# Cellular and molecular mechanisms in breast morphogenesis and epithelial to mesenchymal transition

**Valgarður Sigurðsson**

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Valgarður Sigurðsson

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*Supervised by:*

Þórarinn Guðjónsson, PhD. and Magnús Karl Magnússon, MD.

*Doctoral Committee:*

Þórarinn Guðjónsson, PhD., Magnús Karl Magnússon, MD. Jón Már Björnsson, PhD. Unnur Þorsteinsdóttir, PhD. and Ole William Petersen, MD.


University of Iceland, Faculty of Medicine

Biomedical Center, Stem Cell Research Unit

&

Landspitali LSH

Department of Laboratory Hematology

Valgarður Sigurðsson

# Frumu- og sameindalíffræðileg stjórnun greinóttrar formgerðar og bandvefsumbreytingar í brjóstkirtli

*Leiðbeinendur:*

Þórarinn Guðjónsson, PhD. og Magnús Karl Magnússon, MD.

*Doktorsnefnd:*

Þórarinn Guðjónsson, PhD., Magnús Karl Magnússon, MD. Jón Már Björnsson, PhD. Unnur Þorsteinsdóttir, PhD. og Ole William Petersen, MD.

Háskóli Íslands, Læknadeild

Lífvísindasetur Læknagarðs, Rannsóknastofa í stofnfrumufræðum

&

Landspítali LSH

Rannsóknastofa í blóðmeinafræði

*Fyrir Arngunni, Iðunni,*

*Þorra og Nínu Rakel*

# ÁGRIP Á ÍSLENSKU

Brjóstkirtillinn er samsettur úr greinóttum þekjuvef sem umlukinn er æðaríkum stoðvef. Stofnfrumur þekjuvefjar sjá um vöxt og viðhald kirtilsins og eru boð gegnum týrosín-kínasa viðtaka talin veigamikil í þessu ferli. Innanfrumustjórnprótín sem tilheyra Sprouty fjölskyldunni hafa áhrif á virkni boða gegnum týrósín kínasa og hefur verið sýnt fram á nauðsyn þeirra við stórnun greinóttrar formgerðar í ýmsum vefjum. Í myndun greinóttrar formgerðar taka þekjufrumur að hluta til upp tjáningarmynstur bandvefsfruma (e.Epithelial to mesenchymal transition,EMT) til að öðlast þá eiginleika sem þarf til að ryðja sér leið inn í umliggjandi stoðvef. Þetta ferli er vel þekkt í fósturþroska en í krabbameinum eru slíkar frumur illa skilgreindar en hafa nýlega verið tengdar við tilurð krabbameins stofnfruma. EMT svipgerð sést oftar innan basal-líkra brjóstakrabbameina en þessi æxli eru talin eiga upptök sín í stofnfrumum brjóstkirtilsins. Boð gegnum týrosín kínasa gegna lykilhlutverki í að hvata bæði greinótta formgerð og EMT. Markmið þessa verkefnis var að kanna hlutverk æðaþels og sprouty í greinóttri formgerð og EMT í brjóstkirtli. Til þess hef ég einkum notað samræktun brjóstaæðaþels með brjóstaþekjuvefsfrumum (þar með talið brjóstaþekju-stofnfrumum) í þrívíðu (3D) frumuræktunarkerfi, auk þess að skoða tjáningu í eðlilegum og illkynja brjóstavef.

Niðurstöður sýndu að æðaþel hafði mikil frumufjölgandi áhrif á eðlilegar og illkynja brjóstaþekjuvefsfrumur í 3D samræktun. Auk þess bentu samræktir til örvunar á bæði greinóttri formgerð og EMT-umbreytingu í brjóstastofnfrumulínunni D492. D492 myndaði frumuklasa með bandvefslíkum frumum sem voru einangraðar og síðar ræktaðar sem D492M (e.mesenchymal) undirlína. Með því að kanna tjáningu kenniþróteina þekjuvefs og bandvefsfruma kom í ljós að D492M hafði undirgengist EMT-umbreytingu. Hún sýndi ennfremur svipgerð og starfhæfa eiginleika krabbameinsstofnfruma, en slíkir eiginleikir hafa verið tengdir EMT. Ég kannaði jafnframt tjáningu EMT kenniþróteina í basal-líkum brjóstaæxlum og gat sýnt fram á hærri tjáningu slíkra kenniþrótína þar sem aukin þéttni af æðaþeli var til staðar. Að lokum sýndi ég einnig fram á að tjáning Sprouty-2 (Spry-2) í manna og músa brjóstvef sveiflast í takt við formgerðarmyndun. Niðurstöður með Spry-2 gena

bælingu (e.KD) gefa sterklega til kynna að Spry-2 hafi mikilvæg temprandi áhrif á stjórnun greinóttarar formgerðar brjóstkirtils. Jafnframt sýndu D492 Spry-2 KD frumur mun meiri tilhneigingu til að mynda bandvefslíkar frumuklasa í 3D samrækt með æðaþelsfrumum.

Samantekið þá hef sýnt fram á að æðaþælsfrumur eru mikilvægar fyrir vöxt og formgerð þekjuvefjar brjóstkirtilsins og álykta að þær séu hluti af stofnfrumuvist kirtilsins og stuðli þannig að myndun greinóttrar formgerðar og bandvefsumbreytingu. Ég álykta ennfremur að æðaþelsfrumur geti þjónað áður óþekktu hlutverki í framþróun og meinvörpun basal-líkra brjóstakrabbameina. Ég sýndi jafnframt fram á að Spry-2 er mikilvægt stjórnprótein greinóttrar formgerðar brjóstkirtils og að bæling á því geri frumur móttækilegri fyrir bandvefsumbreytingu.

Lykilorð: Greinótt formgerð, Æðaþelsfrumur, Bandvefsumbreyting, Sprouty-2, Brjósta stofnfrumur, Basal-lík brjósta krabbamein.

