stromal cells can be attributed to one of two possible causes, namely the animal system or the cells used. Therefore, change in either one or both of these components of the model system may provide more success in the future. Thus, in an attempt to overcome the problems previously encountered in developing a fully humanized model several new ideas and

concepts can be considered. These relate to both the animal hosts and the cells to be engrafted.

Animals

Before discussing the advantages and disadvantages of different varieties of alternative cells available for use, the most appropriate animal carrier system needs to be identified. With the increasingly greater frequency of xenograft studies incorporating tumour and stromal stem cells (e.g. CEPs), as well as the future prospect of humanized mammary fat pads created from stem cells, the best possible animal system for success needs to be designed or discovered. A growing number of studies are reporting that NOD/SCID mouse provides the best animal carrier system for human stem cell studies (Rice et al. 2000, van der Loo et al. 1998, van Hennik et al. 1999). The NOD/SCID mutation results in an immunodeficient mouse with multiple defects in adaptive as well as non-adaptive immunologic function. This strain of mice lack functional lymphoid cells, serum complement activity and show little or no serum immunoglobulin with age. The multiple defects in innate and adaptive immunity unique to the NOD/SCID mouse provide an excellent *in vivo* environment for reconstitution with human haematopoietic cells.

In addition to its greater level of immunosuppression the systemic hormonal environment of NOD/SCID mice during puberty or pregnancy has been demonstrated to be sufficient to promote human mammary epithelial cell proliferation and lactogenic differentiation (Parmar et al. 2002). The use of NOD/SCID mice rather than nude mice now means ectopic oestrogen supplementation is not required for mammary epithelial cell proliferation *in vivo* (Parmar et al. 2002). Unlike nude mice, whose reproductive system and thus hormonal environment are compromised (Kopf-Maier and Mboneko 1990),

NOD/SCID female mice are fertile, with a fully functional reproductive system. This may suggest that the generally observed graft-host interspecies incompatibilities, observed in previous nude mice based studies, originate from paracrine heterotypic interactions rather than from incompatibilities in the systemic hormonal environment.

This strain of mice can more readily accept and cultivate hard to grow cells and so it is possible that they will be more amenable to the growth of the SV40 LT immortalized human mammary fibroblasts and endothelial cells. Furthermore, as discussed in detail in the discussion of Chapter 6, the residual NK cell activity in nude mice (Cudkowicz and Hochman 1979, Kiessling et al. 1977, Yu et al. 1992) was raised as a significant factor that may have caused the death of the immortalized stromal cells. As NOD/SCID mice have little or no residual NK cell activity the use of this strain could quickly resolve the issue of whether NK cell activity was an important factor. Alternatively the population of NK cells in nude mice could be depleted by treatment with anti-asialo-GM1 antibody as others have demonstrated prior to administration of the human cells (Barry et al. 1991, Shpitz et al. 1994, Lacerda et al. 1996).

Stromal cells

Before discussing the alternative cells available for use in the model there is another method of testing the hypothesis of NK cell induced cell death in nude mice; by changing the method of immortalization. NK cells are attracted to cells expressing viral and bacterial proteins. Therefore an alternative method of immortalization such as viral or bacterial constituents (e.g. not SV40 LT) may provide more success. For example, the human mammary stromal cells could be immortalized by other methods such as ionizing radiation and chemical carcinogens (Newbold et al. 1982), although these methods are clearly very

inefficient compared with viral genes used in the present study. These cells will not be subject to attack by the NK cells. Presently there are no human adult mammary fibroblasts and endothelial cells that have been manufactured in this manner. Although human cells apparently immortalized with telomerase only have been reported (e.g. BJ foreskin human fibroblasts) these have also been engineered using viral type vectors and include bacterial selection markers. When selecting alternative cells for use in the development of the 3-compartment xenograft model, two important factors must be considered. These are the engraftability/survivability *in vivo* and secondly the authenticity of the cells as representative of real tumour associated stroma.

