Modelling breast epithelial-endothelial interaction in three-dimensional cell culture

A thesis submitted for the degree of Master of Science

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Ágrip

Brjóstkirtillinn samanstendur af tveimur megingerðum þekjuvefsfruma, kirtilþekju- og vöðvaþekjufrumum. Saman mynda þessar frumugerðir hina greinóttu formgerð brjóstkirtilsins. Kirtilvefurinn er umlukinn æðaríkum stoðvef sem inniheldur margar mismunandi frumugerðir, þ.m.t. bandvefsfrumur og æðaþelsfrumur. Þroskun og sérhæfing kirtilsins er mjög háð samskiptum hans við millifrumuefni brjóstsins og frumur stoðvefjarins. Mest áhersla hefur verið lögð á rannsóknir á bandvefsfrumum í þessu tilliti, en minni athygli beint að æðaþelsfrumum, sem voru lengi taldar gegna því hlutverki einu að miðla súrefni og næringu um líkamann. Á síðustu árum hefur verið sýnt fram á að nýmyndun æða í krabbameinsæxlum spili stórt hlutverk í framþróun æxlisvaxtar og hefur það verið tengt slæmum horfum. Nýlegar rannsóknir hafa sýnt fram á mikilvægt hlutverk æðaþels í þroskun og sérhæfingu ýmissa líffæra, til dæmis í heila, lifur og beinmerg sem og í framþróun krabbameins. Nýleg þekking bendir einnig til mikilvægra áhrifa æðaþels á þroskun eðlilegs og illkynja brjóstvefjar. Markmið verkefnisins er að kanna áhrif brjóstaæðaþels á eðlilegar og illkynja brjóstaþekjufrumulínur og nota til þess þrívíð ræktunarlíkön sem þróuð voru á rannsóknastofunni, sem og að endurbæta þessi líkön til frekari rannsókna á samskiptum æðaþels og þekjufruma. Flestar frumulínur eru af kirtilþekjuuppruna en skortur er á frumulínum sem endurspegla svipgerð vöðvaþekjunnar. Búin var til ný frumulína af vöðvaþekju uppruna úr vef fengnum úr brjóstaminnkunaraðgerð. Frumulínan hefur svipgerð samskonar þeirri sem sjá má í vöðvaþekjufrumum. Í samrækt með

æðaþeli mátti sjá mikla aukningu á stærð frumuþyrpinga, sem og á fjölda frumuþyrpinga miðað við viðmið án æðaþels, bæði hjá eðlilegum og illkynja þekjufrumum. Til að kanna betur þessi áhrif var þróað nýtt samræktunarlíkan sem nota má til að greina áhrif leysanlegra þátta á þekjufrumur. Niðurstöður benda til að áhrifum æðaþelsfrumanna sé miðlað af leysanlegum þáttum sem dreifast um gelið, en ekki af beinni snertingu á milli frumugerðanna. Áframhaldandi rannsóknir á samskiptum æðaþels og þekjufruma og greining á þáttum sem eiga þar hlut munu varpa mikilvægu ljósi á hlutverk æðaþels í þroskun brjóstkirtilsins og í framþróun krabbameins.

Abstract

The branching epithelial structure of the breast is surrounded by a highly vascularised stroma that is rich in collagen. The role of stroma (mesenchyme) in organogenesis and development has been widely recognized. However, the complexity of the stroma, with its many different cell populations makes it challenging to identify the modulating factors that affect breast development. Development of the breast gland is under tight control, both from systemic hormones, as well as by interaction with cell populations within the breast, such as fibroblasts and myoepithelial cells. The enveloping architecture of the myoepithelial cells in the normal breast suggests they might act as natural tumour suppressors, by maintaining tissue polarity. There is a distinct lack of myoepithelial cell lines available for research. Fibroblasts have received much attention regarding breast morphogenesis, not much attention has been given to the other major cell type of the mammary stroma, the endothelial cells. In addition to supplying organs with oxygen and nutrients and removal of waste products, data is emerging showing endothelial cells as major players in the development of many organs such as the bone marrow, liver and pancreas. Endothelial cells are also important factors in cancer progression by vascularising the tumour, supplying it with nutrients and removing waste. New data is emerging showing that endothelial cells also affect growth of tumours by production of growth factors. The aim of this project is to develop a new three-dimensional co-culture system that can be used to analyse the proliferative and morphogenic effect of breast endothelial cells on normal and

malignant breast epithelial cells. In co-cultures, a significant increase in epithelial colony size was observed as well as an increase in cloning efficiency when compared with endothelial-free control. To further analyse the proliferative and morphogenic effects seen I developed a new co-culture system that can be used to explore the effect of soluble factors in co-culture. Results indicate that the proliferative effects of endothelial cells are caused by soluble factors that spread through the culture; whereas the morphogenic signal could be caused by a different mechanism, for example direct cell-cell contact. Future research in the laboratory aims to identify the factors involved as well as to improve the co-culture models for further research.

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Abbreviations

αSMA	Alpha smooth muscle actin
BRENC	Breast endothelial cell
BSA	Bovine serum albumin
CCL5	Chemokine ligand 5
CDM3	Chemically defined medium 3
Ck	Cytokeratin
DCIS	Ductal carcinoma *in situ*
DMEM	Dulbecco's modified eagle's medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EHS	Engelbreth Holm-Swann
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMT	Epithelial-mesenchymal-transition
ERα	Oestrogen receptor alpha
ESA	Epithelial specific antigen
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
HER	Human epidermal growth factor receptor
HGF/SF	Hepatocyte growth factor/scatter factor
HIF1α	Hypoxia inducible factor 1 alpha
HMW	High molecular weight
HPV	Human papilloma virus
HUVEC	Human umbilical vascular endothelial cells
IGF	Insulin-like growth factor
IDC	Invasive ductal carcinoma
ISCN	International system for human cytogenetic nomenclature
IU	International units
LEP	Luminal epithelial cell
MACS	Magnetic cell sorting
MAPK	Mitogen-activated protein kinase
MEP	Myoepithelial cell
MMP	Matrix metalloprotease
MUC-1	Sialomucin-1
NOD	Non obese diabetic
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
rBM	Reconstituted basement membrane
RNAi	RNA interference
RPM	Rotations per minute
SCID	Severe combined immunodeficiency
SHH	Sonic hedgehog
TDLU	Terminal duct lobular unit
TGFβ	Transforming growth factor beta
TIMP-1	Tissue inhibitor of matrix metalloprotease-1
TW	Transwell filter
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
WT1	Wilm's tumour protein

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Figures and tables

I – Introduction

1 – Breast development

The breast gland is a modified sweat gland that develops early in embryonic development with the thickening of the epidermis. This is followed by an invasion into the underlying mesenchyme. At birth there is no discernible difference between the sexes, the gland has a basic structure of branching ducts, with some variation in branching between individuals, likely caused by maternal hormonal levels. Until the onset of puberty there is limited development of the breast gland. With the onset of puberty the female breast undergoes a period of significant changes, including increased cell proliferation and differentiation. The cells in the ductal ends, which from birth have looked similar to the terminal end buds of the mouse mammary gland, proliferate and invade the surrounding stroma. They branch into multiple acini, forming the functional units of the breast, the Terminal Duct Lobular Units (TDLUs). TDLUs are a multiacinar structures resembling a cluster of grapes (figure 1a). (Strange *et al.*, 1992, Cardiff and Wellings, 1999, Howard and Gusterson, 2000, Djonov *et al.*, 2001).

2. – The cellular context of the human adult breast gland

2.1 – Epithelial cells

The branching epithelial compartment of the adult female breast is composed of two distinct epithelial lineages, an inner layer of

luminal epithelial cells (LEP) and an outer layer of myoepithelial cells (MEP) (figure 1b). LEPs are polarized and line the lumen of the acini and ducts. In the lactating breast, LEPs produce milk that is secreted
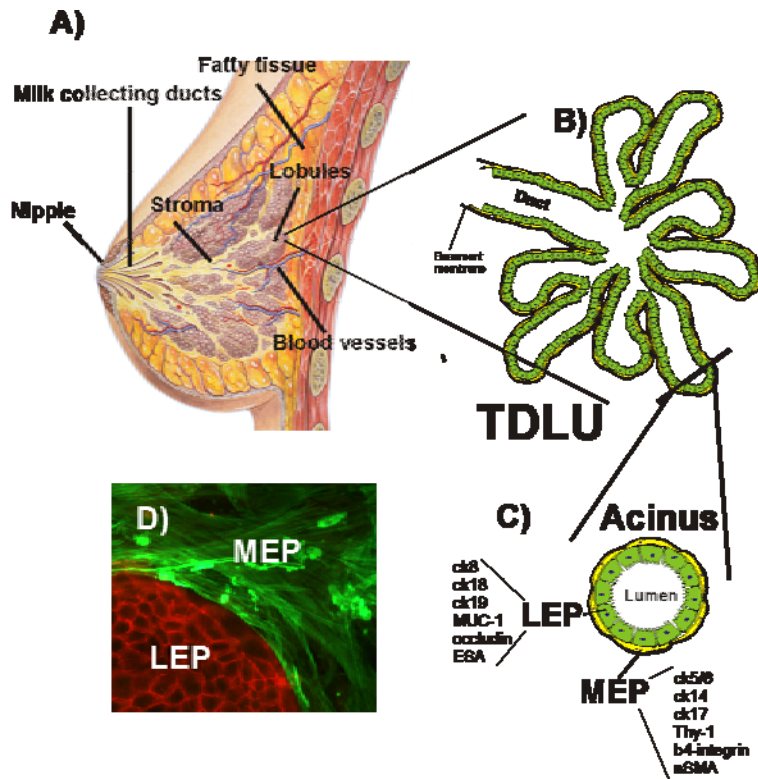
Figure 1: Schematic structure of the human breast gland. (**A**) The human breast gland is composed of a branching epithelial structure starting from the nipple and penetrating into the breast terminating in the acini in the Terminal Duct Lobular Units (TDLUs). (**B**) The TDLUs are composed of multiple acini resembling a cluster of grapes. (**C**) Each acinus is composed of a double layered epithelial structure with an inner layer of luminal epithelial cells (LEP), expressing LEP specific markers, and outer layer of myoepithelial cells (MEP), expressing MEP specific markers. (**D**) Immunofluorescent staining of LEPs (Occludin, red) surrounded by MEPs (alpha smooth muscle actin, green) in monolayer culture.

into the lumen in an apocrine fashion. MEPs are situated between the LEPs and the surrounding stroma and basement membrane (figure 1c). In the acini, MEPs appear stretched and do not form a continuous layer, meaning that some LEPs are in direct contact with the basement membrane (Howard and Gusterson, 2000, Fridriksdottir *et al.*, 2005).

In ducts, MEPs assume a cubodial structure, characteristic of basal cells in other epithelial structures, and form a continuous layer, separating the luminal compartment from the surrounding stroma. LEPs and MEPs are believed to originate from a common progenitor (stem cell) in the epithelium (Pechoux *et al.*, 1999, Fridriksdottir *et al.*, 2005, Polyak, 2007). The two epithelial cell lineages can be distinguished and isolated from one another using several cell surface- and cytoskeletal markers (figure 1c,d). LEPs strongly express cytokeratins ck8, ck18 and ck19. They also express sialomucin-1 (MUC-1) and epithelial specific antigen (ESA). LEPs are polarized and express tight junction proteins such as occludin (figure 1d) and claudin. MEPs express different cytoskeletal markers, high molecular weight (HMW) cytokeratins ck5/6, ck14, ck17 and alpha smooth muscle actin (αSMA) (figure 1d). During lactation, neuroendocrine cells in the brain release oxytocin to the bloodstream stimulating MEPs to contract causing milk in the ducts to be extruded towards the nipple. MEPs are in close contact with the basement membrane and express hemidesomosome proteins such as β4-Integrin. MEPs also express P63, a P53 homolog, which has been shown to take part in epithelial stem cell regulation and differentiation (Barbareschi *et al.*, 2001, McKeon, 2004, Hu *et al.*, 2008). Using antibodies raised against cell surface proteins it became possible to isolate pure populations of LEPs and MEPs (O'Hare *et al.*, 1991, Pechoux *et al.*, 1999). The fact that isolation and culture of different cell populations *in vitro* is possible opens up possibilities to conduct recombination studies to analyse cell-cell interactions in the normal and malignant breast gland.

2.2 - The breast stroma

The branching epithelial structure of the breast is surrounded by a highly vascularised stroma that is rich in collagen. Around the TDLUs, the collagenous stroma is relatively loosely packed, but peripherally the collagen matrix is much denser (Cardiff and Wellings, 1999). Several populations of cells can be found within the stroma, such as cells of the immune system, adipocytes, fibroblasts and endothelial cells, the last of which are in very close proximity with the TDLUs, (figure 2). The stroma is also rich in growth factors and extracellular matrix (ECM) proteins, such as laminin and collagen IV. The stroma is known to be a major player both in breast development and tissue remodelling during pregnancy and lactation by secretion of growth factors and proteases (Kuperwasser *et al.*, 2004, Kass *et al.*, 2007).
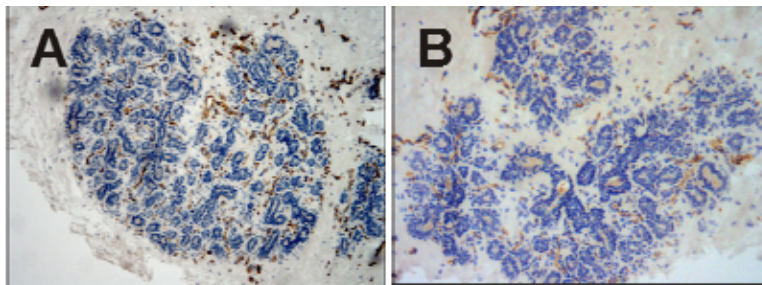
Figure 2: The close relationship of the vasculature and epithelial structures in the adult female breast. Endothelial cells are in close relationship with the acini in TDLUs, encapsulating them in a basket like fashion revealed in staining for (**A**) CD31 and (**B**) VE - cadherin. (adapted from (Sigurdsson *et al.,* 2006))

3 – Tissue remodelling in the human breast

During pregnancy, lactation and post-lactational involution, the breast gland undergoes a major period of cell proliferation and

differentiation, followed by apoptosis (figure 3). During pregnancy, the resting TDLUs (figure 3a) expand into the surrounding stroma due to expansion of the epithelial structure. During lactation the acini are dilated (figure 3b) and filled with milk that is pushed towards the nipple by the contractile function of the myoepithelial cells as described above. After weaning the breast gland goes through a period of involution, the milk producing cells are removed by apoptosis and the gland is reduced to a form similar to what it was before pregnancy (Strange *et al.*, 1992, Howard and Gusterson, 2000). This remodelling process is also seen during each menstrual cycle, however, to a much lesser degree (Andres *et al.*, 1995, Dabrosin, 2003). Like the previous proliferative events, this monthly cycle is caused by several factors, both endocrine and paracrine (Howard and Gusterson, 2000, Neville *et al.*, 2002). Endocrine hormones such as oestrogen and progesterone, which have been shown to affect duct elongation and TDLU expansion respectively, play an important role in control of breast gland remodelling. Paracrine factors are also produced in the breast gland itself, by the epithelial- and stromal cells, such as growth factors and other bioactive peptides (Clevenger and Plank, 1997, Mol *et al.*, 2000, Kass *et al.*, 2007).