# ABSTRACT

The breast is composed of branching epithelial ducts terminating in structures referred to as terminal duct lobular units (TDLUs) surrounded by vascular-rich stroma. Continous cell renewal and expansion during breast morphogenesis is dependent on epithelial stem cells. During branching, epithelial cells acquire partial mesenchymal-like phenotype with increased migration and loss of polarization, similar to epithelial-mesenchymal transition (EMT). In breast cancer, EMT has been linked to basal-like breast cancer, a highly vascularized and aggressive subtype rich in cancer stem cells. Signals through receptors tyrosine kinases (RTKs), including the epidermal growth factor receptor (EGFR) family, have been shown to be critical in branching morphogenesis in different epithelial systems. RTKs are regulated through negative feedback by the sprouty protein family and lack of sprouty has been shown to result in abnormal and increased branching.

The aim of this thesis was to study extrinsic (endothelial) and intrinsic (Sprouty-2) regulation of breast morphogenesis and EMT. Towards that goal I cocultured normal and malignant breast epithelial cells with breast endothelial cells (BRENCs) in 3D cell culture to unravel their morphogenic potential.

I have demonstrated that BRENCs stimulate growth and morphogenesis of breast epithelial cells and in coculture with D492, a breast epithelial stem cell line, a dramtic increase in branching morphogenesis was seen. In addition to branching, BRENCs induced the formation of spindle like colonies in D492. D492M, a (mesenchymal) subline was isolated from a spindle-like colony and characterization showed irreversible EMT, as evidenced by reduced expression of keratins, a switch from E- to N-cadherin and acquisition of cancer stem cell characteristics. The EMT phenotype could be partially blocked by inhibition of hepatocyte growth factor. Furthermore, I showed that tumor cells close to vascular rich areas within basal-like breast cancers had a characteristic EMT phenotype. Finally, I showed that Sprouty-2 expression is linked to critical steps in branching morphogenesis. Sprouty-2 knock down in D492 resulted in more complex branching structures in 3D culture indicating the loss of negative feedback control of branching morphogenesis. Interestingly, using

coculture with endothelial cells I demonstrated that Sprouty-2 knock down cells were more prone to undergo EMT further tightening the link between branching and EMT in breast morphogenesis.

Collectively, I have shown that endothelial cells are potent inducers of breast epithelial growth, branching morphogenesis and in the conversion of cells towards an EMT phenotype. I hypothesize that endothelial cells may be an important regulator of the stem cell niche in the human breast gland and may also play an active role in the progression of basal-like breast cancers through induction of EMT. In addition, I have shown that Spry-2 is instrumental for correct branching morphogenesis of the human breast gland and in safeguarding breast epithelial integrity.

Keywords: Branching morphogenesis, Endothelial cells, Epithelial to mesenchymal transition, Sprouty-2, Breast epithelial stem cells, Basal-like breast cancer.

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*"Science is the father of knowledge,*

*but opinion breeds ignorance."*

*-Hippocrates-*

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# CLARIFICATION OF CONTRIBUTION

**Paper #1**. In this paper we describe improved methods for the isolation and propagation of breast endothelial cells (BRENCs). I designed the study in collaboration with Agla Fridriksdottir and Thorarinn Gudjonsson (TG). Furthermore, I performed all the experiments, analyzed the data and wrote the manuscript in collaboration with TG.

**Paper #2.** In this paper we describe proliferation effects of BRENCs in coculture studies with primary breast epithelial cells and cell lines. I designed the setup of 3D cocultures of epithelial cells in rBM with BRENCs and performed all pilot studies that were replicated and expanded by Sævar Ingþórsson. Furthermore, I participated in the design of experiments, analyzed data and participated in the writing of the manuscript along with TG and Magnus Karl Magnusson (MKM).

**Paper #3.** In this paper we describe the effects of BRENCs on branching morphogenesis and EMT in a breast epithelial stem cell line, D492. I designed all the experiments in collaboration with TG and MKM, perfomed all the experiments and analyzed the data in collaboration with Bylgja Hilmarsdóttir. I wrote the manuscript in collaboration with TG and MKM

**Paper #4**. In this paper we describe expression and functional effects of Sprouty-2 in branching morphogenesis. I designed all the experiments in collaboration with TG and MKM. Furthermore, I perfomed all IHC and expression data on mouse mammary glands and human breast glands. I, analyzed the expression of sprouty in branching morphogenesis of 3D rBM cultures. Sprouty-2 Knock down (KD) cells were made in collaboration with Sigríður Rut Franzdóttir. All functional studies on Sprouty-2 KD cells, including 3D rBM monculture and coculture studies, were performed by me and Bylgja Hilmarsdóttir. Furthermore, I analyzed the data and wrote the manuscript in collaboration with TG and MKM.

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# LIST OF ABBREVIATIONS

2D: Two dimensional

3D: Three dimensional

BLBC: Basal-like breast cancer

BRENC: Breast endothelial cells

E-cad: Epidermal cadherin

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EGM: Endothelial growth medium

EMT: Epithelial to mesenchymal transition

EpCAM: Epithelial specific antigen

ER: Estrogen receptor

ERK: Extracellular signal-regulated Kinase

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

FGFR: Fibroblast growth factor receptor

GFP: Green fluorescent protein

GH: Growth hormone

HGF: Hepatocyte growth factor

HIF: Hypoxia inducible factor

HPV-16: Human papilloma virus 16

HUVEC: Human umbilical vein endothelial cells

IGF-1: Insulin like growth factor 1

IF: Immunofluorescence

IHC: Immunohistochemistry

IL-6: Interleukin 6

K: Keratin

KD: Knock down

KO: Knock out

LEP: Luminal epithelial cell

MAPK: Mitogen-activated protein kinase

MEP: Myoepithelial cell

MDCK: Madin-darby canine kidney cells

miRNA: Micro RNA

Muc1: Sialomucin

MVD: Microvascular density

N-cad: Neuronal cadherin

PDGF: Platelet derived growth factor

p-EGFR: Phosphorylated epidermal growth factor receptor

PI-3 kinase: phosphatidylinositol 3-kinase

PR: Progesterone Receptor

Q-rt-PCR: Quantitative real time PCR

RB: Retinoblastoma

rBM: Reconstituted basement membrane

RTK: Receptor tyrosine kinase

Spry: Sprouty

TDLU: Terminal duct lobular unit

TF: Transcription factor

TGF-β1: Transforming growth factor beta 1

Thy-1: Thymocyte differentiation antigen 1

TJ: Tight junction

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

WB: Western blot

# LIST OF FIGURES

# LIST OF PAPERS

**This thesis is based on the following original papers:**

**1.** Human breast microvascular endothelial cells retain phenotypic traits in long-term finite life span culture. Sigurdsson V, Fridriksdottir AJ, Kjartansson J, Jonasson JG, Steinarsdottir M, Petersen OW, Ogmundsdottir HM, Gudjonsson T. In Vitro Cell Dev Biol Anim. 2006 Nov-Dec;42(10):332-40.