With the recent availability of the tumourigenic mammary fibroblast cell line, HMF3wtX, it can now be xenografted in combination with breast tumour epithelial and the immortalized endothelial cells. The HMF3wtX fibroblasts have been demonstrated to consistently survive *in vivo* and form tumours. The rationale of this study would be to determine whether this line affected breast epithelial tumour take rates or whether it would overpower the co-engrafted tumour epithelial cells to form a predominantly fibroblast rich sarcoma rather than a solid epithelial carcinoma. However if this was the case the growth of the HMF 3wtX's could be attenuated, for example by irradiation or anti-mitotics prior to engraftment. Also it could be determined whether the HMF3wtX fibroblasts would interact and stimulate the endothelial cell to firstly survive and secondly differentiate to form complex structures (e.g. blood vessels) If, however, the tumour growth is promoted and the xenograft remains predominantly epithelial then we would have developed a truly 'humanized' two compartment xenograft tumour model. This cell line needs to be characterized in

more detail to fully determine its authenticity in terms of its retention of normal fibroblast function.

It is well recognised that stromal cells found in tumours (e.g. breast, prostate etc.) differs from those found in the normal stromal microenvironment. Therefore it is important to note that the 3-compartment model described in this thesis adopted the use of immortalized normal mammary fibroblasts as opposed to tumour associated fibroblasts which may be a crucial factor in determining their survival as well as their effect on tumour cell growth. As the technique for immortalization is available and easily reproducible the generation of immortalized tumour associated mammary fibroblasts and endothelial cells would not be too difficult. Hence the use of immortalized tumour associated fibroblasts and endothelial cells in the 3-compartment models could provide a more physiologically relevant interaction improving their growth and authenticity. However such a model would require the use of NOD/SCID mice to avoid the problems anticipated with NK cell activity.

As introduced in Chapter 1, both fibroblasts and endothelial cells represent a very diverse and heterogeneous population of cells possibly derived from multi-potent stem cells capable of organ and tissue specific differentiation. For example, cells labelled as common fibroblasts can differentiate into a variety of cell types e.g. fat, cartilage, muscle etc. Circulating endothelial progenitor cells found in several different types of haemopoietic tissue e.g. bone marrow, foetal blood can differentiate into organ specific endothelial cells. Due to the fact that CEPs are ontogenically less mature than angioblasts, they can be useful for the characterization of endothelial cell commitment and differentiation. Moreover the fact that they do form new vessels provides exciting prospects. The therapeutic potential of endothelial progenitor cells was recently demonstrated by

the improved blood flow rates and capillary density following the transplantation of endothelial progenitor cells in hind limb ischaemic mice (Kalka et al. 2000). In another study by Levenberg et al. (2002) showed that when CEPs were transplanted *in vivo*, into SCID mice, the cells appeared to form microvessels containing mouse blood cells. There is clear evidence that these cells have the capacity to functionally contribute to the growth of certain tumours (Rafii et al 2002). By isolating these multi-potential cells and then engrafting into multi-compartment tumour models we may be able to overcome the problems of cell survival and provide an alternative means of introducing stromal elements. Furthermore these cells wherever they are placed would differentiate into organ specific fibroblasts and endothelial cells enhancing model authenticity.

One important issue that requires clarification is how recruited CEPs and pro-angiogenic haematopoietic cells are temporally and spatially related to tumour growth. Based on mouse tumour models, it seems that these cells are most likely to contribute to the early stages of tumour growth, but are only involved in the angiogenic phase. It is also not known whether CEPs are required to maintain the long-term integrity of established tumour vessels.

It is also unclear which tumour types are most dependent on CEPs and haematopoietic cells for their growth. Based on the available studies, mobilization and functional incorporation of CEPs into the tumour vasculature is essential for the growth of a few tumour types (e.g. lymphomas, Lewis lung carcinoma and colon cancers). Therefore additional studies are required to identify the tumour types that are partially or fully dependent on the recruitment of CEPs. One possible hypothesis is that unique chemokines/cytokines and tissue-specific ECM that are produced by each tumour cell type are the main determinants for the recruitment of CEPs. Identification of such unique ECM and

chemo/cytokines will provide new targets to tailor the treatment of each specific malignancy. The ability to target these cells in patients with malignancies is still a distant prospect. Further understanding of the biology of CEPs and endothelial cells is essential for the development of new methodologies to treat cancer, promote vascular healing, provide suitable coating for vascular grafts, and for the delivery of toxins to tumour vascular beds.