3.1 - The role of the stroma in breast development and organogenesis

The role of stroma (mesenchyme) in organogenesis and development has been widely recognized (Robinson *et al.*, 1999, Kuperwasser *et al.*, 2004, Proia and Kuperwasser, 2005). In mice, it has been shown that non-mammary embryonic epithelium can be

induced by mammary mesenchyme to form milk producing mammary structures (Cunha *et al.*, 1995). Moreover, it has recently been shown that increased sonic hedgehog (shh) expression in the mesenchyme in conjuncture with shh ablation in the epithelium can cause hair follicles to form mammary gland-like structures in mice (Gritli-Linde *et al.*, 2007). It has also been shown that abnormal stroma can facilitate cancer formation in phenotypically normal breast epithelium displaying the important role of stromal tissue in breast development and cancer (Kuperwasser *et al.*, 2004). The complexity of the stroma, with its many different cell populations makes it challenging and important to identify the modulating factors that affect breast development.
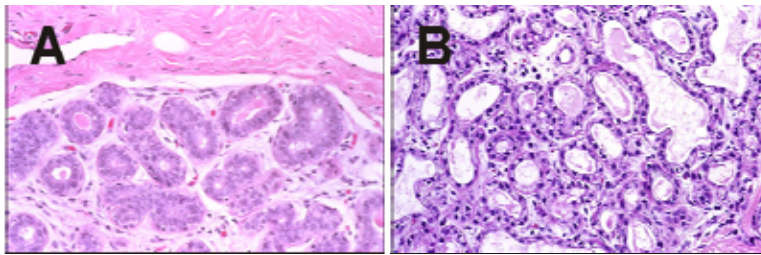
Figure 3: Changes to the breast gland during lactation. (**A**) resting acini, no milk production. (**B**) Lactating breast, acini are dilated and producing milk, which is being released into the lumen. (adapted from (Ross *et al.*, 2002))

3.2 - Fibroblasts

As mentioned earlier, the stroma contains several different cell types, of which fibroblasts have received most attention, in part due to their availability and ease of culture. In the loosely packed intralobular stroma surrounding TDLUs, fibroblasts arrange themselves around the acini (figure 4), whereas extralobular fibroblast arrangement is not as organized (Howard and Gusterson, 2000). It has been shown that fibroblasts produce several different growth factors, including

hepatocyte growth factor/scatter factor (HGF/SF). HGF has been shown to induce mammary epithelial morphogenesis in *in vitro* experiments both in mouse and human mammary gland by inducing proliferation and preventing apoptosis (Niranjan *et al.*, 1995, Sunil *et al.*, 2002, Leroy *et al.*, 2006). HGF has been shown to be negatively regulated by transforming growth factor beta (TGFß), which is secreted by epithelial cells and inhibits the formation of lateral branches during mammary gland morphogenesis (Cheng *et al.*, 2005, Cheng *et al.*, 2007). While fibroblasts have received much attention regarding breast morphogenesis, not much attention has been given to the other major cell type of the mammary stroma, the endothelial cells.
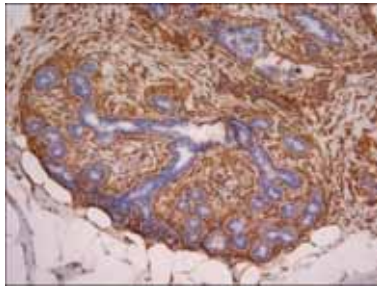
Figure 4: Intra- and extra lobular localization of fibroblasts. In TDLUs, fibroblasts arrange themselves in and around acini following the acinar structure revealing the epithelial cells' close relation-ship with the stroma. Thy-1 staining (brown) reveals both MEPs, a part of the epithelial structure, and the fibroblasts of the stroma. Epithelial cells can be seen as blue (nuclear stain) (Ingthorsson, unpublished).

3.3 - Endothelial cells

During the life cycle of the mammary gland it undergoes a series of major events, such as cell proliferation, differentiation and involution. This variability is reflected in a highly varied need for oxygen and nutrients. Nutrients are provided by the vascular system which compensates for the increased need for nutrients and oxygen

with a massive expansion of microvessels. Expansion is especially concentrated around the TDLUs where the microvessels assume a basket like pattern, encapsulating the acini. This expansion of the microvessels is caused by pro-angiogenic signals derived from the epithelium such as vascular endothelial growth factor (VEGF). During involution of the breast gland, the vascular system is also reduced in complexity, returning to its resting state (Djonov *et al.*, 2001).

Endothelial cells in the body are a heterogeneous group, based on the type of blood vessel, as well as the function of each organ. Microvessels in organs such as the brain and heart have a continuous/tight surface, whereas the microvasculature of endocrine glands and intestinal villi have a fenestrated surface, allowing for a faster transfer of nutrients and chemicals (Pasqualini *et al.*, 2002). This variability in endothelial cell properties makes the source of endothelial cells used in research relevant. Microvascular endothelial cell cultures are difficult to maintain *in vitro*, they have high requirements for nutrients and serum content, proliferate slowly and are difficult to isolate due to overgrowth of fibroblasts in the culture. This has made *in vitro* culture and research on tissue specific endothelial cells problematic. Our lab has recently published a paper describing a protocol for isolation and long term culture of finite lifespan breast endothelial cells *in vitro* (Sigurdsson *et al.*, 2006).

3.4 - Endothelial cells in organogenesis and development

In addition to supplying organs with oxygen and nutrients and removal of waste products, data is emerging showing endothelial cells as major players in the development of many organs. Examples are the liver and pancreas (Lammert *et al.*, 2003). Studies on mouse hepatic development have shown that endothelial cells are a vital factor in the invasion of hepatic precursor cells into the surrounding mesoderm (Matsumoto *et al.*, 2001). Lammert *et al.* (2001) were able to induce ectopic pancreatic islet formation in mouse embryos by inducing ectopic vascularisation in the foregut, indicating the important role of the endothelium in organ development. Endothelial cells have also been implicated in maintaining the niche of neural stem cells in the brain (Gilbertson and Rich, 2007, Riquelme *et al.*, 2008) and haemapoetic stem cells in the bone marrow (Psaila *et al.*, 2006, Yin and Li, 2006, Colmone and Sipkins, 2008). Considering the close contact between mammary epithelial and endothelial cells it is likely that endothelial cells affect mammary gland development in similar ways to what has been seen in other organs.

4 - Breast cancer

Breast cancer is the most common type of cancer affecting women worldwide. 10% of women may expect to get breast cancer during their lifetime (Dalgin *et al.*, 2007). The vast majority of breast cancers originate in the epithelial compartment, predominantly in the luminal epithelial cells. Breast cancer most often originates in the ducts of TDLUs, first as abnormal, benign neoplasia, progressing to

ductal carcinoma *in situ* (DCIS), expanded duct and lobules with limited metastatic ability. DCIS progresses into invasive ductal carcinoma (IDC) upon the breaching of the myoepithelial cell layer surrounding the luminal cells and an invasion into the stroma (Allred *et al.*, 2001, Burstein *et al.*, 2004, Dalgin *et al.*, 2007). Prognosis is highly correlated with the phenotype of the tumour, for example whether the tumour cells express the oestrogen receptor and respond to anti-oestrogen treatment (Gruvberger *et al.*, 2001).

4.1 – Breast cancer subgroups

Recently, breast cancer has been classified into five groups based on their molecular gene expression pattern (Perou *et al.*, 2000, Sorlie *et al.*, 2001, van't Veer *et al.*, 2002, Sorlie *et al.*, 2003, Sorlie *et al.*, 2006), Luminal A, Luminal B, HER-2+, Normal-like and Basal like These groups have furthermore been shown to correlate with prognosis, with Luminal A cancers having the best prognosis and Basal-like the worst. About 60-70% of breast cancers express the oestrogen receptor (ER), and have a phenotype similar to the luminal epithelial cells; cancers of this type (Luminal A) have the best prognosis, as they respond well to anti-oestrogen treatment. The other ER positive group (Luminal B) has worse prognosis than Luminal A but better than the oestrogen receptor negative groups, HER-2 (also known as EGFR2) over-expressing group, the Normal-like group and the Basal-like group. Breast cancers classified as Basal-like have the worst prognosis, they often respond well to initial treatment, but have the highest metastatic rate. Phenotypically they resemble myo-

epithelial/basal cells, expressing ck5/6, ck14 and ck17 as well as Wilm's tumour protein (WT1), which has been linked with poor prognosis in breast cancer.

4.2 The role of microenvironment in cancer progression

Breast cancer progression depends on changes in homeostasis, both genetically and to the microenvironment of the cells. Recent evidence has shown that the molecular profile of tumour stroma can also affect prognosis and clinical outcome (Shekhar *et al.*, 2007, Bergamaschi *et al.*, 2008). During initial changes the paracrine signalling between cellular compartments of the breast are changed, resulting in a loss of tissue polarity (Bergamaschi *et al.*, 2008). Loss of cell polarity changes the behaviour of cells which can lead to changes in genetic expression. As the tumour grows, the need for nutrients and oxygen causes an increase in angiogenesis which then leads to increased growth and an increased chance of metastasis.

4.2.1. Myoepithelial cells

Majority of breast cancers originate from the luminal epithelial cells. As described earlier, LEPs are in very close contact with myoepithelial cells in the breast gland, with MEPs situated between LEPs and the surrounding stroma. During the initial stages of breast cancer progression, the myoepithelial cells maintain their position on the periphery of the epithelial structure, in contact with the stroma. However, when the tumour becomes malignant the myoepithelial envelope becomes discontinuous and in invasive tumours the

myoepithelial cells are hard to identify but appear in a non-organized fashion within the tumour (Gusterson *et al.*, 1982, Sternlicht and Barsky, 1997). The enveloping architecture of the myoepithelial cells in the normal breast suggests they might act as natural tumour suppressors, by maintaining tissue polarity, and protecting the luminal epithelial cells from the surrounding stroma. Furthermore, the myoepithelial cells produce several protease inhibitors, such as Maspin and tissue inhibitor of matrix metalloprotease-1 (TIMP-1), inhibiting both angiogenesis and invasion of epithelial structures (Ronnov-Jessen *et al.*, 1996, Sternlicht and Barsky, 1997, Sternlicht *et al.*, 1997, Bissell and Radisky, 2001, Petersen *et al.*, 2001, Barsky and Karlin, 2005). Gudjonsson *et al*. (2002a) demonstrated that myoepithelial cells maintain the correct polarity of luminal epithelial cells when co-cultured in collagen-1 matrix by secretion of laminin α1 (figure 5). Co-culture with myoepithelial cells
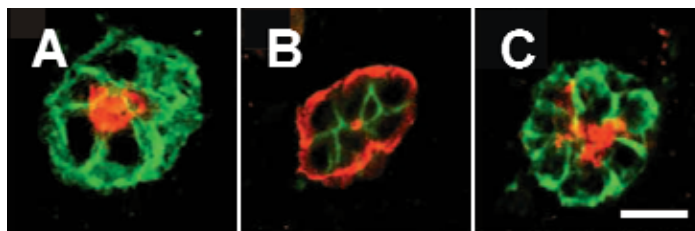
Figure 5: Myoepithelial cells correct the polarity of luminal epithelial cells in co-culture. (**A**) When cultured in Matrigel, LEPs form polarized colonies with Sialomucin-1 (red) expression seen apically. (**B**) When cultured in collagen 1 gel, LEPs form colonies with reversed polarity, Sialomucin-1 expression is seen on the outside of colonies. (**C**) When co-cultured with MEPs polarity of LEP colonies is corrected as evidenced by apical Sialomucin expression. Bar=25µm (adapted from (Gudjonsson *et al.*, 2002a))

derived from tumours did not give the same results, indicating that changes in myoepithelial function can promote tumour progression resulting in a loss of polarity. Interestingly, cancer derived

myoepithelial cells showed reduced expression of Laminin α1 (Gudjonsson *et al.*, 2002a). Loss of the myoepithelial lining during cancer progression exposes the luminal cells to the stroma and its components, which can lead to increased tumour growth and metastasis.

4.2.2 Fibroblasts

Normal breast development requires epithelial structures to invade the surrounding stroma (often referred to as mesenchyme tissue). This is achieved by the secretion of matrix metalloproteases (MMPs) and a temporary epithelial-mesenchymal-like transition (EMT) of the invading epithelium (Petersen *et al.*, 2003). As previously discussed, fibroblasts secrete a number of growth factors affecting normal epithelial cells, this also holds true for cancer cells. Invasive breast tumours often contain a substantial amount of stroma containing activated fibroblasts that secrete growth factors in response to an apparent wound healing signal from the tumour cells, increasing MMP activity and promoting angiogenesis as well as stromal and tumour proliferation (Dvorak, 1986, Bissell and Radisky, 2001, Kuperwasser *et al.*, 2004, Mueller and Fusenig, 2004, Hu *et al.*, 2008). Fibroblasts within tumour stroma have recently been shown to promote breast cancer metastasis by expressing chemokine ligand 5 (CCL5), a molecule that both promotes angiogenesis as well as attracting macrophages to the tumour area, further increasing the wound healing proliferative signal (Karnoub *et al.*, 2007). It has recently been shown that fibroblasts derived from tumours can

directly decrease the sensitivity of cancer cells to Tamoxifen treatment (Shekhar *et al.*, 2007).

4.2.3 Endothelial cells

The most important role of the vascular system is the supply of oxygen and nutrients and the removal of waste products, such as carbon dioxide. This holds especially true for tumours, which often harbour a very high metabolism, requiring large amounts of oxygen and nutrients (Djonov *et al.*, 2001). A large threshold to be overcome in cancer progression is achieving angiogenic potential. The tumour environment is hypoxic, which results in a low pH in the tumour. Low pH induces angiogenesis through the activation of Hypoxia inducible factor 1α (HIF1α) (Zhou *et al.*, 2006). Several known factors have been shown to be under HIF1α control resulting in increased angiogenesis within tumours. One such factor is VEGF, which stimulates the growth of new blood vessels into the tumour. Angiogenesis is closely connected with activated fibroblasts (myofibroblasts) in the invasive tumour, and the wound healing signal, as discussed earlier (Dvorak, 1986, Bissell and Radisky, 2001, Mueller and Fusenig, 2004). For tumour cells to metastasise they need to acquire the ability to infiltrate the blood vessels, survive the transport to a new location, and then again infiltrate the blood vessel in the new location. One way for tumour cells to achieve this mobility is the expression of vascular endothelial-cadherin (VE-Cadherin) (Labelle *et al.*, 2008). Inhibiting angiogenesis in a tumour would theoretically hamper its growth potential. For this reason, therapeutic

agents with anti-angiogenic properties have received much attention for several years with some hopeful results (Grothey and Ellis, 2008).

Keeping in mind that endothelial cells have been shown to play a key developmental role in several organs by the release of growth factors and other proliferative signals, the close relationship endothelial cells have with both the normal and malignant breast gland makes endothelial cells a very interesting subject for research.

The literature regarding the effects of endothelial cells on epithelial cells in the breast is very limited. This is in part due to lack of tissue specific, breast endothelial cells, so researchers have applied endothelial cells from other sources/organs. Shekhar *et al.* (2000, 2001) co-cultured normal MCF10a cells and pre-neoplastic MCF10a-EIII8 cells with normal Human umbilical vascular endothelial cells (HUVEC). They demonstrated that normal MCF10a could maintain the proliferation of HUVECs for a week whereas the EIII8 cells could maintain endothelial growth for much longer. They also demonstrated that HUVECs had a profound effect on EIII8 cells, inducing them to undergo ductal alveolar morphogenesis in co-cultures. These interactions indicate a role for endothelial cells in cancer progression, besides those of nutrient supply/waste removal, but further studies are needed especially using tissue specific endothelial cells. To be able to capture critical aspects of this endothelial-epithelial interaction, it becomes pivotal to utilize appropriate experimental models.

5 – Three-dimensional cell culture

To be able to interpret and extrapolate experimental results it is necessary to choose an appropriate research model. Animal models, such as knockout mice have proven very useful in the research of many diseases, for example in the research of breast cancer (Kuperwasser *et al.*, 2004, Karnoub *et al.*, 2007). There are however many differences in the physiology and tissue architecture of the mammary gland of mice and humans. Hormonal levels and dependencies are different and *in vivo* experimental setups make controlling the experiment problematic (Cardiff and Wellings, 1999, Cardiff, 2001, Cardiff *et al.*, 2001). In some cases, *in vivo* approaches are impossible, for example when focus is on human development, meaning that *in vitro* models must be applied. For decades, *in vitro* cell culture in a monolayer has been the accepted method of choice, and has been very useful in answering basic biological questions. It is however widely accepted that two dimensional cell culture does not recapitulate the true nature of the cells. *In vivo*, cells are organized in three-dimensional structures necessary for correct form and function of tissues and organs. Cells are dependent on ECM and neighbouring cells in their environment for correct differentiation. These environmental cues are lost when cells are cultured in monolayer which can result in abnormal differentiation (figure 6). In contrast, three-dimensional culture methods can maintain and/or restore critical aspects of tissue architecture making these models attractive for studies on tissue morphogenesis and cancer (Bissell and Barcellos-Hoff, 1987, Kim, 2005, Smalley *et al.*, 2006).