**2.** Endothelial cells stimulate growth of normal and cancerous breast epithelial cells in 3D culture. Ingthorsson S, Sigurdsson V, Fridriksdottir AJ, Jonasson JG, Kjartansson J, Magnusson MK, Gudjonson T. BMC Res Notes. 2010 Jul 7;3:184.

**3.** Endothelial induced EMT in breast epithelial cells with stem cell properties. Sigurdsson V, Hilmarsdottir B, Sigmundsdottir H, Fridriksdottir AJ, Rigner M, Villadsen R, Borg A, Agnarsson BA, Petersen OW, Magnusson MK, Gudjonsson T. PLoS One. 2011 sep;6(9):e23833

**4.** Branching morphogenesis in the mammary gland is regulated by Sprouty-2. Sigurdsson V, Hilmarsdottir B, Ingthorsson S, Franzdottir SR, Steingrimsson E, Magnusson MK, Gudjonsson T. Manuscript.

# INTRODUCTION

## 1. Contextual histology of the human breast

The control of branching morphogenesis remains one of the most challenging questions in developmental biology. This is particularly relevant for the human breast gland that is composed of a complex tree of branching epithelial ducts separated from the surrounding vascular-rich stroma by a basement membrane. The adult female breast gland is under continuous remodeling during each menstruation cycle and during pregnancy as evidenced by the cycling process of cell proliferation, differentiation and apoptosis. These processes are mediated by cell-cell and cell-stroma interactions as well as by systematic hormones and local growth factors.

Modeling the contextual histology of the human breast gland in culture is a prerequisite for uncovering the function of distinct cell types in breast tissue morphogenesis and for capturing critical steps in breast cancer formation and progression.

### 1.1 Breast development

The human breast gland is a modified sweat gland that develops as an appendage from the epidermis in embryogenesis (reviewed in (Ronnov-Jessen, Petersen et al. 1996; Cowin and Wysolmerski 2010)). Breast development occurs in three distinct phases, embryonic, postnatal and in adulthood, with the most significant changes occurring at the onset of puberty and during pregnancy (Russo and Russo 2004; Nelson and Bissell 2005). During embryogenesis, the development of the breast is dependent on heterotypic interactions between the branching epithelium and the surrounding fetal mesenchyme that induces proliferation and invasion of the epithelium. The epithelial cells invade and branch into the underlying stroma, resulting in the generation of rudimentary epithelial ducts (Ronnov-Jessen, Petersen et al. 1996; Howard and Gusterson 2000). From birth until the onset of puberty the breast gland is essentially the same in both males and females (Russo and Russo 2004). At puberty, further development occurs in females influenced by increased estrogen and progesterone hormonal signals (Howard and Gusterson 2000). Epithelial budding forms

lobules from the rudimentary ducts which leads to the formation of terminal duct lobular units (TDLUs). This process is accompanied by stromal expansion, including proliferation of resident stromal cells and increased vasculature (Ronnov-Jessen, Petersen et al. 1996; Russo and Russo 2004). Estrogen plays an important role in ductal elongation whereas progesterone is important for formation and differentiation of the TDLUs (reviewed in (Fendrick, Raafat et al. 1998; Neville, McFadden et al. 2002)). The breast gland cycles through continuous cell turnover during each menstrual cycle as evidenced by cell proliferation, differentiation and apoptosis (Going, Anderson et al. 1988). During pregnancy, increased production of ovarian hormones results in dramatic expansion of the epithelial compartment that develops further into the functional lactating breast at parturition (Russo, Moral et al. 2005). Complete functional differentiation of the breast epithelium is limited to the lactation period and with cessation of lactation, the breast gland involutes to its resting pre-pregnancy state (Russo, Calaf et al. 1987). This continuous cycle is seen in every menstruation period and on a large scale in each pregnancy until the ovary function declines at menopause (Going, Anderson et al. 1988).

**1.2 Breast epithelial cells**

The adult breast epithelial ducts are composed of an inner layer of luminal epithelial cells (LEP) and an outer layer of myoepithelial cells (MEP) (figure. 1) (Ronnov-Jessen, Petersen et al. 1996). Specific techniques can be used to separate LEP and MEP cells based on marker expression (Gudjonsson, Villadsen et al. 2004). In general, the LEP cells express cytokeratins (K) such as K8, K18 and K19, and other markers like sialomucin (Muc1), epithelial specific antigen (EpCAM) and components of tight junction complexes (occludin and claudins) (Gudjonsson, Villadsen et al. 2004). Only a sub-population (3-10%) of the luminal epithelial cells express the estrogen receptor (ER) (Petersen, Hoyer et al. 1987; Clarke, Howell et al. 1997). In contrast, MEP cells express K5, K14 and K17 and other proteins like alpha smooth muscle actin, Thy-1 and beta4-integrin (Gudjonsson, Ronnov-Jessen et al. 2002; Gudjonsson, Adriance et al. 2005). The LEP and MEP cells reside throughout the whole ductal tree of the breast including the TDLUs that are the functional units of the breast gland (Figure 1). In the