Tumour cells

Human breast tumours are comprised of phenotypically diverse populations of breast cancer cells expressing a variety of cell surface markers including (CD44, CD24, and B38.1) (Ahrens et al. 2001, Uchida et al. 2000, Kufe et al. 1983). It has, therefore, been hypothesized that only a rare, phenotypically distinct subset of cells within breast cancers have the capacity to significantly proliferate and form new tumours, but that cells within this subset do so very efficiently (Reya et al. 2001). Therefore, the traditional thinking that a large numbers of cells must typically be inoculated to give rise to tumours in xenograft models may be clumsy and inefficient.

Very recently it has been reported that subsets of immunophenotypically distinct ($CD44^+/CD24^-$) breast cancer cells within primary breast tumours had the ability to form new tumours and have enhanced take rate as xenografts (Al-Hajj et al. 2003). As few as 100 cells with this phenotype were able to form tumours in mice, whereas tens of thousands of breast cancer cells, from the same tumour, with alternate phenotypes failed to form tumours. This tumourigenic sub-population could also be serially passaged: each time cells within this population generated new tumours containing additional $CD44^+CD24^{-/low^-}$ lineage tumourigenic cells as well as the phenotypically diverse mixed populations of non-tumourigenic cells present in the initial tumour. It is hoped that by isolating

these cells from primary breast tumours they can be used to improve xenograft take rates as well as accelerate the development of much needed xenograft models.

These results, therefore, demonstrate that there is a hierarchy of breast cancer cells in which some cells have the ability to proliferate extensively, whereas the majority of tumour cells that can be derived from this population have only limited proliferative potential *in vivo*. This sub-population of proliferative cells may represent multi-potent epithelial progenitor cells. The ability to prospectively identify tumourigenic cancer cells will facilitate the elucidation of pathways that regulate their growth and survival. Furthermore, because these cells drive tumour development, strategies designed to target this population may lead to more effective therapies. Traditionally drug therapies have been developed based on their ability to cause tumour regression in animal models. Due to the fact that the majority of cancer cells within a tumour may be of the non-tumourigenic type, therapies directed at these cells would cause tumour regression. However, if therapies fail to target the tumourigenic cells, then these cells would persist after therapy and be able to regenerate the tumour, resulting in tumour relapse.

It is recognised that normal stem cells have mechanisms that make them relatively resistant to cytotoxic drugs, such as increased expression of BCL-2 family proteins, increased expression of membrane transporters like breast cancer drug resistance protein, and multiple drug resistance (Lagasse and Weissman 1994, 1997, Domen et al. 1998, Zhou et al. 2002). The expression of such proteins in tumourigenic breast cancer stem cells may make them inherently more resistant to current therapies. The prospective identification of this tumourigenic population of cancer cells should allow the identification of

molecules expressed in these cells that could be targeted to eliminate this crucial population of cancer cells, leading to more effective cancer therapies.

8.4 Future prospects

The concepts described above are logical extensions of the work in this thesis with a similar but refined strategy. There remain several additional ideas that could further enhance the value of human xenograft system for modelling tumour development and treatment. These include humanizing the animal immune system and humanizing its mammary gland.

Development of a humanized immune system in animals

A fundamental limitation inherent to all xenograft models is the lack of an immune response against the tumour cells. With the development and testing of new therapeutic strategies being a key function of these models it is unfortunate that the action of therapeutic strategies that incorporate the immune response in the fight against cancer will be impaired. For example tumour vaccines and gene therapy strategies enlist immune cells to attack and kill tumour cells. As animal models used in xenografts are immunodeficient by definition they lack all or a significant aspect of their immune function. However, there are several potential solutions to the immune response problem in the context of modelling immunotherapies. For example, it has been shown that non-disrupted pieces of tumour biopsy tissues implanted into SCID mice resulted in the co-engraftment of tumours plus tumour infiltrating lymphocytes (TIL), with TILs within the tumour graft remaining functional and responding to lymphocyte cytokines (Sugiyama et al. 2001). Human peripheral blood lymphocytes, injected subcutaneously with a human lung tumour into SCID mice, also engraft and

display antitumour cytotoxic activity (Iwanuma et al. 1997). One could, therefore, envisage the use of immunodeficient mice (e.g. nude or SCIDs) with humanized immune systems following engraftment with human bone marrow stem cells (Lubin et al. 1991).