The use of three-dimensional substrates has been widely used in the research of cell-stroma and cell-cell interaction. The most widely used substrates are hydrated collagen 1 gels (Elsdale and Bard, 1972) that effectively mimic the basic three-dimensional environment of many organs for example the breast, and reconstituted basement membrane matrix (rBM) manufactured from a special type of mouse tumour, the Engelbreth Holm-Swann (EHS) tumour (Commercially available as Matrigel®) (Kleinman *et al.*, 1986, Kleinman and Martin, 2005).
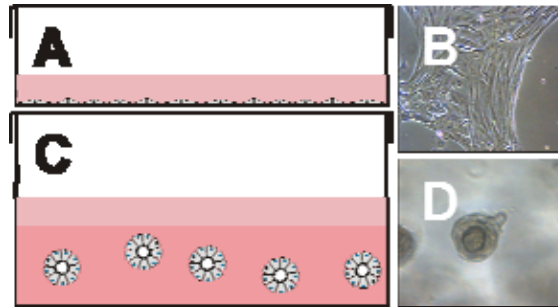
Figure 6: Two-dimensional vs. three-dimensional cell culture. When cultured in monolayer (**A**, **B**), luminal epithelial cells adopt a very different phenotype to the one they form when embedded in 3D rBM (**C**, **D**).

5.1 – Cell differentiation and morphogenic models

Research in mammary gland development and cancer progression using 3D models has been ongoing for several years. Isolated luminal epithelial cells embedded into rBM retain critical phenotypic characteristics of their *in vivo* form. They form round polarized colonies with a hollow central lumen. Characteristic feature of these structures is the apical expression of Sialomucin-1 (Petersen *et al.*, 1992). When embedded in collagen, LEPs form colonies with inside-out morphology, sialomucin is expressed on the outside of the

colony, not the inside. The main difference between collagen and rBM is that rBM is rich in basement membrane material, which in part maintains correct polarity *in vivo*. When myoepithelial cells are embedded in rBM they form solid round colonies expressing myoepithelial markers. When LEPs are embedded in collagen with MEPs, the MEPs are able to rescue the LEP phenotype, producing the correct luminal colony phenotype (Gudjonsson *et al.*, 2002a, Gudjonsson *et al.*, 2003, Gudjonsson *et al.*, 2005). The myoepithelial cells produce laminin-α1 which is a major protein in rBM and this is believed to be a key reason for the correction of the LEP phenotype. Gudjonsson *et al.* (2002b) isolated and immortalized two different epithelial cell populations from healthy breast tissue, one with a luminal epithelial phenotype, termed D382 and one with stem cell properties, termed D492. When embedded in rBM D492 formed TDLU like structures, expressing both myoepithelial and luminal epithelial markers whereas D382 formed round colonies with solely LEP phenotype (Gudjonsson *et al.*, 2002b). The D492 cells are thought to have progenitor cell properties whereas the D382 cells are more differentiated towards the LEP lineage. D382 will be used in the experimental chapter of this thesis. Sigurdsson (2005) optimized the isolation procedure of microvascular organoid isolation as described by Hewett and Murray (Hewett and Murray, 1993). His optimizations made tissue specific breast endothelial cells (BRENCs) available for long term *in vitro* culture (Sigurdsson *et al.*, 2006). When embedded in rBM, BRENCs remain non-proliferative but viable for a long time.

In contrast, when BRENCs were seeded on top of rBM they form tubular like net
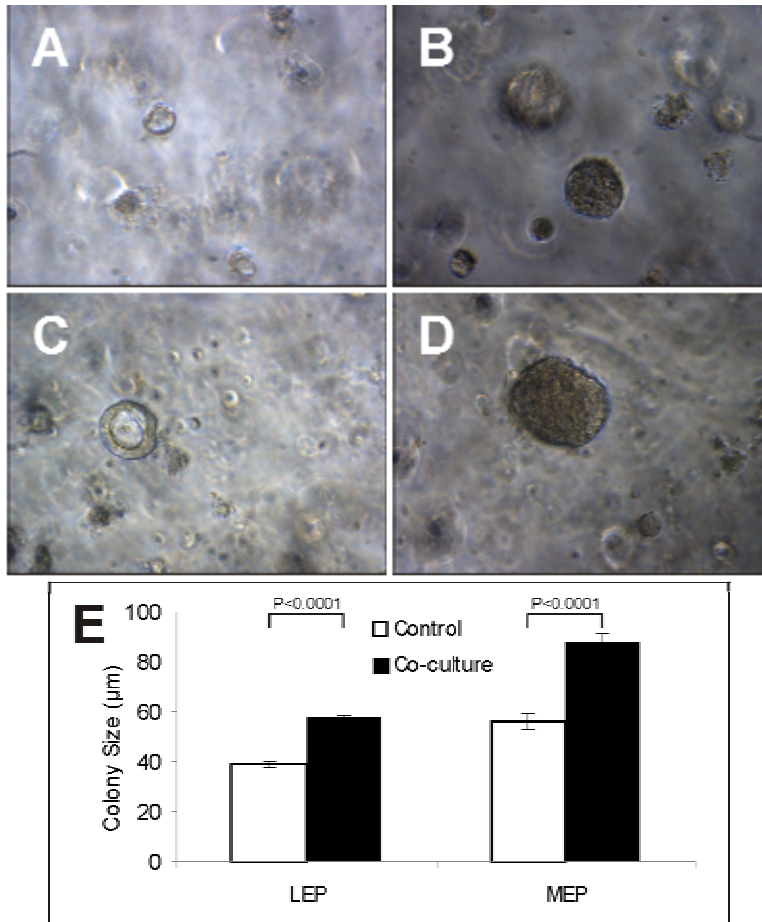
Figure 7: Endothelial cells have a proliferative effect on primary luminal- and myoepithelial cells in co-culture. When LEPs were co-cultured with BRENCs (**C**) a significant proliferative effect was seen in LEP co-cultures compared with control (**A**) Both colony size and lumen diameter was increased (**C** and **E**). When comparing MEP control (**B**) and co-culture (**D**) there was also a significant proliferative effect (**E**). (adapted from (Sigurdsson, 2005))

structures that disintegrate within 24-72 hours, making them not suitable for long term culture experiments. When purified primary luminal or myoepithelial cells are seeded in rBM they form colonies as described by Petersen *et al*. (1992). However, when co-cultured with BRENCs there was a significant increase in MEP colony diameter in co-cultures indicating a proliferative effect produced by the BRENCs (figure 7). LEP colonies were also increased in size, and additionally there was a significant increase in the size of the central lumen. According to these results it appeared that BRENCs conferred both proliferative and morphogenic signals to primary breast epithelial cells (Sigurdsson, 2005).

The use of primary cells in research has its benefits, but it also introduces problems. Primary cells have a finite life span *in vitro* making them hard to rely on in long term *in vitro* culture. Immortalization of primary cells can to some extent fix this problem by extending the lifespan of the cells. Many different immortalization methods are used in order to establish continuous cell lines. One of the most widely used approaches to date is transfection using the E6 and E7 oncogenes from the human papilloma virus (HPV-16). The transfection disrupts the function of the tumour suppressors P53 and retinoblastoma. This enables transfected cells to acquire changes required for extended lifespan, for example activated telomerase to prevent chromosome shortening (reviewed by (Gudjonsson *et al.*, 2004)). Long term culture of immortalized cells may cause changes to karyotype, potentially causing changes in marker expression. When creating cell lines this must be kept in mind.

5.2 – Cancer progression models

Several research groups are currently utilizing three-dimensional culture models to analyse the behaviour of immortalized normal and malignant cell lines in three-dimensional culture (Schmeichel and Bissell, 2003, Debnath and Brugge, 2005). The MCF10a cell line has proven very useful in the modelling of initial stages of cancer progression. MCF10a is an immortal cell line that was immortalized without the usage of transfection (Soule *et al.*, 1990). It has given great insight into the molecular mechanics of lumen formation in mammary epithelial cells, as well as some of the factors disrupting lumen formation, including overexpression of HER-2 and Cyclin D1 (Debnath *et al.*, 2002, Debnath and Brugge, 2005). Many research groups use cancer progression models, where cell lines of increasing malignancy are utilized to analyze the steps in cellular changes involved in cancer progression. The series of HMT3522 cell lines have proven very useful in that respect. The series is composed of a number of cell lines of increasing malignancy, from the non-malignant S1 and S2 sublines which in 3D culture form regular acinus like structures to the malignant T4-2 subline which forms disorganized large structures in 3D (Briand *et al.*, 1987, Kenny *et al.*, 2007, Rizki *et al.*, 2008). This correlates with *in vivo* tumourigenicity, where the S1 and S2 sublines do not form tumours in mice while T4-2 is tumourigenic. The phenotype of T4-2 cells could be reversed to an organized form using inhibitory antibodies against β1-integrin (Weaver *et al.*, 1997). The same effect could be seen when the

function of EGFR was inhibited using a neutralizing antibody (Wang *et al.*, 1998).

5.3 – Cancer cell lines

Three-dimensional culture models have been used to analyse the morphology of several cell lines. Kenny *et al*. (2007) grouped several cell lines according to their morphology in 3D. The groups were termed Round, Mass, Grape-like and Stellate with Round being most normal like, and Stellate the most abnormal, EMT-like. Each group had a well defined pattern of expression of selected molecular markers according to their three-dimensional growth pattern. Notably, the cells forming grape-like colonies had high expression of EGFR1 and HER-2, whereas the stellate colonies lacked EGFR1/HER-2 expression, as well as E-Cadherin, falling under the Basal-like category of cancers. The malignant MDA-MB-231 cell line falls under this basal-like category. Wang *et al*. (2002) showed that the stellate phenotype of MDA-MB-231 could be reversed with the inhibition of β1-integrin in combination with phosphatidylinositol 3-kinase (PI3K) or mitogen-activated protein kinase (MAPK) (Wang *et al.*, 2002). There are several different cancer cell lines in regular use by researchers, ranging across a spectrum of malignancy potential and marker expression (Neve *et al.*, 2006). In the experimental part of this thesis, I will use three different cancer cell lines, MCF7, T47-D and MDA-MB-231 (table 1). Most immortal breast cell lines are derived from the luminal epithelial compartment of the breast. There is a distinct lack of immortal non-malignant cell lines derived from myoepithelial cells.

As stated earlier, the vast majority of cancers originate in luminal epithelial cells, but not in myoepithelial cells, perhaps putting a focus on the luminal compartment which can explain the apparent lack of myoepithelial derived cell lines. However, the role of myoepithelial cells in maintaining breast gland homeostasis as discussed earlier makes them a very interesting subject for research.

II - Aim of the study

Endothelial cells have been recognized as vital players in organ development in many organs such as the liver and pancreas. Recent studies have also shown that endothelial cells play important role in the regulation of stem cells in many organ systems, such the nervous system and bone marrow. Moreover, formation of new blood vessels – *angiogenesis* – is well known as an important factor during cancer progression in many organs including the breast. However, little is known about the morphogenic effects of breast endothelial cells on normal and cancerous breast epithelial cells, though preliminary data suggests a stimulatory effect of endothelial cells on the epithelium (Shekhar *et al.*, 2000, Shekhar *et al.*, 2001, Sigurdsson, 2005). The study of cell-cell interaction depends on the availability of suitable models that represent the subject at hand. Furthermore, in the case of the breast, there has been a distinct lack of cell lines that represent the myoepithelial lineage, most cell lines are derived from the luminal epithelial compartment. The aim of the present work was to design a co-culture system to analyze the proliferative and morphogenic effect of breast endothelial cells on normal- and cancerous breast epithelial cells, and to establish a new cell line derived from the myoepithelial compartment of the breast gland.

Specific tasks:

1. Establish and characterize a myoepithelial-derived cell line
2. Design a novel co-culture assay that captures some of the interaction between breast endothelial cells and epithelial cells
3. Analyse the proliferative and morphogenic effect of endothelial cells on breast epithelial cells

III – Materials and methods

1 – Cell culture and isolation

Primary epithelial- and endothelial cells were isolated from breast tissue samples from reduction mammoplasties, obtained through collaboration with the Department of Pathology and the Department of Plastic Surgery, Landspitali-University Hospital. Before receiving the tissue, written informed consent was obtained from the women undergoing reduction mammoplasties.

1.1 – Primary breast epithelial cells

Breast tissue obtained from reduction mammoplasties was processed as previously described (Pechoux *et al.*, 1999). The tissue was minced finely and digested under gentle rotation overnight with 900 IU/ml Collagenase type III (Worthington), in DMEM/F12 basal medium (Gibco) containing 50 IU/ml penicillin and 50 µg/ml streptomycin (Gibco). The digestive mixture was centrifuged at 1,000 rpm for 40 seconds, producing a pellet containing epithelial organoids. The organoid pellet was re-suspended in PBS, and the organoids washed 6 times using PBS to remove fibroblasts from the suspension. The epithelial organoids were seeded on collagen coated T25 culture flasks (BD Biosciences) and cultured in the chemically defined medium CDM3, consisting of DMEM/F12 basal medium supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin, 10 µg/ml ascorbic acid, 100 ppm bovine serum albumin (BSA), 10 nM dibutyryl cyclic AMP, 100 ng/ml EGF, 0.1 nM estradiol, 0.1 mM

ethanolamine, 20 µg/ml fetuin, 100 ng/ml fibronectin, 0.5 µg/ml hydrocortisone, 3 µg/ml insulin, 2.6 ng/ml Na_2SeO_3, 0.1 mM phosphoethanolamine, 25 µg/ml transferrin and 10 nM triiodo-thyronine. (EGF purchased from Peprotech, all other growth factors purchased from SIGMA).

1.2 – Primary breast endothelial cells

Primary breast endothelial cells were isolated as described by (Sigurdsson *et al.*, 2006). Breast tissue from reduction mammoplasties was minced finely and digested under gentle rotation overnight in DMEM/F12 basal medium supplemented with 900 IU/ml Collagenase III, 50 IU/ml penicillin and 50 µg/ml streptomycin. The digestive mixture was centrifuged for 40 seconds at 1,000 rpm and the supernatant centrifuged further for 5 minutes at 1,000 rpm, yielding a pellet of microvessels. This microvessel pellet was incubated with anti CD31 (PECAM) Dynabeads (Invitrogen) for 20 minutes at 4°C under gentle rotation. Cells and microvessels bound to the dynabeads were isolated from the suspension using a magnetic concentrator. Endothelial organoids were cultured on collagen-1 (Inamed Biomaterials) coated T25 culture flasks using the EBM-2 basal medium (Lonza), containing 50 IU/ml penicillin, 50 µg/ml streptomycin and ascorbic acid, EGF, bFGF, Heparin, IGF and VEGF of unknown concentrations (Lonza). The EBM medium was supplemented with 30% foetal bovine serum (FBS) (Gibco), but the concentration of the serum was lowered to 5% for co-culture

experiments (EBM-2+growth factors will be referred to as EGMX, X denoting the percentage of FBS in the culture medium).

1.3 – Cell lines

The MCF10a and D382 cell lines were cultured in the chemically defined medium H14 (Briand *et al.*, 1987) consisting of DMEM/F12 basal medium supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin, 10 ng/ml EGF, 0.1 nM estradiol, 0.5 µg/ml hydrocortisone, 250 ng/ml insulin, 2.6 ng/ml Na_2SeO_3, 5 µg/ml prolactin and 10 µg/ml transferrin. The T47-D and MDA-MB-231 cell lines were cultured in RPMI1640 basal medium (Gibco) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 10% FBS. The MCF7 cell line was cultured in DMEM/F12 basal medium supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 5% FBS. The SI28 cell line was cultured in CDM3 culture medium as listed earlier. Cell lines are listed in table 1.

Table 1: List of cell lines used in the study

Cell line	Origin	Culture medium	Reference
MCF10a	F	H14	(Soule *et al.*, 1990)
D382	RM	H14	(Gudjonsson *et al.*, 2002b)
SI28	RM	CDM3	(Ingthorsson, unpublished)
MCF7	IDC (PE)	DMEM/F12 + 5% FBS	(Soule *et al.*, 1973)
T47-D	IDC (PE)	RPMI + 10% FBS	(Keydar *et al.*, 1979)
MDA-MB-231	AC (PE)	RPMI + 10% FBS	(Cailleau *et al.*, 1978)

F: Fibrocystic disease, **RM:** Reduction mammoplasty, **IDC:** Invasive ductal carcinoma, **PE:** Pleural effusion, **AC:** Adenocarcinoma

2 – Magnetic cell sorting

To obtain a pure population of myoepithelial cells we used magnetic cell sorting (MACS, (Miltenyi Biotec)) which utilizes superparamagnetic microbeads that can be coupled with monoclonal antibodies against cell surface antigens.