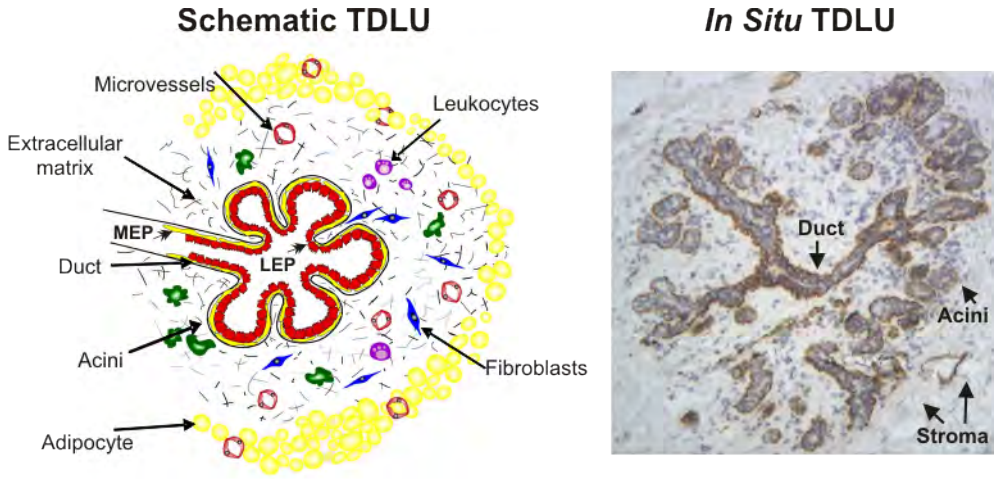


**Figure 1. Histology of the human TDLU.**
*Left.* A schematic figure of TDLU. Breast epithelial ducts terminate in lobules composed of acini and are collectively called terminal duct lobular units (TDLU). The breast epithelium is composed of an inner layer of luminal epithelial cells (LEP-red) and an outer layer of myoepithelial cells (MEP-yellow). The epithelial compartment is separated from the surrounding stroma by a basement membrane. The collagen rich stromal compartment is composed of fibroblasts, adipocytes, immune cells and endothelial cells in microvessels along with extracellular matrix. *Right.* In situ figure of TDLU. Immunohistochemistry staining of cryosectioned human TDLU stained for β4-integrin (brown) outlining the connection of the epithelium to the basement membrane. Counterstain with hematoxylin (blue).

TDLUs, LEP cells produce and secrete milk during lactation and MEP cells (upon activation from oxytocin) contract and pump the milk to the collecting ducts (reviewed in (Lochter 1998)). Evidence show that LEP and MEP cells arise from a common stem / progenitor cell that reside within the luminal epithelial compartment (Kao, Nomata et al. 1995) (Stingl, Eaves et al. 1998) (Pechoux, Gudjonsson et al. 1999; Stingl, Eaves et al. 2001) (Gudjonsson, Villadsen et al. 2002) (Villadsen, Fridriksdottir et al. 2007).

### 1.3 Stem cells of the human breast gland

The existence of breast epithelial stem cells has been postulated for many years due to the dramatic tissue remodeling (proliferation – differentiation – apoptosis) during breast morphogenesis associated with menstruation and pregnancy. Pioneering, experiments were conducted in mouse models using serial transplantation studies and immunostaining for postulated stem cell related markers (Deome, Faulkin et al. 1959; Daniel, Young et al. 1971; Smith and Medina 1988). Two papers in Nature eliminated any doubt over the existence of the mouse mammary stem cells (Shackleton, Vaillant et al. 2006; Stingl, Eirew et al. 2006). These research groups utilized a unique marker profile composed of antibodies against

CD24, CD49f and CD29 surface markers to isolate cells that were highly enriched in mouse mammary epithelial stem cells. They were able to recreate a fully functional mammary gland in a cleared mammary fat pad from single stem cells (Shackleton, Vaillant et al. 2006). The mouse mammary stem cells overlap the basal compartment (including MEP cells) and these cells do not express the estrogen receptor (Stingl 2009). The identification of human breast stem cells has been a more difficult task mainly due to the lack of functional assays to test the stemness of suspected cell populations. However, several breast epithelial populations have been postulated to have stem cell properties. These include side population cells (effluction of Hoechst dye), suprabasal cells (luminal cells with suprabasal position, $K14^+/K19^+$), ER positive cells (luminal compartment), cells positive for K5 (Basal compartment including MEPs) and cells showing higher expression and activity of aldehyde dehydrogenases (Bocker, Moll et al. 2002; Clayton, Titley et al. 2004; Clarke, Spence et al. 2005; Ginestier, Hur et al. 2007; Villadsen, Fridriksdottir et al. 2007). It is likely that these different proposed stem cell groups overlap each other in their marker profile and function and that these methods are identifying stem and progenitor cells at different developmental stages within the hierarchical tree (Stingl 2009). Interestingly, during human embryogenesis, all epithelial cells of the breast express the LEP marker K19 (Anbazhagan, Osin et al. 1998). The candidate stem cell population in the adult human breast gland, that is most relevant to the findings of the Nature papers described above, are the suprabasal cells (Gudjonsson, Villadsen et al. 2002; Villadsen, Fridriksdottir et al. 2007). These cells are a part of the luminal compartment but localize in a suprabasal position close to the myoepithelial cells. The suprabasal cells contact neither the lumen or the basement membrane and express markers of both LEP cells (EpCAM, K19) and some markers of MEP cells (K14) (Gudjonsson and Magnusson 2005). These cells do not express markers of fully differentiated LEP cells, like Muc1. Gudjonsson et al. utilized that knowledge to isolate $EpCAM^+$ / $Muc1^-$ suprabasal cells and $EpCAM^+$ / $Muc1^+$ fully differentiated luminal epithelial cells (Gudjonsson, Villadsen et al. 2002). Subsequently, the two populations were immortalized with retroviral transduction using the E6 and E7 oncogenes from human papilloma virus 16. The suprabasal cell line, D492, shares common markers of LEP (K8, K19, EpCAM and E-cadherin) and MEP cells (K5/6, K14, Vimentin and Thy-1) while the Muc1+ cell line (D382) has a restricted LEP phenotype (Gudjonsson, Villadsen et al. 2002). Furthermore, the D492 cells could maintain

their original phenotype and differentiate into LEP-like and MEP-like cells but D382 cells could only give rise to more LEP-like cells (Gudjonsson, Villadsen et al. 2004). What may be most interesting about the D492 cell line is the morphology in three dimensional (3D) cell culture within a reconstituted basement membrane (rBM). While the D382 cell line recapitulates acinar-like formation in this assay the D492 cell line forms elaborate colonies with ducts and lobular-like structure on the branching tips, similar to the *in vivo* TDLU (Figure 2) (Gudjonsson, Villadsen et al. 2002).