Development of a humanized mammary gland in animals

Although the mouse and human mammary glands share a reasonable level of similarity they also have some marked differences (Ronnov-Jessen et al. 1996). For example, due to the fact that the mouse mammary gland is composed entirely of adipocytes with fibroblasts prominent only in narrow sheaths around the ducts, whereas in the human gland the ducts are embedded in large tracts of connective tissue (composed predominantly of fibroblasts) that are adjacent to deeper layers of adipose tissue (Hovey et al. 1999), it is reasonable to suggest that the environment of the mouse mammary fat pad in mice is quite different to the human breast (Neville et al. 1998). Also human mammary epithelial cells are never found in the adipose tracts of the breast. Therefore, both human mammary stromal and epithelial cells may require and in turn thrive in the specialized stromal microenvironment of the human gland rather than the largely adipose tissue microenvironment of the mouse mammary gland.

In addition the actions of several steroid and peptide hormones appear to be mediated by an array of growth factors, proteases and ECM components synthesized by constituents of the mammary fat pad (Hovey et al. 1999). Furthermore, mammary adipose tissue represents a significant store of lipid that, by itself and through its derivatives, could influence the growth of mammary epithelium in diverse ways. In summary the vital role of the mammary fat pad during mammary gland development, emphasizes the point that the great species

differences must be addressed if local growth and morphogenic mechanisms within the mammary gland are to be resolved.

In an attempt to overcome these problems cleared mammary fat pads were commonly used in early xenotransplantation studies (DeOme 1959). This technique took advantage of the fact that the mouse mammary gland is not fully developed at 3 weeks of age, making it possible to excise the rudimentary mouse mammary epithelium from the fat pad yielding a cleared fat pad devoid of any epithelium. However cleared fat pads still contain several other cell types, such as fibroblasts, endothelial cells and cells of the immune system, all of which could influence mammary gland development. Attempts to recapitulate human breast epithelial morphogenesis by introducing human mammary epithelial cells into the cleared mammary fat pads of mice have not yielded similar results. Unlike their murine counterparts, the introduced human mammary epithelial cells fail to colonize these fat pads and thus do not develop into ductal and lobular structures (Outzen and Custer 1975, Sheffield 1988) To get round this issue, researchers have attempted to recreate functional tissues by transplanting normal breast tissue fragments or collagen-embedded dissociated human mammary epithelial cells either subcutaneously or beneath the renal capsule of immunocompromised nude mice (Parmar et al. 2002, Yang et al. 1994). Although the tissue fragments and dissociated cells survive and respond to hormone stimulation, no outgrowth or expansion of the transplanted tissues has been observed.

For these reasons, one possible way forward would be to develop a new protocol for the establishment of human mammary stroma within the mouse mammary fat pad, anticipating that the resulting stroma would provide the proper environment for the development of human mammary epithelium.

To be able to study normal breast homeostasis as well as tumourigenesis with the ultimate physiological relevance, a model must mimic not just the tissues *in vivo* but the entire organs involved (Bissell and Radisky 2001). In order for this to happen we must develop a 'humanized' mammary gland by replacing mouse mammary gland components with their human counterparts thereby reconstructing an organ that is comparable to the human gland in its architecture, organization and composition. In attempts to recreate breast tissues in mice past studies have attempted to introduce tissue fragments or dissociated mammary epithelial cells into the cleared mouse mammary fat pads of immunocompromised mice (Outzen and Custer 1975, Sheffield 1988). These attempts were unsuccessful in recreating functional, properly differentiated breast tissues, presumably due to the inadequate stromal environment provided by the mouse mammary fat pad. We now have the relevant cells and techniques (genetic and tissue engineering) to create a humanized mammary gland model in mouse, specifically in the mammary fat pad. Human mammary stromal fibroblasts grafted into the fat pads of nude mice support elaboration of transplanted human mammary epithelial cells into an infiltrating ductal tree that is highly differentiated and responsive to lactogenic cues (Kuperwasser et al. 2004). Once a humanized mammary fat has been established genetically modified epithelial or stromal cells can be incorporated thereby providing a model for the controlled analysis of specific molecules and pathways in an *in vivo* context. Furthermore, the humanized mouse could be used for other applications such as the development of strategies for reconstructing human breast after surgery (Huss and Kratz 2001).