Organoids isolated from reduction mammoplasties were cultured for 4-5 days on T25 flasks, during which the cells in the organoids proliferate and spread out from the organoids on the culture surface forming a monolayer. This monolayer was trypsinized using 1 ml 0.25% Trypsin/EDTA for 5 minutes. After the cells were fully freed from the surface the trypsin was neutralized using 25 µl of 1% soybean trypsin inhibitor (SIGMA). The cell suspension was pipetted vigorously to break up any un-trypsinized lumps of cells and then passed through a 30 µm filter yielding a single cell suspension. Myoepithelial cells were isolated by a sequential positive selection of anti-β4-integrin and anti-Thy-1 mouse monoclonal antibodies, (dilutions 1:250 and 1:100 respectively). The single cell suspension was incubated with anti-β4-integrin for 30 minutes at 4°C. The cells were then washed 2x using MACS buffer (PBS containing 2 mM EDTA and 0.1% BSA) and incubated with goat anti mouse IgG microbeads for 20 minutes at 4°C (dilution 1:5 in MACS buffer). The cells were then washed again 2x with MACS buffer and then run through a MACS MS column mounted on a MiniMACS Separator. Positive cells were re-seeded on collagen coated T25 culture flasks and cultured in the CDM3 culture medium. Cells were then cultured

for 6 days and re-selected twice for Thy-1 positive cells following the same protocol.

3 – Immortalization of primary epithelial cells

Purified primary myoepithelial cells (β4-integrin$^+$thy-1$^+$) were immortalized using supernatant from the PA317 LXSN 16E6E7 packaging cell line containing retroviral particles with the E6 and E7 oncogenes from the HPV-16 retrovirus (figure 8). Cell cultures were treated overnight at 50% confluency with supernatant containing 8 μg/ml polybrene. Transfected cells were selectable via a neomycin resistance gene, which was selected for using 500 μg/ml Geneticin (G418) in the CDM3 culture medium.

Figure 8: The retroviral construct used for transfection of primary epithelial cells. The construct contained both E6 and E7 genes of the HPV-16 virus, promoted by SV40 and selected for via Neomycin resistance.

4 – Karyotype analysis

Karyotype analysis was performed at The Cytogenetics Laboratory at the Department of Genetics and Molecular Medicine, Landspitali-University Hospital. Cells cultured in monolayer were incubated with metaphase arresting solution (MAS, Genial Genetic Solutions, Ldt.) for three hours in order to increase the proportion of cells in metaphase in the culture. The cells were then treated with a hypotonic solution (7.5 mM KCl) for 20 minutes at 37°C, then fixed with an acetic acid/methanol solution (3/1) and G-banded with

Trypsin and Leishman's stain. 30 cells were analysed for statistical purposes and karyotypes described using International System for Human Cytogenetic Nomenclature (ISCN) standards.

5 – Immunocytochemistry

Monolayer cell cultures were fixed with either methanol for 10 minutes at -20°C or with 3.5% Formaldehyde in PBS for 5 minutes followed by 2x7 minute incubations with 0.1% Triton X-100 in PBS at room temperature, depending on the primary antibody used (table 2).

Table 2: List of antibodies used for immunocytochemistry

Antibody	Clone	Species	Isotype	Dilution	Fixation	Company - Order number
αSMA	1A4	Mouse	IgG2a	1:100	Methanol	Sigma-Aldrich - A2547
β4-integrin	3E1	Mouse	IgG1	1:250	Methanol	Millipore - MAB1964
CD31	JC/70A	Mouse	IgG1	1:50	Methanol	DAKO – M0823
ck5/6	D5/16B4	Mouse	IgG1	1:100	Methanol	Invitrogen - 18-0267
ck8	M20	Mouse	IgG1	1:50	Methanol	abcam - 9023
ck14	LL002	Mouse	IgG3	1:25	Methanol	abcam – ab7800
ck17	E3	Mouse	IgG2b	1:50	Methanol	DAKO – M7046
ck18	CD10	Mouse	IgG1	1:50	Methanol	DAKO – M7010
ck19	RCK108	Mouse	IgG1	1:50	Methanol	DAKO – M0888
cl-caspase-3	asp1750	Rabbit	IgG	1:50	Formaldehyde	Cell Signalling - 9661
ESA	VU1D9	Mouse	IgG1	1:50	Methanol	NovoCastra - NCL-ESA
ki67	polyclonal	Rabbit	IgG	1:25	Formaldehyde	Abcam - ab833
MUC-1	115D8	Mouse	IgG2b	1:50	Methanol	Biogenesis - 1510-5025
P63	7JUL	Mouse	IgG1	1:25	Formaldehyde	NovoCastra - NCL-P63
thy-1	ASO02	Mouse	IgG1	1:100	Methanol	Dianova - Dia 100
Vimentin	V9	Mouse	IgG1	1:100	Methanol	DAKO – M0725
WT-1	6F-H2	Mouse	IgG1	1:50	Methanol	DAKO – M3561

Primary antibodies were pre-mixed in PBS containing 10% FBS and incubated for 30 minutes. The cells were then washed x2 with PBS for 5 minutes and incubated with Rabbit anti-mouse immunoglobulins

(DAKO, Z0259, 1:25 dilution) for 30 minutes. Rabbit primary antibodies required an intermediate incubation with mouse anti-rabbit immunoglobulin (DAKO, M0737 1:50 dilution) before incubation with Z0259. Cells were then washed x2 with PBS for 5 minutes and incubated for 30 minutes with PAP, mouse, monoclonal (DAKO, P0850, consisting of soluble immunocomplexes of horseradish peroxidase and monoclonal mouse anti-horseradish peroxidase). Visualization was performed using 3.3-diaminobenzidine (DAKO DAB, S3000) activated with 0.5 mg/ml 30% H_2O_2. Nuclear staining was performed using Harris's Haematoxylin. Image acquisition was performed using a Leica DFC320 digital camera.

6 – Confocal microscopy

Co-culture gels were frozen in n-hexane at the end of the culture period. For cryosectioning, gels were mounted in tissue freezing medium and sectioned in 9 µm slices in a cryostat. Cryostat sections were fixed on microscope slides using either methanol for 10 minutes at -20°C, or with 3.5% Formaldehyde in PBS for 5 minutes followed by 2x7minute incubations with 0.1% Triton X-100 in PBS at room temperature, depending on the primary antibody used (Table 2). Microscope slides were then incubated with primary antibodies mixed in PBS containing 10% FBS for 30 minutes. The slides were then washed twice for 5 minutes and incubated with isotype specific fluorescent antibodies (Alexa fluor, 488 (green), 546 (red) Invitrogen) mixed in PBS containing 10% FBS for 30 minutes in the dark. The specimens were then washed twice for 5 minutes and incubated with

fluorescent nuclear counterstain (TOPRO-3, Invitrogen) for 10 minutes in the dark. Samples were again rinsed x2 for 5 minutes and then mounted with coverslips using Fluoromount-G (Southern Biotech) for preservation of the fluorescent signal. Immunofluorescence was visualized using a Zeiss LSM 5 Pascal laser scanning microscope.

7 – Three-dimensional cell culture

Reconstituted basement membrane (rBM, Growth factor reduced Matrigel matrix, BD-Biosciences) was used for co-culture experiments. Matrigel is a liquid at 0-4°C; cells can therefore be seeded into the Matrigel at that temperature. Above 4°C it starts to gelatinize and cells stay embedded in the gel, allowing them to proliferate in a three-dimensional environment.

To analyze the phenotype of SI28 10,000 cells were seeded alone into 300μl rBM, the seeded gel was placed in an incubator at 37°C for 30 minutes to ensure gelatinization of the rBM and then supplemented with CDM3 culture medium for 2 weeks, renewing the medium every 3 days. Gels were then photographed in an inverted phase contrast microscope and frozen in n-hexane and stored at -80°C for immunocytochemistry.

For co-culture experiments, normal and malignant epithelial cells (table 1) were seeded with BRENCs into 300μl rBM in a 24-well plate. 200,000 endothelial cells were seeded with 500 epithelial cells. The seeded gels were then placed in an incubator at 37°C for 30 minutes and then supplemented with EGM5 culture medium. These

co-cultures were maintained for 14 days. Colony size and number was measured on days 5, 9, and 13. On day 14 the gels were frozen in n-hexane and stored at -80°C for immunocytochemistry. To analyse how strong the endothelial effect was in different concentrations, MCF10a cells were also seeded with endothelial cells in different densities. 1,000, 5,000, 10,000, 50,000, 100,000 and 200,000 endothelial cells were seeded with 250 MCF10a cells. Colony size and number was measured on day 10.

To analyze the effect of soluble factors 70,000 BRENCs were embedded into 100μl of rBM and seeded in a 4-well chamber slide. 3,000 epithelial cells were seeded in separate 100μl rBM and seeded in the same well as the BRENCs, allowing the gels to merge in the centre, achieving a gradient in the densities of the two cell types. The chamber slide was then placed in an incubator at 37°C for 20 minutes and supplemented with 1 ml EGM5. Cultures were maintained for 10 days. Colony sizes were measured on days 5 and 10.

To inhibit direct cell-cell contact, BRENCs at a density of 50,000 were seeded on a 0.4μm pore size transwell (TW) filter and cultured with EGM5 in a 12 well plate for 3 days to reach confluency. 500 epithelial cells were then seeded into 100 μl rBM in a separate plate and placed in an incubator at 37°C for 10 minutes. The gels were then supplemented with 1.5 ml EGM5 culture medium and the BRENCs on the TW filters transferred to the wells containing the gels. Cultures were maintained for 8 days. Colony number and sizes were measured on days 5 and 8.

8 – Statistical analysis

All three-dimensional culture experiments were performed in triplicate for statistical accuracy. A minimum of 30 colonies/cells were measured in each well using Adobe Photoshop. Populations were compared using an unpaired two-tailed t test in Microsoft Excel. Graphs were created in Excel. Error bars represent the standard error of the mean (SEM)

IV – Results

1 – Establishment and characterization of a novel breast myoepithelial cell line

1.1 – Establishment of the SI28 cell line

As discussed in the introduction, there is a lack of representative myoepithelial cell lines from the human breast. Therefore, the first task of my project was to establish a myoepithelial cell line to use in a 3D co-culture model with endothelial cells and for future use in the laboratory. Myoepithelial cells were initially purified from primary culture of breast epithelial cells derived from reduction mammoplasty. Myoepithelial cells were labelled with anti-β4-integrin antibody and sorted in a MACS separator (see materials and methods for details). Positively selected cells were cultured for 6 days and then re-selected twice with anti-thy-1 antibody. This resulted in a phenotypically pure culture of myoepithelial cells. The purified myoepithelial cells were transfected at passage 4 using a retroviral construct containing the E6 and E7 oncogenes and the neomycin resistance gene. Positive selection of transfected cells was conducted by adding neomycin (Geneticin) to the culture medium. The selected cells gave rise to the established myoepithelial cell line referred to as SI28.

1.2 – Marker expression of SI28 in early passage (pre crisis)

In early passage (before passage 10) SI28 cells grew in monolayer in a pattern with little cell-cell contact, displaying a typical

myoepithelial phenotype. Cells expressed the myoepithelial markers β4-integrin, thy-1, ck5/6, ck14 and ck17 but little or no staining of the luminal markers ESA, ck19 or MUC-1 (figure 9). Cultures were split in a 1:10 ratio every 5-6 days at 90% confluency. At passage 13 the cells entered crisis. Cell death was increased and proliferation was negligible. Cells formed dense colonies with large amount of cellular debris released. Crisis lasted for 2 months, until passage 16 when cell proliferation started to increase again.

1.3 – Marker expression and karyotype status of SI28 in late passage (post crisis)