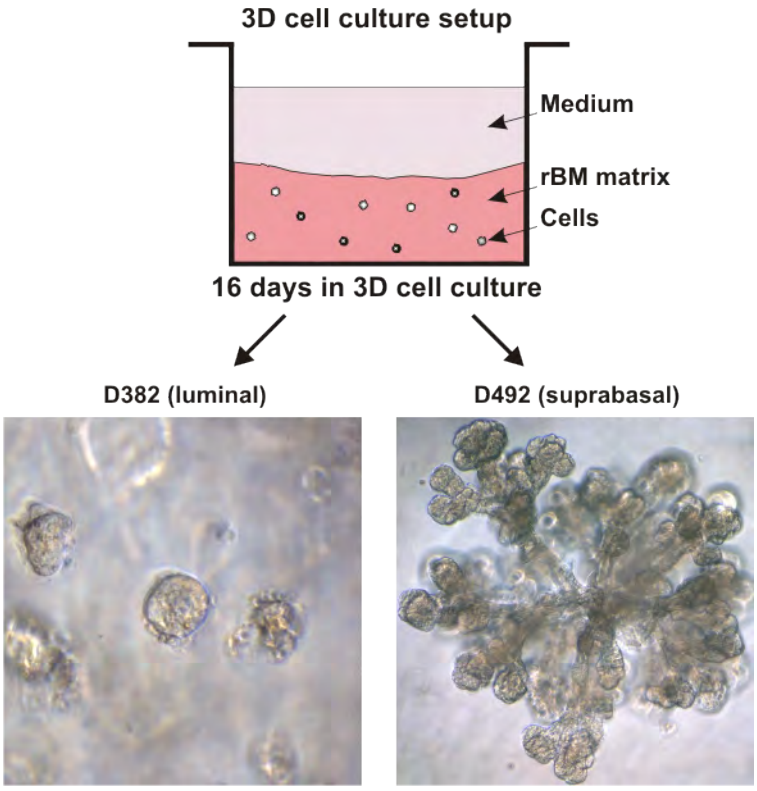


**Figure 2. Phenotypic properties of breast epithelial cell lines in 3D cell culture.**
*Top.* Schematic figure of 3D cell culture within rBM matrix (Matrigel). *Lower left.* The D382 cell line has a restricted LEP phenotype and can form polarized, acinus-like, colonies with a central lumen in 3D culture. *Lower right.* The suprabasal breast epithelial cell line, D492, has the abilities to form elaborate, TDLU-like, branching structures in 3D culture.

Recently, Villadsen et al. isolated $K14^{+}/K19^{+}$ suprabasal cells from primary breast epithelial cells with a similar flow cytometry profile used to identify the mouse mammary stem cells described above (Villadsen, Fridriksdottir et al. 2007). In addition, they could show that these cells reside within the luminal compartment in terminal ducts. Collectively, all evidence suggests that the breast gland is maintained by tissue specific stem cells. This

might not be surprising considering that during the luteal phase of the menstrual cycle the growth fraction can reach to over 30% and in pregnancy there is at least a ten fold increase in the number of lobules and a complementary expansion of the stroma (Shetty, Loddo et al. 2005). In fact, the majority of the tissue in the resting breast gland belongs to the stromal compartment that is instrumental in breast development and proper tissue homeostasis.

**1.4 Stromal cells in the normal breast gland**

Normal human breast epithelium is surrounded by vascularized intralobular loose stroma. The loose stroma is separated from fat tissue by dense dense collagen rich stromal tissue. (reviewed in (Sternlicht, Kouros-Mehr et al. 2006)). The stroma is composed of cellular components such as fibroblasts, fat cells, immune cells and endothelial cells. The stroma is also rich in bioactive extracellular matrix components such as basement membrane proteins (laminin and collagen IV), and interstitial collagen (Noel and Foidart 1998; Nelson and Bissell 2006). Crosstalk between the epithelium and stroma are crucial for proper development and homeostasis of the normal breast gland (Weaver, Fischer et al. 1996; Nelson and Bissell 2006). The epithelial compartment proliferates and differentiates during development but the stromal compartment contributes instructive signals to this process (Wiseman and Werb 2002). Breast stromal cells (e.g., fibroblasts, myofibroblasts, leukocytes and endothelial cells) are known to produce variety of cytokines and growth factors, which can influence proliferation, migration, morphology, differentiation and biosynthesis (Weaver, Petersen et al. 1997; Hu and Polyak 2008). Many growth factors have been implicated in epithelial–stromal interactions in the mammary gland such as transforming growth factor beta (TGF-β1), Epidermal growth factor (EGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF) and insulin like growth factor I (IGF-I) (Coleman, Silberstein et al. 1988; Robinson, Silberstein et al. 1991; Ulich, Yi et al. 1994; Niranjan, Buluwela et al. 1995; Walden, Ruan et al. 1998). These factors among others are produced by the stroma and mediate short-range signals influencing mammary epithelial growth (reviewed in (Sternlicht, Kouros-Mehr et al. 2006)). Unraveling the hierarchical complexity of differentiated and stem-like epithelial cells and their heterotypic interaction with stromal cells is fundamental for understanding morphogenesis of the human breast gland. Branching morphogenesis is a

highly complex process that creates the tree-like structure of the breast gland that is vital for its correct function. Aberrant regulation of branching morphogenesis including loss of cellular adhesion and polarization is one of the hallmarks of breast cancer.

## 2. Branching morphogenesis

Branching morphogenesis can be viewed as a strategy to pack a large cell surface area into a limited tissue volume and is a recurring theme in organ development (Sternlicht, Kouros-Mehr et al. 2006). Key mechanisms seem to be conserved between most branched organs, from trachea of the fruit fly to lungs, kidneys and breast glands in humans (Lu, Sternlicht et al. 2006; Lu and Werb 2008). However, during formation and maintenance of branched organs, tissue specific factors initiate and facilitate the branching process, like in the breast gland where hormonal and stromal derived signals influence the outcome (Sternlicht, Kouros-Mehr et al. 2006).