Future versions of humanized mammary fat pads could incorporate human mammary progenitor cells. The existence of mammary epithelial stem

cells has been the subject of much debate, but recent evidence demonstrates the existence of precursor cells within the luminal epithelial pool (Smith 1996, Stingl et al. 1998, Smalley et al. 1999). Recently, (Gudjonnson et al. 2002a) have isolated and immortalized a human mammary progenitor cell that gives rise to structures that resemble terminal ductal lobular units (TDLU) when implanted either in Matrigel or orthotopically into nude mice (Gudjonsson et al. 2002b). Thus it seems reasonable that introduction of human progenitor cells in the mouse mammary gland may be a useful strategy for generating chimeric animals with extensively humanized mammary organs.

Ultimate model

It is natural to assume that the more human components are used the greater the resemblance to the human situation. Once these issues are overcome then the more complex problems of growth and serum factors as well as hormonal influences and dependency have to be addressed. The closer this model resembles human breast cancer *in vivo* the more credible preclinical trials will become.

To conclude, as metastasis is the primary cause of mortality in breast cancer the ultimate goal is to develop authentic models of local invasion and metastases. If and when a fully humanized xenograft model is built containing human stroma including human blood vessels, one can commence development of chemotherapeutic strategies that target these components that permit local invasion and metastases. As these targets within the mouse model will be of human origin with typical human antigen expression profiles the relevance of this model as a pre-clinical model will be undoubted.

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Appendix I Source of reagents

Reagent	Source
Acetone	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Benzamidine hydrochloride	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
β-mercaptoethanol	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Borate: di-sodium tetraborate AnalaR	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Bovine serum albumin (BSA)	Sigma, Poole, Dorset, UK
Calcium chloride	Sigma, Poole, Dorset, UK
Chloroform AnalaR	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
'Chloros' (10%) Sodium hypochlorite	Haychlor-Hayes Chemicals West Yorkshire, UK
Chymotrypsin	Sigma, Poole, Dorset, UK
Citifluor antifadant AF1	Citifluor Ltd, University of Kent, Canterbury, Kent, UK
Deoxycholate AnalaR	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
diaminobenzidine tetrahydrochloride, 3-3' diaminobenzidine (DAB)	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK

Dimethyl Sulphoxide	Sigma, Poole, Dorset, UK
DEPC water	Sigma, Poole, Dorset, UK
DNTPs	Perkin Elmer, via Applied Biosystems, Birchwood Science Park, North Warrington, UK
DPX mountant	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Dulbecco's PBS (EDTA)	Biowhittaker
EGM-2 Bullitkit	Biowhittaker
Eosin	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Ethanol AnalaR	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
ethylenediaminetetraacetic acid in Ca++ and Mg++ free Dulbecco's PBS (EDTA)	Imperial, Andover, Hampshire, UK
Ficoll-Hypaque	Sigma, Poole, Dorset, UK
Foetal calf serum (FCS)	Imperial, Andover, Hampshire, UK
Formaldehyde AnalaR	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Glutaradehyde	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Glycine	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Ham's F12	Biowhittaker

Hanks balanced salt solution	Gibco, via Life Technologies, Paisley, Renfrewshire, UK
Harris type haematoxylin	Sigma, Poole, Dorset, UK
Hoechst 33258	Hoechst, now Aventis, Strasbourg, F67917, Germany
Hydrochloloric acid (HCl)	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Hydrogen peroxide AnalaR	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Hydrocortisone	Sigma, Poole, Dorset, UK
Hydromount	National Diagnostics, Hull, Humberside, UK
Insulin	Sigma, Poole, Dorset, UK
Isopropanol AnalaR	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
L-glutamine	Sigma, Poole, Dorset, UK
Matrigel	Becton Dickinson Labware, Bedford MA, USA
Methanol AnalaR	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Methylene blue	Sigma, Poole, Dorset, UK
O.C.T. compound	Tissue-Tek RA Lamb Ltd Eastbourne, East Sussex
RPMI 1640 Medium	Gibco, via Life Technologies,