At passage 25, SI28 grew in a cobblestone pattern in monolayer. Cells maintained their expression of the myoepithelial markers β4-integrin and ck14 at similar levels as pre-crisis but thy-1 expression was largely lost. The luminal markers MUC-1 and ck19 are still minimally expressed but weak ESA expression is seen in 50% of the cells (figure 10). To further analyse the phenotype of SI28 I looked at other basal/myoepithelial markers. SI28 expresses myoepithelial/basal markers such as ck5/6, ck17 and vimentin as well as the tumour suppressor proteins P63 and WT-1 all of which are connected with the myoepithelial and basal phenotype as discussed in the introduction. Partial positivity to selected luminal markers, such as keratins 8, 18 and ESA suggest some biphenotypic properties (figure 11).
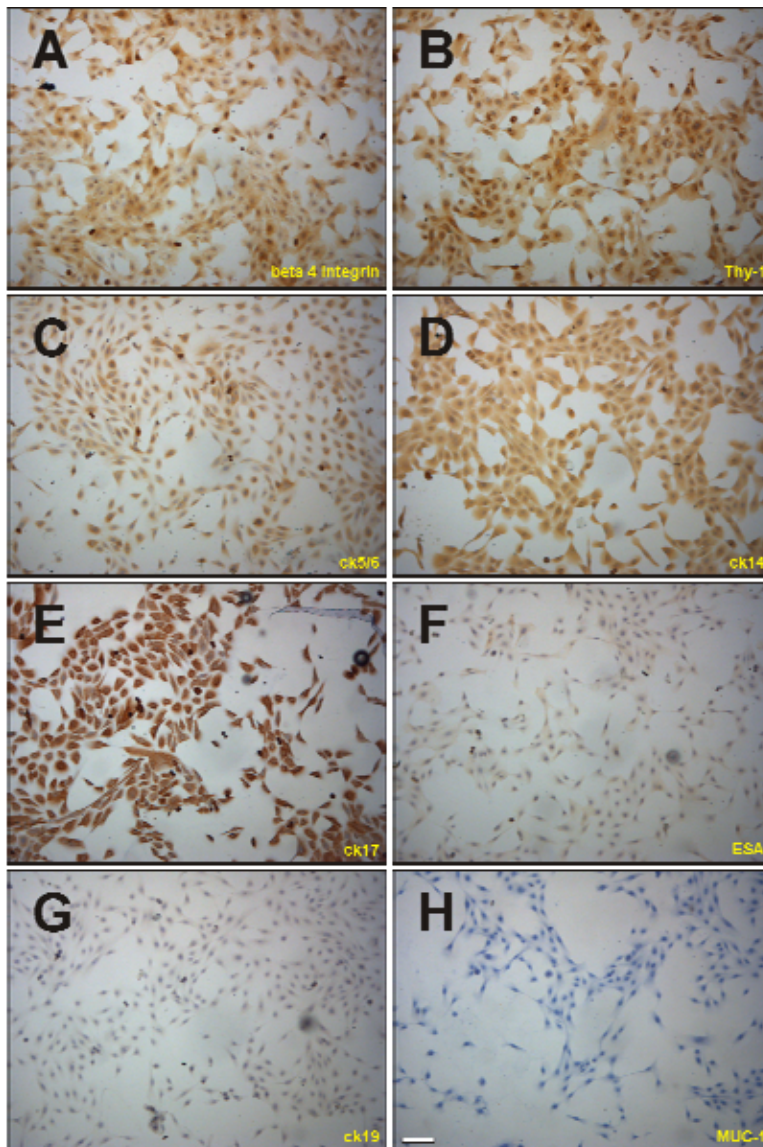
Figure 9: Marker expression of SI28 cells before crisis. In monolayer, SI28 cells grow with limited cell contact. SI28 cells express β4-integrin in all cells (**A**) as well as thy-1 (**B**). SI28 express the basal cytokeratins ck5/6 (**C**), ck14 (**D**) and ck17 (**E**) in all cells. Weak ESA staining can be seen in some cells (**F**) but no staining of the luminal epithelial cell markers ck19 (**G**) or MUC-1 (**H**) was seen. Bar=100µm
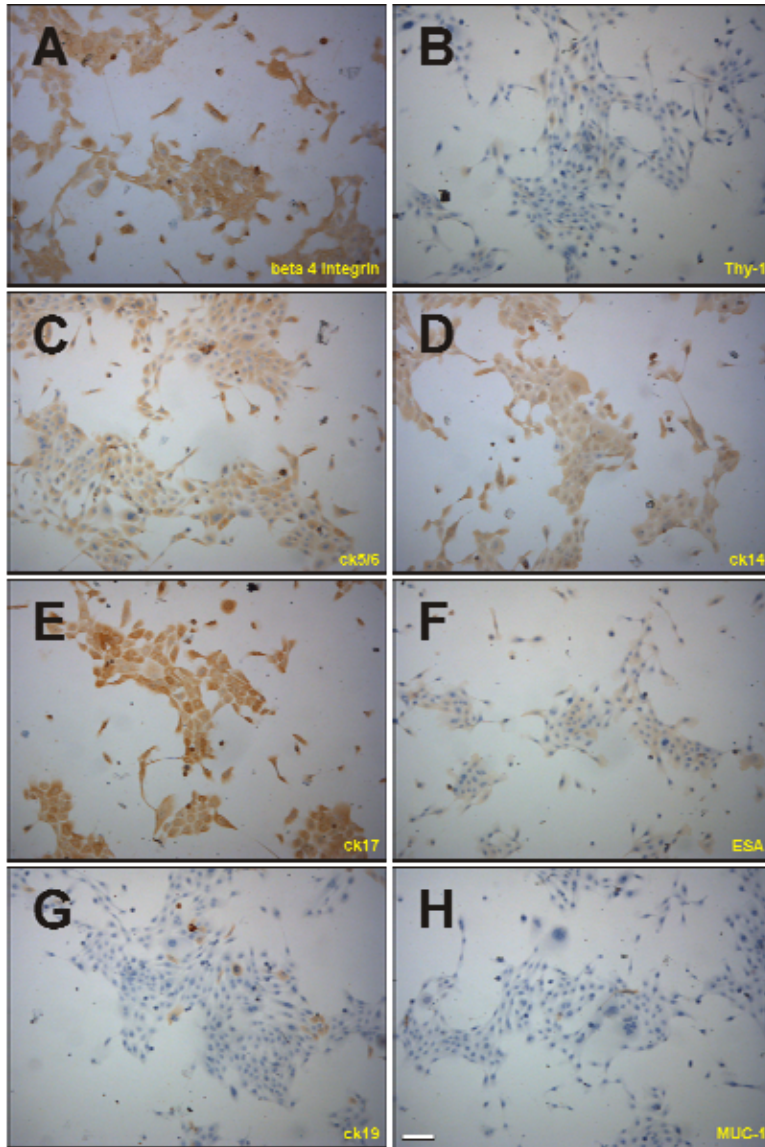
Figure 10: Marker expression of SI28 cells after crisis. In monolayer, SI28 cells grow within colonies of loosely connecting cells. SI28 cells express β4-integrin in all cells (**A**) whereas thy-1 expression has largely been lost (**B**). SI28 maintains expression of the basal cytokeratins ck5/6 (**C**), ck14 (**D**) and ck17 (**E**) in all cells. Weak ESA staining can be seen in a proportion of cells (**F**). Staining of the luminal epithelial cell markers ck19 (**G**) or MUC-1 (**H**) is still very limited and only seen in a very low proportion of cells. Bar=100μm
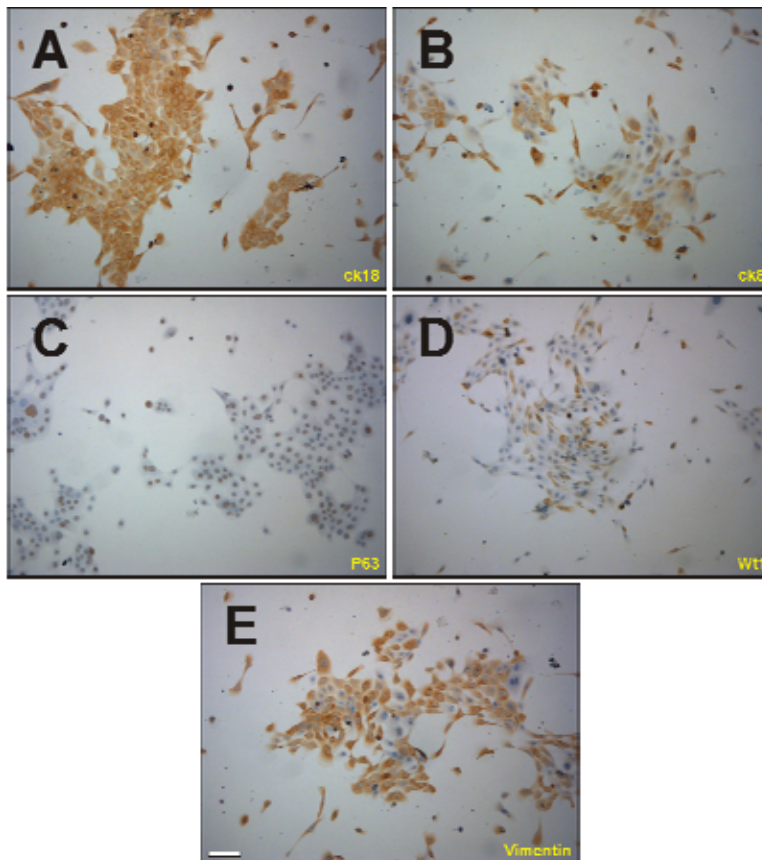
Figure 11: Post-crisis characterization of SI28 continued. SI28 cells express the simple luminal epithelial keratin 18 in all cells (**A**), whereas about 50% express ck8 (**B**). SI28 cells widely express the basal specific nuclear antigen P63 (**C**) and a proportion of cells (c. 60%) express the Wilm's Tumour-1 antigen (**D**). Vimentin expression is universal (**E**), although expression levels vary. Bar=100µm

At passage 10 (pre-crisis), SI28 cells have a normal karyotype, 44 autosomal chromosomes and 2 X chromosomes (figure 12a). Post-crisis, at passage 25 the prevailing karyotype was 43-44 autosomal chromosomes and 2 X chromosomes, with an extra copy of 13q added to chromosome 20 (figure 12b).
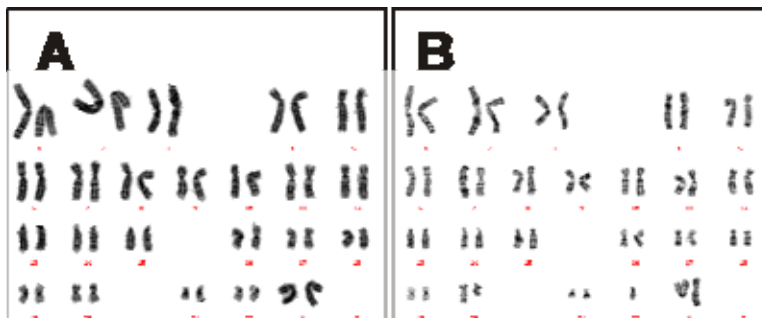
Figure 12: Karyotyping of SI28 reveals a near normal karyotype. At passage 10 SI28 has a normal karyotype, 46XX (**A**). At passage 25 some changes to karyotype have occurred, the prevailing karyotype is 45-46,XX,add(20)(q13) (**B**).

1.4 – SI28 shows a myoepithelial/basal phenotype when cultured in 3D

Primary myoepithelial cells form solid round colonies when cultured in 3D (Gudjonsson *et al.*, 2002a). When SI28 cells are embedded into rBM at a high density (100,000 cells per 300µl rBM) and cultured for 2 weeks they also form solid round colonies (figure 13). Colonies arise from single cells and given enough time reach much larger sizes than primary myoepithelial cell colonies. Immunostaining of cryosectioned cultures reveals that expression of markers is similar to that of monolayer cultures (figure 13). Colonies are partially polarized, expressing β4-integrin on the outside of the cells in contact with the rBM (figure 13c). Following establishment of the SI28 cell line I wanted to see how this cell line and other epithelial (normal and cancerous) cell lines reacted in co-culture with breast endothelial cells.
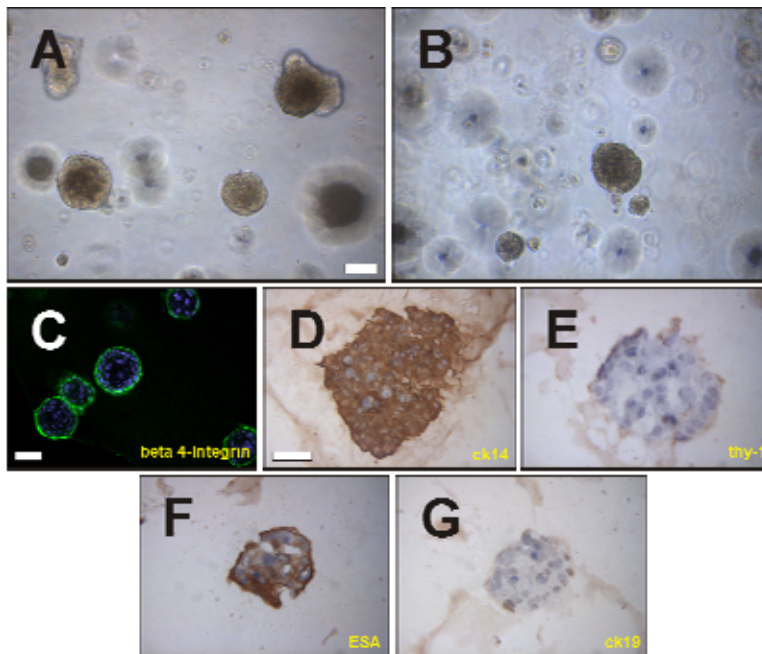
Figure 13: SI28 cells form solid round colonies in 3D. SI28 cells were embedded in Matrigel and supplemented with CDM3 (**A**) and EGM5 (**B**) culture medium. In 3D, SI28 forms solid round colonies with β4-integrin expression on the outside (**C**). ck14 (**D**), thy-1 (**E**), ESA (**F**) and ck19 (**G**) expression pattern is similar to that seen in figure 3. Bar **A-B**=100µm; **C-G**=50µm

2 – Three-dimensional co-culture

To analyze the effect of breast endothelial cells on epithelial cells in co-culture I used a number of cell lines, both normal-like and malignant, to better represent the different cell types in the breast gland, as well as different types of cancers (table 1). As shown by Sigurdsson in his thesis (Sigurdsson *et al.*, 2006), BRENCs form capillary like structures within a few hours when seeded on top of rBM (figure 14a). These delicate structures dissociate after approx. 24-72 hours (figure 14b) making long term co-cultures in this setup difficult. In contrast when embedded into rBM, BRENCs appear non-proliferative but viable (figures 14c, d) for long time in culture. This

provides the opportunity to analyse how endothelial cells affect proliferation and morphogenesis of breast epithelial cells over long periods of culture time.
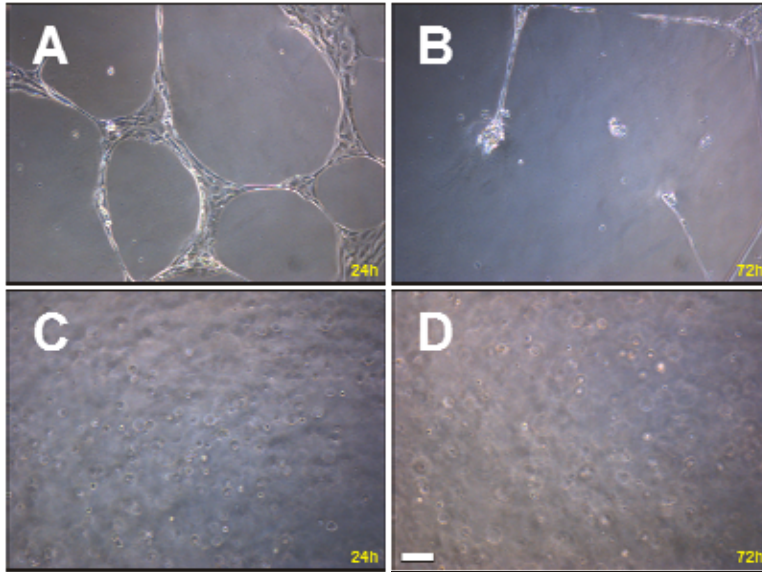
Figure 14: Behaviour of BRENCs when seeded on or in Matrigel. When seeded on top of rBM, BRENCs form capillary-like structures (**A**) that dissociate after 48-72 hours (**B**). When BRENCs are embedded into rBM, BRENCs stay viable for a long time in culture (**C** and **D**) Bar=100µm

2.1 – BRENCs have a proliferative effect on epithelial cell lines in co-culture

To ensure clonal growth and to reduce the possibility of cell aggregation in co-cultures, only 500 epithelial cells were seeded together with 200,000 BRENCs and supplemented with EGM5 culture medium. In 3D culture the most common phenotype of colonies is a round colony without an apparent lumen formation. This phenotype was seen in cultures with the non-malignant cell lines D382 (luminal) and SI28 (myoepithelial). MCF10a formed clusters of solid round

colonies (figure 15). The malignant cell lines T47-D and MCF7 also formed solid round colonies; whereas the highly malignant MDA-
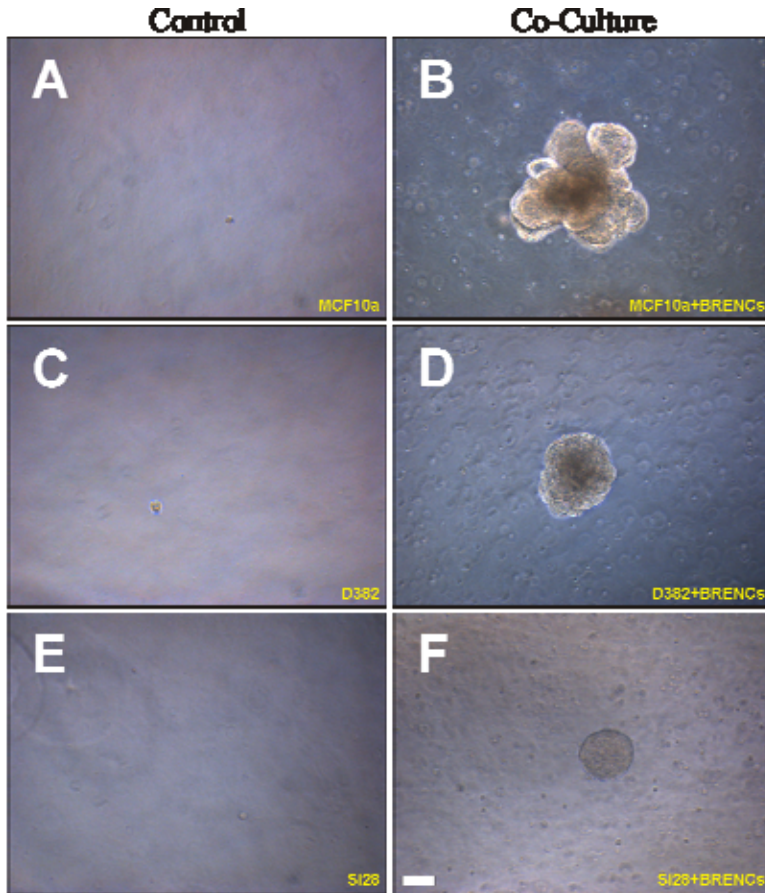
Figure 15: BRENCs induce proliferation of nonmalignant cell lines in co-culture. MCF10a, D382 and SI28 cells were seeded at a clonal dilution, alone or in co-culture with BRENCs. Alone there was very limited colony formation, most seeded cells staid as non-proliferative single cells (**A**, **C**, **E**, respectively). In co-culture MCF10a cells formed multiacinar-like structures (**B**), whereas both D382 and SI28 formed solid round colonies (**D**, **F**, respectively). Bar=100μm