### 2.1 Branching morphogenesis in the human breast gland

The human breast gland is a unique organ that largely develops through branching morphogenesis after birth. From the onset of puberty, the female breast gland goes through robust branching morphogenesis creating ductal structures with lobular units on the ends with a complementary stromal expansion (Hovey, Trott et al. 2002; Russo and Russo 2004). This process is induced by hormonal signals at the onset of puberty, during each menstruation cycle and during pregnancy (Fendrick, Raafat et al. 1998; Sternlicht, Kouros-Mehr et al. 2006). Ovariectomy and hypophysectomy in mice show that ovarian and pituitary hormones are important for early development of the mammary gland (Daniel, Silberstein et al. 1987; Feldman, Ruan et al. 1999; Kleinberg, Feldman et al. 2000). However, embryonic branching occurs without any need for hormonal signals in contrast to adolescent and adult branching (Cowin and Wysolmerski 2010). Growth hormone (GH), Estrogen and estrogen receptor (ER) are needed for adolescent branching while additional signals from progesterone and progesterone receptor (PR) are crucial in adult tertiary side

branching (Sternlicht, Kouros-Mehr et al. 2006). Mouse experiments have shown that GH acts on its receptor located on stromal cells in the mammary gland. In turn, the stromal cells produce insulin-like growth factor-1 (IGF-1) that interacts with its receptor on mammary epithelial cells and induces branching. Induction of IGF-1 by GH was furthermore enhanced by estradiol (reviewed in (Kleinberg, Feldman et al. 2000)). Although hormonal signals are essential for branching morphogenesis, it is believed that they mediate their branching effects by inducing powerful signals through receptor tyrosine kinases (Sebastian, Richards et al. 1998; Rosario and Birchmeier 2003).

**2.2 Receptor tyrosine kinases in branching morphogenesis**

Branching epithelial morphogenesis in various tissues depends on heterotypic signaling between epithelium and stroma (Ronnov-Jessen, Petersen et al. 1996; Hsu and Yamada 2010; Little, Georgas et al. 2010; Warburton, El-Hashash et al. 2010). Receptor tyrosine kinases (RTKs) are key mediators of signal transmission in response to extracellular cues that regulate proliferation, differentiation and branching morphogenesis in the breast gland (Dillon, Spencer-Dene et al. 2004; Zahnow 2006). Extracellular factors such as FGFs, HGF and EGF act via their respective receptors (FGFRs, c-MET and EGFRs) to activate, among other pathways, the mitogen-activated protein kinase (MAP-kinase) pathway and the phosphatidylinositol 3-kinase (PI-3 kinase) pathway. MAP-kinase and PI-3 kinase pathways are critical for the development of many branched organs and generally act to promote branching (Davies 2002). Interestingly, in ovariectomized and ERα-deficient mice, ligands of EGFR receptors can rescue mammary gland development, emphasizing their importance in mammary branching (Coleman, Silberstein et al. 1988; Kenney, Bowman et al. 2003). Growth and morphogenic signaling must be precisely regulated both spatially and temporally to ensure normal homeostasis. Indeed, disruption in the regulation of RTK signaling is a major factor in cancers, including breast cancers (Gastaldi, Comoglio et al. 2010; Gutierrez and Schiff 2011). In particular, members of the EGFR family (ErbB2) and c-MET/HGF have been suggested as major players in the progression of breast cancers (reviewed in (Gastaldi, Comoglio et al. 2010; Gutierrez and Schiff 2011). Kuperwasser et al. demonstrated the ability of human derived, normal and aberrant fibroblasts to support both

branching morphogenesis and cancer formation from normal appearing cells through TGF-β1 and HGF (Kuperwasser, Chavarria et al. 2004). This highlights the determinative role for stroma in normal and malignant breast morphogenesis. Activation of different RTKs is important in breast morphogenesis but it is also likely that inhibitors of RTK signaling can modulate the signal transduction in numerous ways. In fact, the recently discovered sprouty proteins, which are endogenous negative feedback regulators of RTK signaling, have been shown to have negative effects on proliferation and migration induced by HGF (Lee, Putnam et al. 2004).

**2.3 Sprouty proteins in branching morphogenesis**

Recently, studies have highlighted the importance of negative feedback control of RTK signaling in ensuring correct cell fate (Dikic and Giordano 2003; Amit, Citri et al. 2007). Studies have started elucidating a novel mechanism of tyrosine kinase signal regulation through a small family of sprouty (Spry) proteins (reviewed in (Kim and Bar-Sagi 2004)). Spry was initially discovered in genetic screens for mutations in drosophila that impact tracheal and eye development (Hacohen, Kramer et al. 1998; Casci, Vinos et al. 1999). In the case of tracheal branching, loss of Spry resulted in extra secondary branches. Hacohen et al. showed that this was due to inhibiting effects of Spry on Btl signaling (homolog to mammalian FGFR) in tracheal epithelium resulting in unrestrained Bnl-Btl (FGF-FGFR) signaling when it was lost (Hacohen, Kramer et al. 1998). Spry is now known to act as a conserved inhibitor / modulator of RTK signaling in higher eukaryotes (Gross, Bassit et al. 2001; Impagnatiello, Weitzer et al. 2001; Lee, Schloss et al. 2001; Sasaki, Taketomi et al. 2001; Hanafusa, Torii et al. 2002; Tefft, Lee et al. 2002; Yusoff, Lao et al. 2002). Spry proteins have been proposed to act in a negative feedback loop and their expression and activity are increased in response to elevated signaling through RTKs such as FGFR2 and EGFR1 (Figure 3) (Mason, Morrison et al. 2006). There are four known mammalian Spry proteins (Spry-1-4). The detailed molecular mechanism of the different sproutys has not been fully elucidated. However, Hanafusa et al showed that after stimulation by growth factors, Spry-1 and Spry-2 translocate to the plasma membrane and become phosphorylated on a highly conserved tyrosine residue (Hanafusa, Torii et al. 2002).