	Paisley, Renfrewshire, UK
Penicillin	Gibco, via Life Technologies, Paisley, Renfrewshire, UK
Phenol AnalaR	Sigma, Poole, Dorset, UK
Phosphate buffered saline (PBS)	Sigma, Poole, Dorset, UK
Sodium chloride AnalaR	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Sodium citrate AnalaR	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Streptomycin	Gibco, via Life Technologies, Paisley, Renfrewshire, UK
Trishydroxymethyl-methylamine (Tris)-HCl	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Triton-X	Sigma, Poole, Dorset, UK
Trypsin	Sigma, Poole, Dorset, UK
Tween-20 Organiscs	Sigma, Poole, Dorset, UK
Type III trypsin	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Water HiPerSolv for HPLC	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
Vectabond	Vector Laboratories, Orton Southgate, Peterborough, UK
Vectasatin universal elite ABC Kit	Vector Laboratories, Orton Southgate, Peterborough, UK
Xylene AnalaR	BDH Laboratory Supplies, Merck,

	Lutterworth, Leicestershire, UK

Appendix II Source of consumables

Consumable	Source
Cell lifter	Sigma, Poole, Dorset, UK
Coverslips (13mm)	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
0.4 μm pore sized polyethylene terephthalate membranes ($1.6x10^6$ pores /cm^2) (Catalog Number 353095, BD Falcon™ 0.4 μm pore	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
ECL Hyperfilm	Amersham Life Science, Little Chalfont, Buckinghamshire, UK
25cm^2 flasks	Falcon, via Becton Dickinson, Cowley, Oxford, UK
175cm^2 flask	Falcon, via Becton Dickinson, Cowley, Oxford, UK
Hamilton Syringe	Hamilton Company, PO Box 10030, Reno, Nevada, USA
24 well plates	Gibco, via Life Technologies, Paisley, Renfrewshire, UK
2 ml safe-lock tubes	BDH Laboratory Supplies, Merck, Lutterworth, Leicestershire, UK
15ml tube	Falcon, via Becton Dickinson, Cowley, Oxford, UK

Appendix III Source of equipments and computer programs

Program	Source
Anglia Scientific AS300 Microtome	**Anglia** Instruments, Cambridge,UK
Adobe Photoshop	v7.0, Adobe Systems Inc., California, USA
Axiophot microscope	Zeiss, Wellyn Garden City, Hertfordshire, UK
Bright Cryostat	Huntingdon, Cambridgeshire, England
Centrifuge 5410 for 5000rpm	Eppendorf, via Merck Eurolab Ltd, Poole, Dorset, UK
Centrifuge IEC Centra MP4R for 14000rpm	Thermo Quest, Basingspole, Hampshire, UK
Coolview 12 cooled Charge Coupled Device (CCD) camera	Photonic Science, Newbury, Berkshire, UK
High resolution Leaf camera	ISS, Greater Manchester, UK
Image-Pro Plus	v 4.01, Media Cybernetics, Baltimore, Maryland, USA
MRX microplate reader	Dynatech Laboratories, Ashford, Middlesex, UK
PU8625 UV/VIS Spectrophotometer	Philips, Cambridge, Cambridgeshire, UK
Personal computer	Dell, Bracknell, Berkshire, UK
SCSI interface	AHA-2940, Adeptec Inc., California, USA

Shaker	Genetic Research Instrumentation, Felsted, Essex, UK
Shandon Citadel™ 2000 Tissue Processor	Thermoelectron, Bucks
125-ml Wheaton Magna Flex Spinner Flask	(Fisher/Wheaton Scientific, Pittsburgh, PA)
Snapper frame grabber card	Action Imaging, Huntingdon, Cambridgeshire, UK
SPSS version 7.5	Illinois 60606, USA
Windows 95 version 4.0	Microsoft, London, East Anglia, UK
125-ml Wheaton Magna Flex Spinner Flask	(Fisher/Wheaton Scientific, Pittsburgh, PA)