MB-231 formed colonies of apparently loosely connected cells with a diffuse pattern (figure 16). Cells appeared spindle shaped, giving some properties of stromal cells such as fibroblasts (figure 16f). Immunofluorescent staining of cryosectioned MCF10a and MDA-MB-231 co-cultures reveals some of the differences between the solid
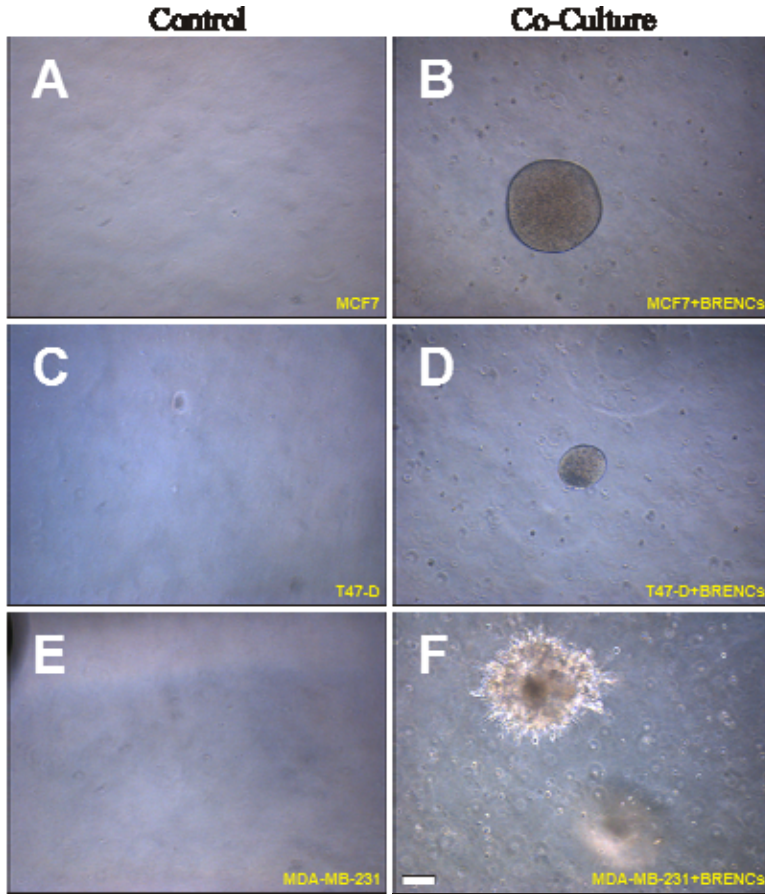
Figure 16: BRENCs induce proliferation of cancer cell lines in co-culture. MCF7, T47-D and MDA-MB-231 cells were seeded at a clonal dilution, alone or in co-culture with BRENCs. When cultured alone very limited colony formation was seen, most seeded cells staid as non-proliferative single cells (**A**, **C**, **E**, respectively). In co-culture, both MCF7 and T47-D formed solid round colonies (**B**, **D**, respectively). MDA-MB-231 formed mesenchymal like colonies when co-cultured with BRENCs. (**F**). Bar=100μm

round and diffuse phenotype (figure 17). Cells in solid colonies are much denser than in the diffuse colonies, so colony diameter does not necessarily reflect the number of cell divisions that have taken place. High levels of apoptosis could also affect colony size, reducing the average size of colonies. In the solid round colonies, β4-integrin expression is seen only on the outside of the colony (figure 17a),
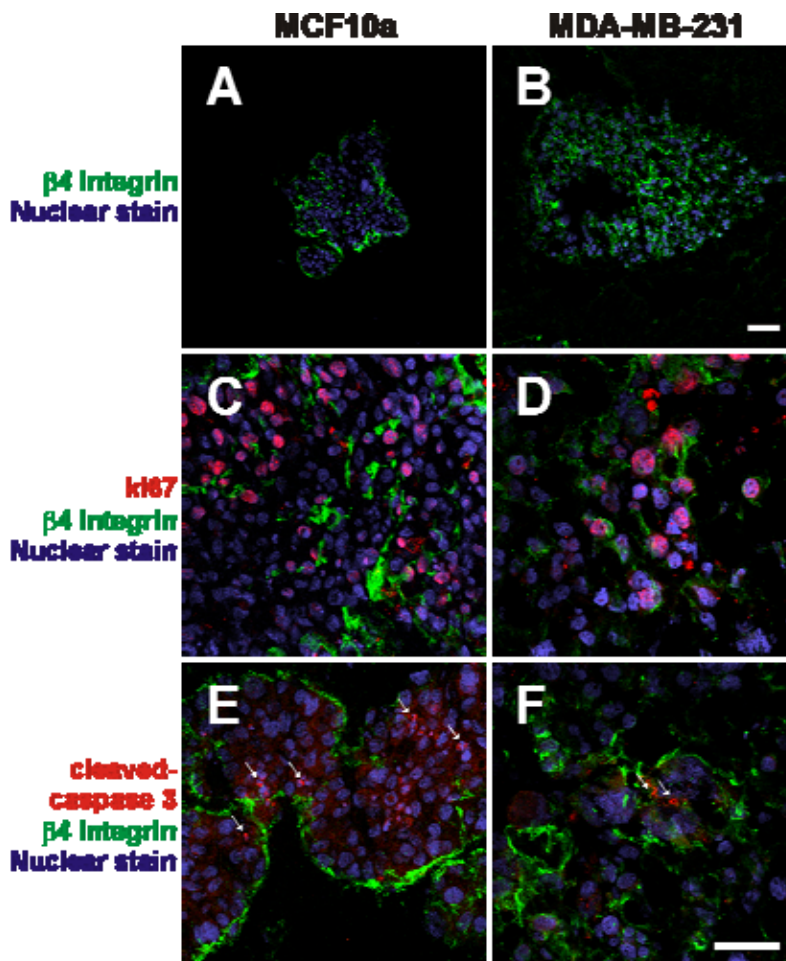
Figure 17: Co-culture colonies have a high ratio of cells in division and a low ratio of cells undergoing apoptosis. β4-integrin staining shows the difference between the diffuse and solid phenotypes. MCF10a colonies express β4-integrin on the outside of colonies (**A**), whereas MDA-MB-231 β4-integrin expression is diffuse and unorganized (**B**). Both colony types harbour a high ratio of mitotic cells as evidenced by ki67 staining (**C**, **D**). Ratio of apoptotic cells is low, arrows indicate sites of staining for cleaved (active) caspase 3 (**E**, **F**). Bar **A-B**=100µm; bar **C-F**=50µm

whereas expression is seen in all cells in the diffuse colonies (figure 17b). Ki67 staining reveals that a large portion of cells are in active proliferation (figures 17c and d). Cleaved caspase − 3 staining indicates there is little apoptosis in colonies (figures 17 e and f, indicated by white arrows).

Compared with BRENCs-free controls there was a significant (P<0.0001) increase in colony size (figure 18) for all cell lines studied. Average colony size of different cell lines varied greatly, with the normal-like MCF10a forming the largest colonies, 320 µm and the
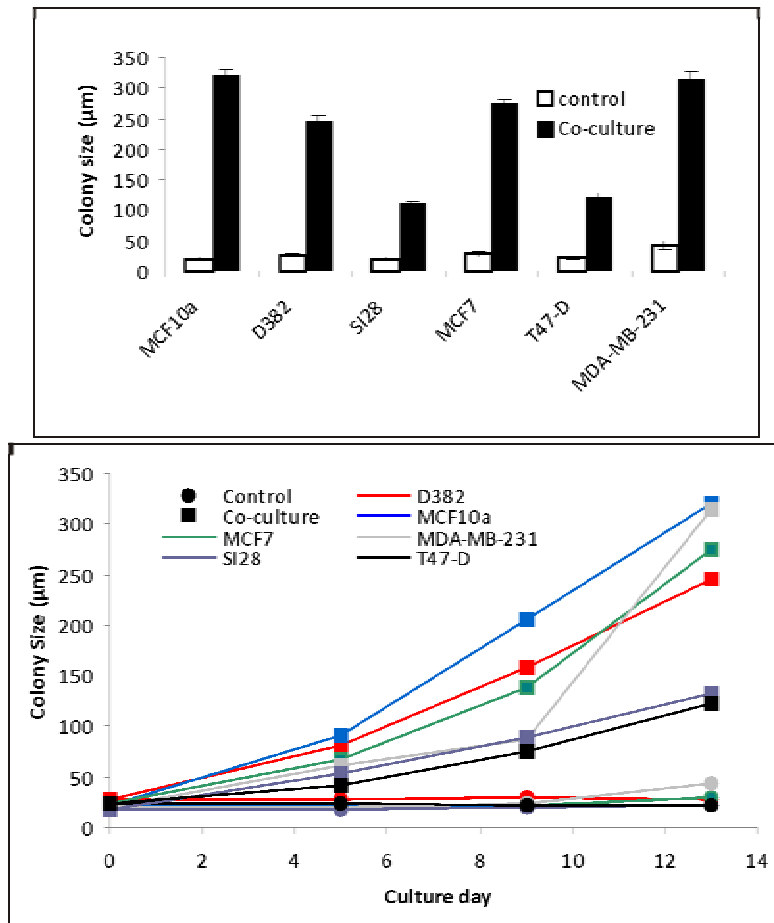
Figure 18: BRENCs cause a significant increase in colony size of epithelial cell lines in co-culture. Colony size of epithelial cells co-cultured with BRENCs increased significantly compared with controls (P<0.0001 for all co-cultures), (upper chart). MCF10a gave the biggest response, with a 15.5 fold increase in colony size, averaging at 320.6 µm diameter. Colony size varied greatly between cell lines. Growth rate of colonies was stable over time, most cell lines followed a linear growth pattern (lower chart). The spindle shaped phenotype of MDA-MB-231 colonies did not appear until after day 8 as revealed in a drastic increase in colony diameter. (Light purple line, lower chart).

malignant T47-D the smallest, 122 µm over a 14 day culture period. Cloning efficiency of epithelial cells was also greatly increased in co-cultures compared with controls. In controls, average ratio of colonies formed/seeded cells was less than 3%. In co-cultures that ratio was increased to over 35% for the non-malignant cells MCF10a and D382, 20% for MCF7 and SI28, 30% for MDA-MB-231. Colony formation in T47-D co-cultures was much lower, 6.8% (figure 19).
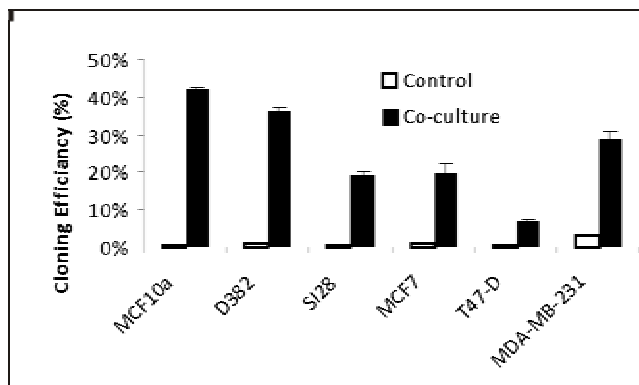
Figure 19: BRENCs increase colony formation in co-culture. Colony formation (cloning efficiency) is greatly increased in co-cultures. MCF10a cells' colony forming ratio increased the greatest, from 1% to 41.9%. Overall, increase in colony formation was greater for the normal cell lines than for the cancer cell lines.

2.2 – Endothelial cell concentration affects colony formation and proliferation

To analyse whether the endothelial cells' proliferative effect was dependent on the amount of endothelial cells in the gel I designed an experiment where I embedded BRENCs at different densities into co-cultures with epithelial cells. MCF10a showed the greatest responsiveness to BRENCs in co-culture and was chosen for that reason. 1,000; 5,000; 10,000; 50,000; 100,000 and 200,000 BRENCs were seeded with 500 MCF10a cells. Cloning efficiency increased as

endothelial density increased in the co-culture (figure 20a). Cloning efficiency reached 25% in co-cultures with 200,000 BRENCs. Colony size however, reached an apparent near-maximum with just 50,000 endothelial cells; colony size in co-culture with higher densities of BRENCs was similar to the 50,000 co-culture. These results suggest that colony formation could depend on close proximity with BRENCs for initial start of proliferation.
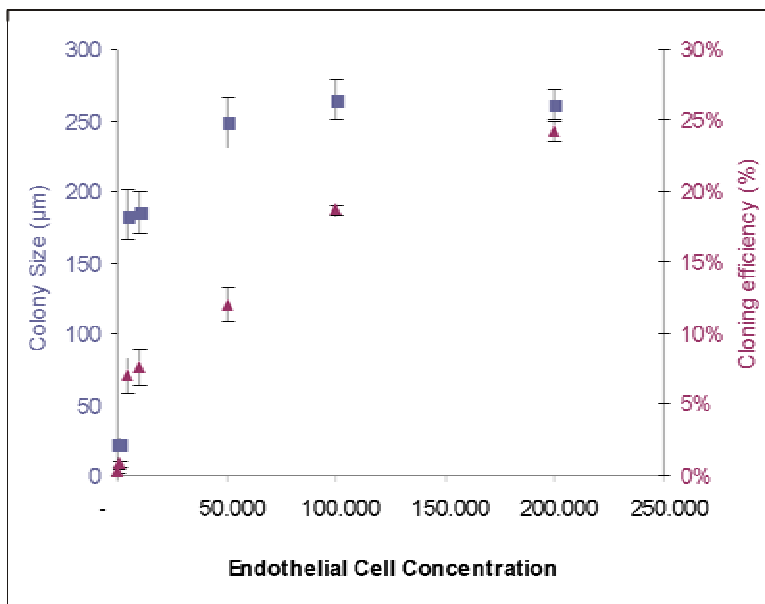
Figure 20: Endothelial cell concentration determines colony number and colony size in co-culture. Colony formation increases with increased concentration of endothelial cells in co-culture (purple marker) Colony size increases too; however, colony size reaches an apparent maximum at 100,000 BRENCs in the co-culture (blue marker)

2.3 – Design of a novel co-culture assay to analyze proximal and distal interactions between endothelial and epithelial cells

The signals from endothelial cells causing increased proliferation in co-cultures can be delivered in different ways. Signals can be directly delivered by cell-cell contact, where a molecule on the

surface of the endothelial cell directly activates a pathway in the epithelial cell, causing an increase in cell proliferation. In the co-culture model this would require that the colonies that arise are derived from cells that were originally in direct contact with an endothelial cell. Signals can also be delivered via a soluble factor(s), for example HGF, which is produced by endothelial cells and then spreads though the gel, activating surface receptors on the epithelial cells, causing increased proliferation. To analyze which of these different type of signalling events occurs, I designed a new co-culture assay. The two cell types were embedded into separate 100 µl of rBM and seeded in opposite ends of a 4-well culture slide chamber, allowing them to merge in the centre of the chamber. This produced a single gel with a gradient in cell densities (figure 21). At one end
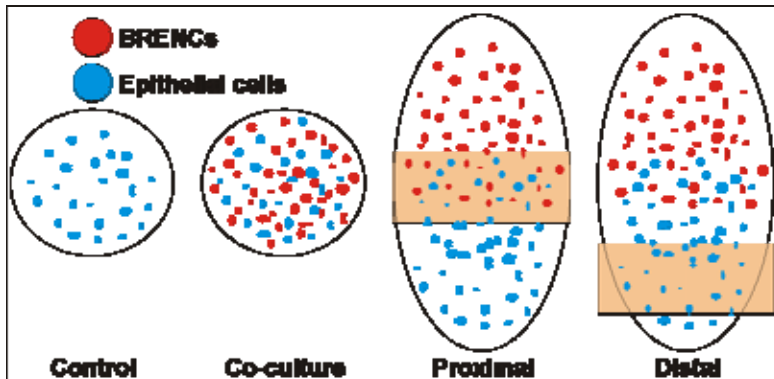
Figure 21: Schematic presentation of the gradient co-culture setup. BRENCs and epithelial cells were embedded into separate 100µl of Matrigel and seeded in opposite ends of a 4-well culture slide chamber, allowing them to merge in the centre of the chamber. This produced a single gel with a gradient in cell densities, at one end 100% endothelial cells, (red dots) at the centre (termed proximal) a mixture of the two cell types, and at the other end (termed distal) 100% epithelial cells (blue dots).

100% endothelial cells, at the centre (termed proximal) a mixture of the two cell types, and at the other end (termed distal) 100% epithelial cells. This approach eliminates the main problem with using

conditioned media where, during the conditioning of the media, nutrients are removed and waste products added.

If the first scenario above applied I expected the effects of endothelial cells to be only visible in the proximal (central) part of the gel, where cell-cell contact was likely to be present. No effect would be seen in the distal part of the gel where there are no endothelial cells.

If the second scenario above applied I expected the effects of endothelial cells to be most visible in the proximal part of the gel, and then gradually decrease towards the distal part.