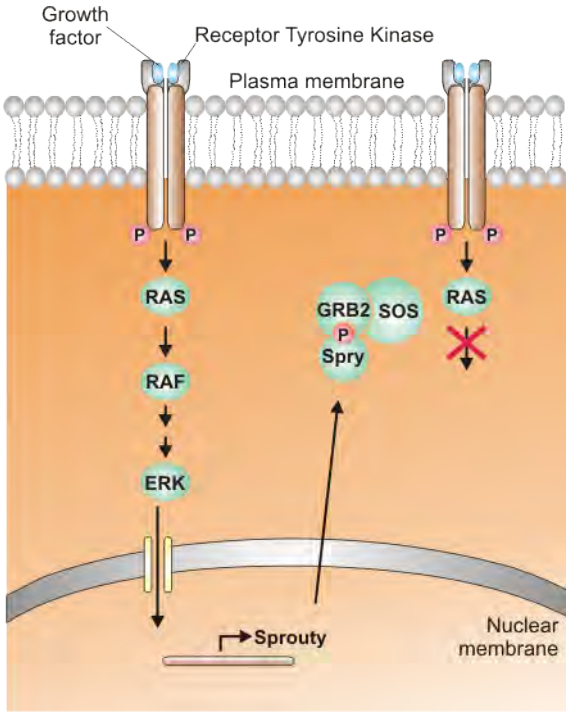


**Figure 3. Negative feedback control of Sprouty proteins on receptor tyrosine kinase signaling.**
Binding of ligands to RTKs leads to activation of RAS-RAF-ERK signaling and downstream targets including upregulation of sprouty expression. Sprouty proteins can subsequently interact with adaptor proteins such as Grb2, a Ras GTPase-activating protein, and negatively affect Ras activation and thus RTK signaling.

These translocation and phosphorylation steps of Spry were shown to block recruitment and activation of key docking and adaptor proteins (Grb2–Sos complex, FRS2 or Shp2), necessary for activation of the ERK-MAPK signaling pathway (Mason, Morrison et al. 2006). Studies on the inhibiting effects of Spry and its activity in mammalian systems have mostly focused on FGFR, EGFR, c-MET and VEGFR regulation in cell culture (reviewed in (Cabrita and Christofori 2008)). Knockout (KO) of Spry-1 and -2 in mice results in branching defects in many different organs (reviewed in (Edwin, Anderson et al. 2009)). Tefft et al. and Mailleux et al. have shown that mouse Spry-2 is expressed in branching epithelial lung buds and seems to negatively regulate lung branching (Tefft, Lee et al. 1999; Mailleux, Tefft et al. 2001). Similarly, the loss of mouse Spry-1 results in abnormal kidney development in the formation of polycystic kidneys (Basson, Akbulut et al. 2005). Spry-2 KO in mice results in enteric hyperplasia and esophageal abnormalities in addition to defects in the development of organ of the corti resulting in hearing loss (Shim, Minowada et al. 2005; Taketomi,

Yoshiga et al. 2005). In contrast, Spry-4 KO results in dwarfism, limb deformation, tooth abnormalities and mandible defects (Hansen, Floss et al. 2003; Klein, Minowada et al. 2006; Taniguchi, Ayada et al. 2007). Double KO of Spry-2/4 suggested that these Spry proteins may have overlapping functions, as the mice were embryonic lethal, showing defects in craniofacial, limb and lung morphogenesis (Taniguchi, Ayada et al. 2007). Spry-3 is a relatively uncharacterized member of the Spry family with low expression in most tissues (Cabrita and Christofori 2008). Recently, Panagiotaki et al. found that Spry-3 is involved in the regulation of axonal branching of motoneurons (Panagiotaki, Dajas-Bailador et al. 2010). Although sprouty proteins have been identified as antagonists of FGFR, c-Met and EGFR signaling in various organs, their role in the human breast gland has not been systematically analyzed. Lo et al. showed that the expression levels of Spry-2 seem to fluctuate in conjunction with critical periods in branching morphogenesis in the mouse mammary gland, showing increased expression during pregnancy (Lo, Yusoff et al. 2004). In addition, they also suggested that Spry-2 could function as a tumor suppressor because it seemed to be downregulated in breast cancers (Lo, Yusoff et al. 2004). In fact, increased and/or abnormal signals through RTKs and loss of their negative regulators is one of the hallmarks of cancer.

## 3. Breast cancer

Stem cells maintain tissue homeostasis throughout the life span of an organism. In the case of the breast gland, stem and progenitor cells proliferate and participate in branching morphogenesis in a controlled manner until the onset of menopause. In malignancy, cancer-initiating cells emerge that have acquired abnormalities in genes necessary for normal homeostasis. Cancerous cells start to proliferate resulting in large populations of cells that have lost their original, tissue specific, role (Hanahan and Weinberg 2011).

### 3.1 Malignant transformation of the breast

Transition towards malignancy is induced by oncogenes (Hanahan and Weinberg 2011). In short, this builds on the observations that carcinogens can alter the function of normal growth controlling genes (proto-oncogenes). This results in oncogenes that can transform

normal cells into cancer cells. Oncogenes can arise from point mutations, chromosomal translocations or DNA amplifications (Lee and Muller 2010). The discovery of oncogenes provided a simple explanation how cancer proliferation is boosted. It however, only explained half the story. Subsequently, another group of growth controlling genes was discovered that suppresses cellular proliferation, the tumor suppressor genes (Reviewed in (Lee and Muller 2010)). The loss of these "gatekeeper" genes, such as p53 and RB, makes cells vulnerable to further genetic insult and thus play a big role in the initiation of cancers. The function of tumor suppressor genes is lost through mutations, methylation or a loss of a chromosomal region harboring the gene (Reviewed in (Shuen and Foulkes 2011)). Malignant transformation of cells results in different types of cancers depending on the cell of origin and the type of genetic abnormalities that take place (Nakshatri, Srour et al. 2009). In the human breast gland most cancers originate in luminal epithelial cells and/or cells harboring stem/progenitor properties within the luminal compartment (Petersen and Polyak 2010; de Ruijter, Veeck et al. 2011). In contrast, differentiated MEP cells are progressively lost through breast cancer progression and some evidence suggests that they may in fact suppress tumor formation (Gudjonsson, Adriance et al. 2005). Although originating almost exclusively within the luminal epithelial compartment breast cancer is a complex disease with number of different subtypes with varying clinical outcomes (Polyak 2007). Pioneering studies by Perou et al. and Sorlie et al. using gene expression profiling showed that breast tumors could be classified into five different molecular subtypes: basal-like, luminal A, luminal B, ErbB2$^{+}$ and normal-like (Perou, Sorlie et al. 2000) (Sorlie, Perou et al. 2001). These observations were confirmed by multiple independent research groups with large sets of breast tumors extending across different ethnic groups (Yu, Lee et al. 2004; Hu, Fan et al. 2006; Sorlie, Wang et al. 2006). These subtypes have been suggested to originate from different breast epithelial cells within the hierarchical developmental tree (Figure 4) (Petersen and Polyak 2010). Measurement of the levels of ER, PR and ErbB2 is of great importance when deciding therapy for the different breast cancer subtypes (De Laurentiis, Cianniello et al. 2010). Clinically, the best prognosis is for the luminal A subtype and the worst for basal-like breast cancers (Polyak 2007). Luminal A and B are the hormone receptor positive subtypes so they can be treated with tamoxifen, aromatase inhibitors or ovarian ablation which improves prognosis substantially (Zwart, Theodorou et al. 2011).