2.4 – Proliferative signals from endothelial cells are conveyed via soluble factors

In this model I used the normal epithelial cell line MCF10a, the myoepithelial cell line SI28 and the malignant cell line MDA-MB-231. Compared with conventional mixed co-culture, the phenotype of the colonies seen was similar (figure 22). When SI28 cells were co-cultured with BRENCs a significant (P<0.0001) increase in colony size was observed when comparing co-culture with endothelial-free control as in the earlier model (figure 23). However, when the control was compared with the distal area the difference was also significant (P=0.003). Comparing co-culture and proximal, no significant difference (P=0.801) was detected. The same proliferative effect was seen when SI28 was exchanged for MCF10a in the model. The difference
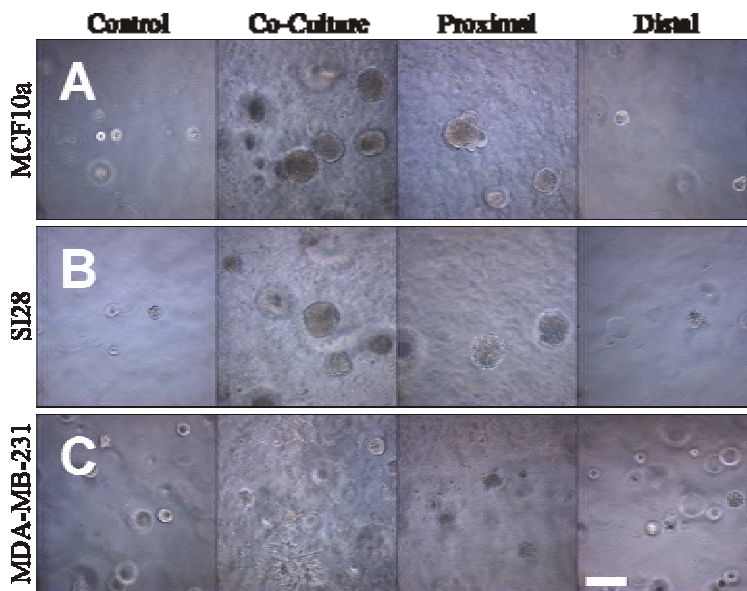
Figure 22: Proliferative effects of BRENCs are mediated via soluble factors. MCF10a (**A**) and SI28 (**B**) cells responded distally to BRENCs co-culture, colony size in the distal compartment was increased (compare control and distal columns), whereas colony size in the proximal compartment was comparable with regular co-culture (compare co-culture and proximal columns). MDA-MB-231 (**C**) did not show increased proliferation in proximal or distal compartments when compared with control. Interestingly, diffuse colonies arose much later in the proximal compartment than in the mixed co-culture, resulting in increased colony diameter (compare co-culture and proximal columns). Bar=200μm

between control and co-culture; control and distal; and distal and proximal was significant (P<0.0001, P=0.007, P<0.0001, respectively). No significant difference (P=0.250) was detected between co-culture and proximal. Co-culturing the highly malignant cell line MDA-MB-231 in this model showed a different outcome. No significant difference was detected between control and distal; control and proximal; and distal and proximal. There was however a significant difference in colony size (P<0.0001) and colony morphology (figure 22) when comparing conventional co-culture and the proximal area. At day 8, the ratio of diffuse colonies/solid colonies was very low in the proximal area. That ratio was much higher in
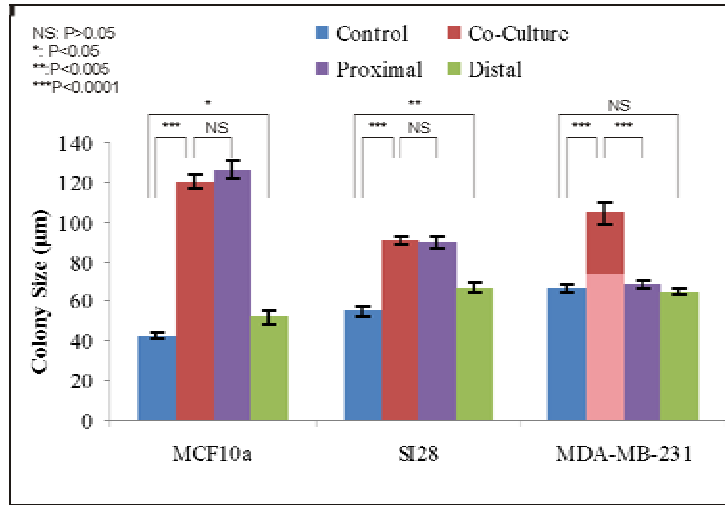
Figure 23: BRENCs cause a significant proliferative effect through the release of soluble factors.
Comparing control and co-culture there is a significant (P<0.0001) increase in colony size of all three cell lines studied. Co-culture vs. proximal was insignificant for MCF10a and SI28, (P=0.25 and 0.80, respectively). A significant (P<0.0001) difference was seen when comparing co-culture and proximal in MDA-MB-231 co-cultures, but not when diffuse colonies were omitted (pink column). Control vs. distal difference proved to be significant in MCF10a (P=0.007) and SI28 (P=0.003) co-cultures. No significant (P=0.600) difference was seen when comparing control vs. distal in MDA-MB-231 co-cultures.

conventional co-culture, as reflected in the increased colony size (figure 23). When diffuse colonies were omitted from the calculation the difference in colony size disappeared (pink insert in fig 23, MDA-MB-231 columns). By day 12 diffuse colonies had appeared in the proximal area, as well as in both control and distal area. These results suggest that morphological effects conferred by BRENCs could be caused by a different mechanism than the proliferative effects, one that perhaps requires a closer proximity between the cell types.

2.5 – Co-culture in a transwell system confirms that proliferative signals are independent of cell-cell contact.

To confirm that the proliferative effects of BRENCs were caused by soluble factors I applied another experimental setup. Using transwell

filter inserts I seeded endothelial cells into the upper chamber, allowing them to reach a confluent state. I then seeded epithelial cells embedded in rBM in the lower chamber underneath the filter inserts (figure 24). This approach eliminated any possibility of direct contact between the cell types. When co-cultured in this setup,
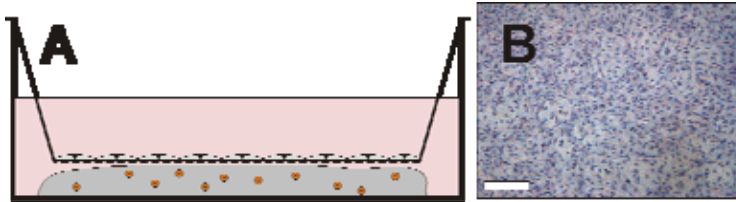
Figure 24: Schematic presentation of the Transwell filter co-culture setup. (A) BRENCs (grey) were seeded on Transwell (TW) filter inserts and allowed to reach confluency. Epithelial cells (orange) were embedded into Matrigel and seeded in the lower chamber. (**B**) Haematoxylin stained BRENCs on a TW insert. Bar=200μm

phenotypes of colonies seen were similar to those in mixed co-culture, apart from MDA-MB-231, which did not show diffuse colonies in co-culture until after 12 days in culture (figures 25 and 26). MCF10a cells showed a dramatic increase in both cloning efficiency and proliferation compared with control (figure 27) ($P<0.0001$). The same proliferative effect was seen with SI28 and D382 (figure 27a). The malignant cell lines showed an increase in proliferation in co-cultures, although to a lesser degree than the normal-like lines. There was however very little difference in cloning efficiency of MDA-MB-231, MCF7 and T47-D between co-culture and control, had an increase in colony number in co-culture (figure 27b).
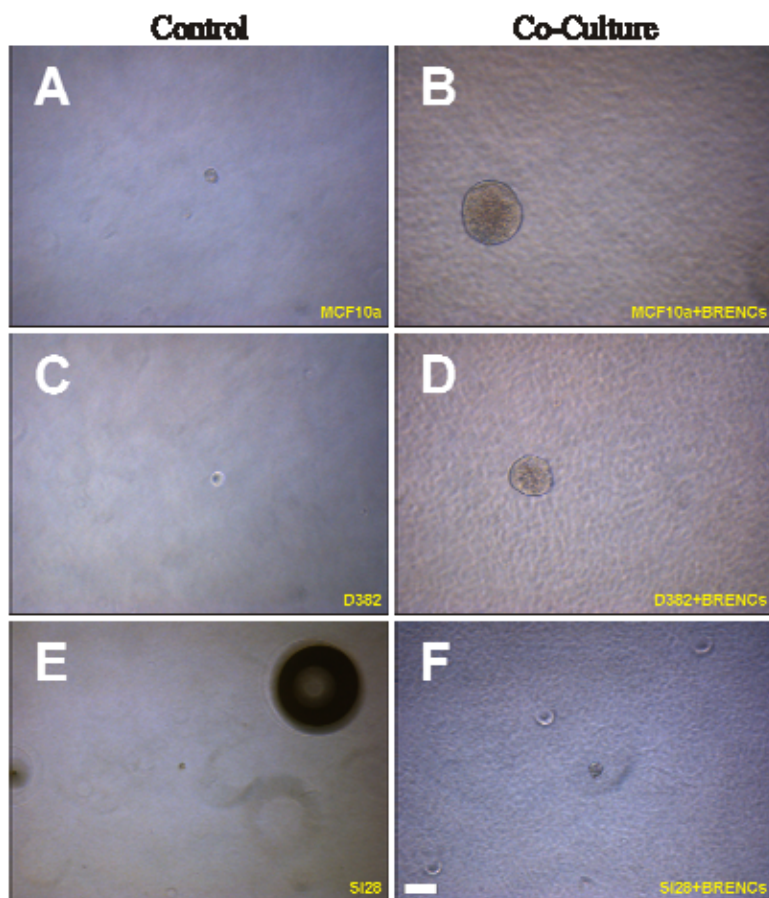
Figure 25: BRENCs enhance proliferation of non-malignant cell lines in a contact independent way.
BRENCs were able to stimulate proliferation of MCF10a (**A**,**B**), D382 (**C**,**D**) and Sl28 (**E**,**F**) when separated by a transwell filter. Each cell line formed larger colonies in co-cultures when compared with control. Bar=100μm
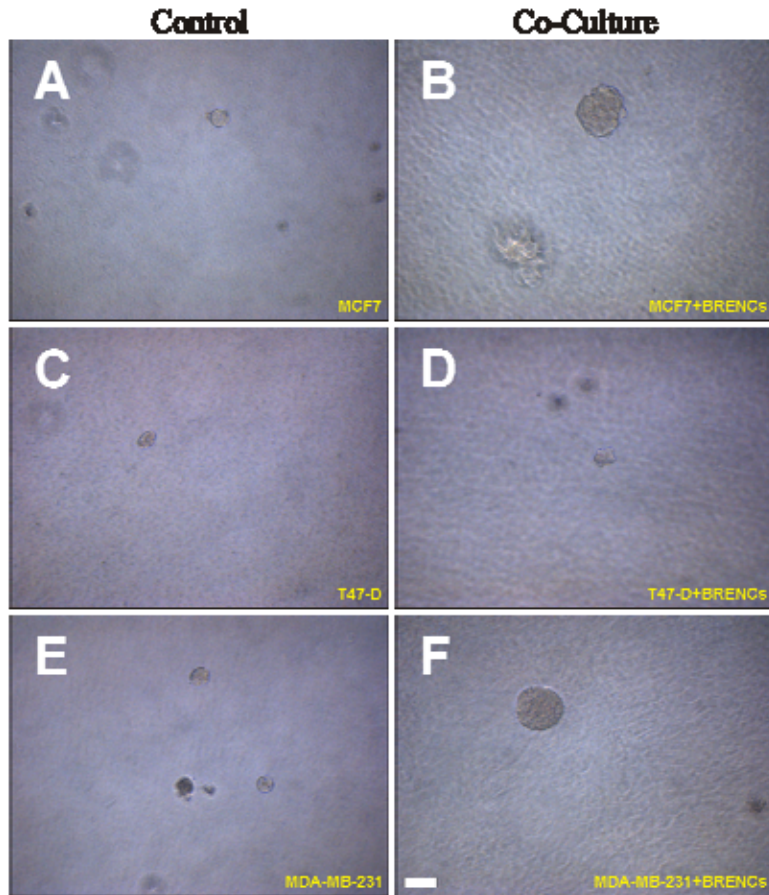
Figure 26: BRENCs stimulate proliferation of cancer cell lines in a contact independent way.
BRENCs were able to stimulate proliferation of MCF7 (**A**,**B**), T47-D (**C**,**D**) and MDA-MB-231 (**E**,**F**)
when separated by a transwell filter. Each cell line formed larger colonies in co-cultures when compared
with control. Bar=100µm
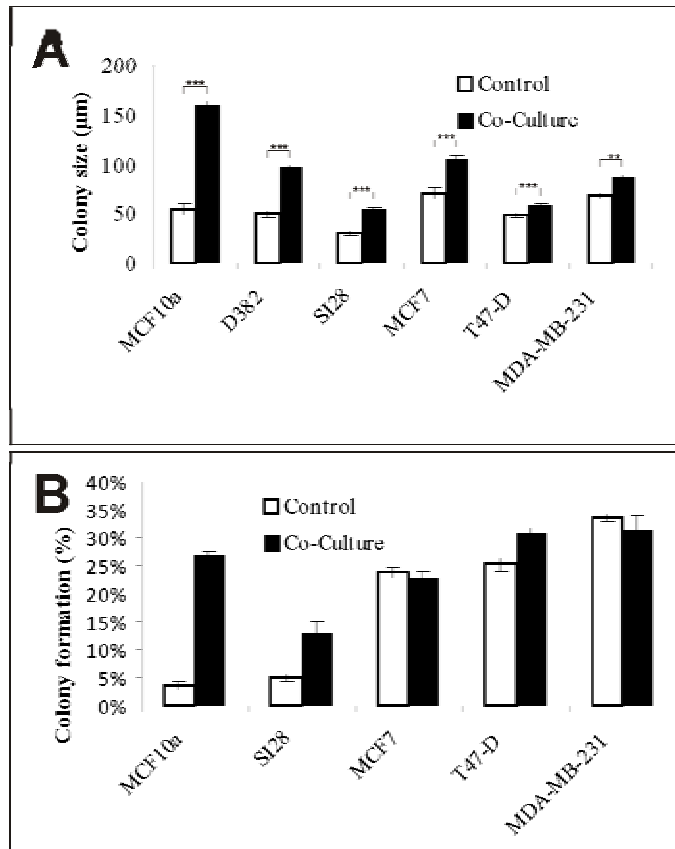
Figure 27: BRENCs have a significant effect on proliferation and cloning efficiency in a transwell setup. (**A**) A significant increase in colony size was seen in co-cultures compared with controls. (**B**) Cloning efficiency (Colony formation) was increased for the non-malignant cell lines only, similar colony count was seen in controls and co-cultures for the cancer lines.

V – Discussion

1 – Summary

In this thesis I have described the establishment and characterization of a new myoepithelial derived cell line from healthy breast tissue. The new cell line, referred to as SI28 displays a myoepithelial/basal phenotype and forms solid round colonies when cultured in rBM. I have co-cultured SI28 and two other (MCF10a and D382) non-malignant cell lines and three (MCF7, T47-D and MDA-MB-231) cancer cell lines with primary breast endothelial cells (BRENCs) and shown that BRENCs have a significant effect on both colony size and cloning efficiency in co-cultures. I have shown that increased density of BRENCs results in both increased colony size as well as increased colony count, showing a plateau at about 100,000-200,000 BRENCs per gel. To further analyse the effects seen in co-cultures I developed two co-culture assays where I could show that factors causing the increased colony formation and proliferation are secreted by the endothelial cells and are soluble, causing increased proliferation even when physically separated by filters. The new co-culture assays have great potential to be useful in future co-culture research, and will be applied to identify the factors involved in breast endothelial-epithelial interaction.

2 – SI28 and the role of myoepithelial cells in the human breast gland.

Understanding the basic communication between cell populations in the breast is pivotal for our understanding of what changes occur during breast cancer progression. Research has revealed the importance of myoepithelial cells for maintenance of correct luminal epithelial cell polarity by production of laminin α1 (Gudjonsson *et al.*, 2002a). Studies have also indicated the role of myoepithelial cells as tumour suppressors in the breast, for example by inhibiting angiogenesis by production of the protease inhibitors Maspin and TIMP-1 (Sternlicht *et al.*, 1997, Hu *et al.*, 2008). Tumours rarely originate from myoepithelial cells and when they do they are usually of low malignancy (Foschini and Eusebi, 1998). Most breast tumours originate in the luminal epithelial compartment of the breast and have different prognosis based on their molecular phenotype. Basal-like cancers are the group associated with the worst prognosis, due to high incidence of metastasis and relapse (Sorlie *et al.*, 2003). Basal-like breast cancers share many phenotypic characteristics with myoepithelial cells, including the expression of high molecular weight (HMW) basal cytokeratins such as ck5/6, ck14 and ck17 (Rakha *et al.*, 2006). This might indicate that basal cancers originate from myoepithelial cells, but research has indicated that basal cancer originates from the undifferentiated breast stem and progenitor cells, that give rise to both myo- and luminal epithelial cells (reviewed by Korsching *et al.*, (2008)). *In situ* breast carcinoma is most often lined with myoepithelial cells, separating them from the surrounding

stroma, whereas invasive carcinomas have a disorganized myoepithelial population, if such a population can be found (Sternlicht *et al.*, 1997). Myoepithelial cells isolated from tumours lack full differentiation program and have a decreased ability to maintain the polarity of luminal epithelial cells, further indicating their role as tumour suppressors (Gudjonsson *et al.*, 2002a).

2.1 – Myoepithelial cell lines and marker expression

Most research on myoepithelial cells has been performed on primary cells, isolated from reduction mammoplasties or tumours. Primary cells have a limited lifespan, so for prolonged research the need for immortal cell lines arises. In my work I have established a new cell line derived from a myoepithelial cell population isolated from healthy breast tissue. The new cell line, SI28, has been cultured stably *in vitro* for over 50 passages and displays a strong myoepithelial/basal phenotype when cultured *in vitro,* as evidenced by the expression of the HMW keratins ck5/6, ck14 and ck17. Expression of P63 and WT1 further identifies SI28 as a myoepithelial derived cell line. It has been postulated that the myoepithelial cells' natural resistance to cancer formation is in part due to their expression of tumour suppressors such as the P53 homologs P63 and P73 (Barbareschi *et al.*, 2001, Yamamoto *et al.*, 2001), Maspin (Sternlicht *et al.*, 1997) and WT1 (Yang *et al.*, 2007). Expression of simple keratins such as ck8 and ck18 indicate that SI28 cells have some properties of luminal epithelial cells as well as the myoepithelial cells'. This is a trait often connected with progenitor cells, although

the high level of ck18 staining might be indicative of either a nonspecific antibody or could be an artefact of monolayer culture. Interestingly, a subpopulation of SI28 cells was positive to ESA and ck19 further suggesting this basal/bipotential property. Gudjonsson *et al.* (2002b) isolated a stem cell population in a suprabasal position in the breast gland, meaning they reside in the luminal compartment, in contact with myoepithelial cells but do not reach into the lumen. These cells (D492 cell line) express a mixture of luminal- and myoepithelial markers including ESA, ck14 and ck19, displaying their bipotential properties.

2.2 – Three-dimensional cell culture

When cultured in three dimensions, D492 forms colonies with a branching morphology similar to the *in vivo* TDLUs (Gudjonsson *et al.*, 2002b). In three-dimensional culture, SI28 cell form simple solid round colonies, similar to that of primary myoepithelial cells. Immunostaining of cryosectioned gels shows that marker expression is similar to monolayer culture. It must be considered that 3D cultured colonies arise from single cells, so immunostaining of single colonies does not fully represent the phenotype of the cell line as a whole. Selection during *in vitro* culture favours those cells that proliferate fast, causing their ratio in culture to increase and the ratio of specialized cells, like fully differentiated myoepithelial cells to decrease. This is demonstrated by the disappearance of αSMA expressing specialized cells in the first passages of myoepithelial cells *in vitro* and explains the lack of αSMA expression in SI28 cultures.

αSMA expression can be induced by adding serum to the culture medium prior to immunostaining. This has not yet been done with SI28 cells. Expression of thy-1 was lost in a similar time period as αSMA, loss of thy-1 has been connected with increased cell proliferation (Hagood *et al.*, 2005, Lung *et al.*, 2005). Transfection with E6 and E7 causes an increase in cell proliferation, possibly explaining the loss of thy-1 expression in SI28 cells.

2.3 – Next steps in SI28 research

Ongoing research is being performed at our lab to further identify SI28 cells' myoepithelial or basal cell properties. Expression of laminin α1 will be analyzed and SI28 will be co-cultured with primary luminal epithelial cells in collagen to see whether it can correct the inverse phenotype of LEP colonies. In the future, the SI28 cell line can provide a stable supply of myoepithelial derived cells for long term *in vitro* research.

3 – Three-dimensional co-culture

It has become accepted that in order to understand organ development and cancer progression it is not enough to look at single cell populations. It is necessary to look at the whole organ as a unit of function. Development of the breast gland takes place in two distinct phases, the first during embryonic development, and the second during puberty (Howard and Gusterson, 2000). Both phases rely heavily on interaction between the epithelium and the surrounding

stroma which produces a number of growth factors regulating proliferation of the epithelium, including HGF.

3.1 – The role of HGF and VEGF in epithelial-stromal interaction

Expression of HGF in fibroblasts has been shown to be positively regulated by oestrogen. Kuperwasser *et al*. (2004) demonstrated the effect of fibroblasts where they were able to induce the formation of human breast epithelial branching structure in NOD/SCID mice, by first humanizing the mouse mammary fat pad with immortalized human fibroblasts and then implanting human breast organoids into the fat pad. Endothelial cells in the liver have been shown to produce HFG when stimulated with VEGF-1. This production of HGF led to increased liver mass due to increased proliferation of hepatocytes (LeCouter *et al.*, 2003). In a novel three-dimensional assay, Milner (Milner, 2007) showed the ability of neurospheres to recruit endothelial cells in co-culture through VEGF-1 signalling. Endothelial cells' responsiveness to steroid hormones has been reported, where stimulation with steroid hormones, such as oestrogen or testosterone has induced epithelial cells to produce VEGF-1, probably though HIF1α, stimulating endothelial proliferation, followed by a proliferation in the epithelial compartment (Franck-Lissbrant *et al.*, 1998, Rudolfsson and Bergh, 2008).

3.2 – Endothelial cells in breast epithelial signalling

In the breast gland, Shekhar *et al*. (2000) showed that endothelial cells were able to induce increased proliferation and

ductal-alveolar formation of MCF10 cells in the presence of estradiol in three-dimensional co-culture. Sigurdsson (Sigurdsson, 2005) reported a significant increase in primary breast epithelial proliferation when co-cultured with breast endothelial cells in a novel three-dimensional co-culture system. I applied the endothelial cell isolation and co-culture techniques developed by Sigurdsson in his thesis (Sigurdsson, 2005, Sigurdsson *et al.*, 2006) to analyse the effect of endothelial cells on normal derived cell lines, including SI28. I demonstrated that when co-cultured with BRENCs the cell lines display a marked increase in proliferation. Considering the data discussed earlier it seems plausible that similar factors apply to the breast gland. That is, systemic hormones (estradiol) induce epithelial cells to produce VEGF-1 causing endothelial cells to produce HGF which eventually causes increased proliferation of epithelial cells.

3.3 – Gradient and transwell models

The number of colonies seen in co-cultures was connected with the number of BRENCs embedded, suggesting the possibility of direct cell-cell contact, as opposed to secreted factors. To address this issue I developed a novel co-culture system, where direct endothelial-epithelial contact was only possible in a certain zone in the culture. Using that model I demonstrated that the proliferative effects seen were caused by soluble factors secreted by endothelial cells. The results were confirmed using a second model where transwell filters were used to physically separate the cell populations without the loss of proliferative effect.

Transswell filters have been used for several years in invasion, migration and chemotaxis assays (Karnoub *et al.*, 2007). The filters have different pore sizes, allowing or preventing cells to migrate from one side of the filter to the other. They have been used in co-culture models to show the presence of soluble factors or direct contact dependency as evidenced by Shen *et al.* (2004) where they were able to promote the growth of neural stem cells in co-culture with endothelial cells.

3.4 – Comparison of the co-culture models

There is a notable difference between the transwell model and the other two models used in this thesis, namely that in the transwell model the endothelial cells form a monolayer and are proliferative, whereas in the other two models they are embedded in rBM which prevents them from proliferating. The reasons why this happens have not yet been determined, but could be attributed to the concentration of the gel matrix in the 3D model. The ratio of epithelial/endothelial cells seeded into rBM does not reflect the ratio *in vivo*. Endothelial cells *in vivo* can be found in very close relationship with the epithelial structures, but are not in greater numbers than the epithelial structures which are densely populated with epithelial cells. However, after two weeks in culture the epithelial cells have formed large colonies and have outnumbered the endothelial cells. Endothelial cells are highly dependent on the culture medium used. In monolayer they are cultured in EGM containing 5% serum. When serum is removed they are unable to proliferate. For that reason EGM5 was used in all co-culture

experiments. The EGM medium contains several growth factors known to affect epithelial cells, including EGF, bFGF and IGF; it also contains VEGF, which is necessary for endothelial cell viability. Currently, the identity of the proliferation factors is unknown, but there is ongoing research at the laboratory in order to identify the factors involved.

4 – Breast Cancer and the stroma

The role of endothelial cells in breast cancer progression has until recently been considered a logistical one, that is to supply the tumour with oxygen and nutrients and to remove waste. The emerging evidence that endothelial cells are not the passive identities they were once considered has created many research opportunities. As discussed in the introduction, angiogenesis is vital for tumour growth, hypoxia in the tumour causes the release of VEGF through HIF1α activation. VEGF in turn causes recruitment of blood vessels into the tumour, both increasing proliferation and an increased risk of metastasis.

4.1 – The role of stromal cells in cancer progression

Considering the proliferative effect of endothelial cells in the liver and prostate (Franck-Lissbrant *et al.*, 1998, LeCouter *et al.*, 2003), as well as in co-culture models with normal breast epithelium (Sigurdsson, 2005) and cell lines (Shekhar *et al.*, 2000) it is easy to wonder if part of that increased proliferation is facilitated though release of endothelial derived growth factors. The wound healing

response of fibroblasts and endothelial cells to tumour invasion is an important component of tumour progression (reviewed by Bissell and Radisky, (2001), Orimo and Weinberg, (2006)). Activated fibroblasts produce both HGF and TGFβ, which might sound contradictory, considering HGF is under negative control by TGFβ. Both of these factors however contribute to the abnormal environment present in an invasive tumour (Liotta and Kohn, 2001, Haslam and Woodward, 2003, Zhang *et al.*, 2003). Returning to the work of Kuperwasser *et al.* (2004) where they were able to induce an apparent cancer growth from a phenotypically normal epithelium by over-expressing both HGF and TGBβ in the fibroblasts used to humanize the mouse mammary fat pad (Kuperwasser *et al.*, 2004). This indicates the important role of the stroma in cancer progression. It does not however mean that abnormal stroma can induce cancer formation from healthy epithelium. Kuperwasser and colleagues were unable to reproduce these results, suggesting that the cancerous growth originated from cells that had already undergone neoplastic changes prior to the experiment.

4.2 – Endothelial cells in co-culture with cancer cell lines

In my co-culture experiments, both the normal cell lines and the cancer cell lines responded to co-culture with endothelial cells with increased proliferation, forming much larger colonies in co-culture than in control. A notable difference in the behaviour of normal and cancer cell lines was the increase in colony formation. At a clonal level, where only 500 epithelial cells were used in co-cultures there

was a marked increase in colony formation. However, in the gradient and transwell assays, where the density of epithelial cells seeded was higher, that increase in colony formation was not seen in co-cultures with cancer cell lines, which formed colonies in controls. An important part of cancer progression is the ability of cancer cells to stimulate proliferation and viability through autocrine and paracrine pathways, this can explain the decreased effect on colony formation when comparing the normal and cancer cell lines.

Shekhar *et al*. (2007) co-cultured fibroblasts isolated from oestrogen receptor (ER) positive and ER negative breast tumours and used them in co-culture with epithelial cell lines in direct contact. Co-culture with fibroblasts from the ER negative tumours resulted in a decreased sensitivity to Tamoxifen treatment, indicating that direct contact with stromal cells conveyed mitogenic effects, as well as pro-liferative effects. One interesting difference between the conventional co-culture and the new gradient co-culture system was the number of diffuse MDA-MB-231 colonies. In co-culture the majority of colonies had a diffuse phenotype, appearing at day 7-8, whereas in both the gradient and transwell co-culture systems the appearance of diffuse colonies was delayed, only appearing at day 12. This suggests that perhaps the factors promoting the appearance of a certain phenotype, as seen by Kuperwasser *et al*. (2004) could be delivered by a different method than the proliferative effect, perhaps requiring much closer or direct cell contact. Much more research needs to be performed on this before any conclusions can be drawn.

5 – Future perspectives

The identity of the endothelial-derived proliferation factor(s) has not yet been established. Initial efforts have included the neutralization of HGF in the culture media using monoclonal neutralizing antibodies. Unfortunately, preliminary results have been inconclusive. I believe that the proliferative effects are provided not by one growth factor, but a mixture of factors. We are currently using Enzyme-Linked ImmunoSorbent Assay (ELISA) to identify factors from conditioned culture medium from co-cultures, compared with control. This approach was used by Karnoub *et al*. (2007) where they identified the chemokine ligand CCL5 as a factor produced by mesenchymal cells affecting the metastatic ability of cancer cell lines, including MDA-MB-231 and MCF7 which were both used in this study. Neutralizing antibodies have been widely used in developmental and cancer research, Wang *et al*. (2002) and Weaver *et al*. (1997) were able to reverse the phenotype of the malignant HMT3522 subline T4-2 by using neutralizing antibodies raised against β1-integrin and EGFR. RNA interference (RNAi) has much potential in order to identify the factors involved. By knocking down the growth factor production of endothelial cells it is possible to avoid the background inevitably created by serum and growth factors in the culture medium.

Our lab has been collaborating with the research group of Kristján Leósson at the Department of Physics, Science Institute, at the University of Iceland during the last 2 years on development of a new approach to immunofluorescent imaging. High quality immuno-

fluorescent images require a high signal-to-noise ratio, meaning that when looking at samples at high magnification a strong signal is needed to reduce the noise from nonspecific binding of fluorescent particles. Leósson has been developing integrated optical waveguide circuits on a chip that can be used for surface-bound evanescent-wave excitation, in contrast to conventional epifluorescent imaging. The technique can be used for biological samples including live cells. Illumination is restricted to a few hundred nanometers from the surface of the chip. This means that the noise generated by a relatively thick sample that is illuminated from above is greatly reduced. This approach has many potential uses in cell biology, it allows high resolution imaging of cell membrane proteins, both in fixed cells that have been stained using immunofluorescent antibodies as well as studying GFP-tagged proteins in live cells. Due to unforeseen problems with the supply of waveguide chips by the company Lumiscence A/S in Denmark, the weight of this project in my thesis was reduced. Recently, however, chip fabrication has been resumed at the University of Iceland and continued collaboration will hopefully result in the use of this system in the study of membrane proteins involved in cell-cell interaction in the co-culture models.

6 – Conclusion

To summarize, I have created a new epithelial cell line with myoepithelial properties named SI28. The cell line has a myoepithelial expression pattern, with some mixed luminal epithelia properties, characteristic of basal or progenitor cells in the breast. SI28 has a near-normal karyotype, and forms solid round colonies when cultured in three-dimensional rBM. I believe that SI28 cell line will be a valuable tool in our laboratory for future research on myoepithelial differentiation program, breast morphogenesis, stem cell biology and cancer. I have also isolated and cultured tissue specific breast endothelial cells as described (Sigurdsson, 2005) and used them in co-culture experiments with normal and malignant epithelial cell lines in three-dimensional rBM. I have demonstrated that endothelial cells have a significant proliferative effect on both normal and malignant cell lines, indicating their important role in both normal breast development and cancer. I developed a new co-culture system where differences in cell population densities form a gradient, making it a useful tool in chemokine research. I have used this model to demonstrate that the proliferative effects seen in co-culture are delivered by soluble factors secreted by the endothelial cells. Initial results also indicate that morphogenic signals are delivered via a separate mechanism, which will have importance in developmental research, such as branching morphogenesis. Further research is ongoing in order to identify the factors involved in the proliferative signals produced by the endothelial cells.

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