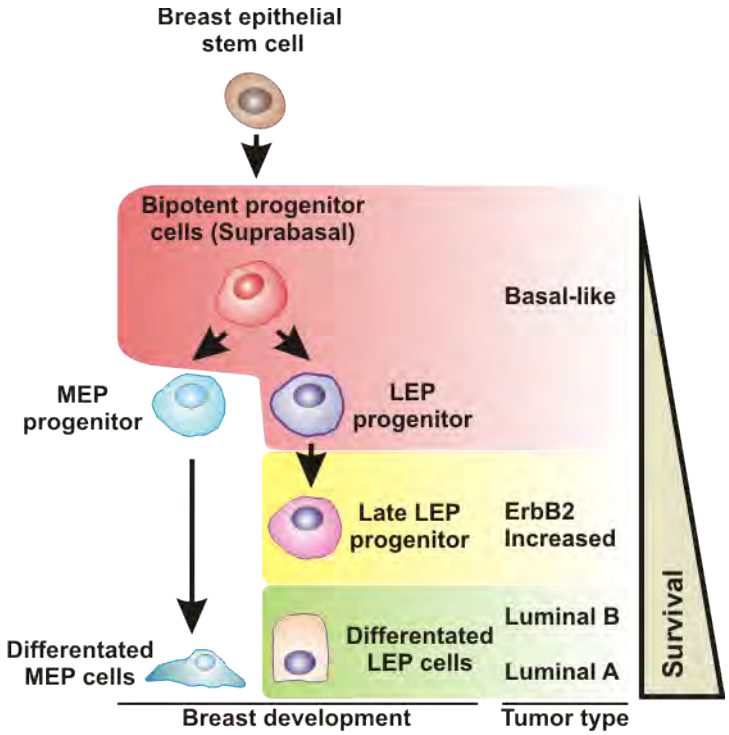


**Figure 4. Hierarchy of breast epithelial cells in development and relation to different breast cancer subtypes.**
The molecular classification of breast cancers by gene expression into Basal-like, ErbB2, Luminal A and Luminal B subtypes has been suggested to have relation to different breast epithelial populations. Breast cancers originating in myoepithelial cells are rare. The basal-like cancers have been proposed to originate in bipotent (suprabasal) progenitor cells or luminal progenitor cells while the ErbB2 subtype has been suggested to arise from late luminal progenitor cells. In contrast, the Luminal A and B subtypes are likely originating from more differentiated luminal epithelial cells. Different breast cancer subtypes also correlate to clinical outcome resulting in the worst survival within the basal-like subtype.

Furthermore, the use of trastuzumab (ErbB2 inhibiting antibody) has provided patients with the ErbB2 overexpressing subtype with significantly improved survival (Damasceno 2011). Actually, if left untreated, the different subtypes have a similar prognosis but the basal-like tumors lack a therapeutic target so ultimately they end up with the worst prognosis (De Laurentiis, Cianniello et al. 2010). Basal-like breast cancer (BLBC) are generally not $ER^+$, $PR^+$ or have an amplification of ErbB2, so they are referred to as triple negative breast cancers (Badve, Dabbs et al. 2011). This triple negativity has proven to be a fairly poor classification for the BLBCs because not all triple negative cancers are of this subtype so additional markers have been included. They are now characterized by having similar markers as suprabasal progenitors/stem cells in the normal breast gland, expressing K5/6, K14, K17, K19, Vimentin and EGFR1 (Nielsen, Hsu et al. 2004; Rakha, Elsheikh et al. 2009). BLBC represent 15-20% of all breast cancers and tend to occur in younger women with a

significantly higher incidence among African Americans (Stead, Lash et al. 2009). BLBCs often show the presence of spindle-like cells and squamous metaplasia (de Ruijter, Veeck et al. 2011). They have a high proliferation index, high grade and frequently show pushing borders with surrounding lymphocyte-rich stroma and increased microvascular density (de Ruijter, Veeck et al. 2011). In fact, stromal derived cells are believed to be important components of tumor growth and progression. In vivo and in vitro experiments have shown that the stromal cells within the breast along with molecules in the extracellular matrix can influence growth, survival and invasive properties of breast cancer cells (Howlett and Bissell 1993; Hanahan and Weinberg 2011).

### 3.2 Stromal cells in breast cancer progression

At the cellular level, focus of the last two decades has been on interactions of tumor cells and fibroblasts and their impact on tumor progression (Elenbaas and Weinberg 2001; Nielsen, Ronnov-Jessen et al. 2002). Studies have shown that co injection of tumor cells with irradiated fibroblasts or conditioned media from fibroblasts has growth promoting effects in mouse xenograft models (Camps, Chang et al. 1990; Noel, De Pauw-Gillet et al. 1993). Additionally, the TGF-beta type II receptor gene has been knocked out in fibroblasts and subsequently cotransplanted with mammary cancer cells in nude mice resulting in increased tumor growth, invasion and metastasis (Cheng, Bhowmick et al. 2005). Cheng et al. showed that this was the result of increased fibroblast derived TGF-$\alpha$, MSP and HGF (Cheng, Chytil et al. 2007). Additionally Kuperwasser et al. were able to construct a functionally normal breast gland in mouse mammary fat pads when they prepared the mouse tissue by injecting human stromal fibroblasts prior to the epithelial engraftment. However, when these fibroblasts were made to express higher levels of TGF-$\beta$1 or HGF these apparently normal derived epithelial cells formed in some cases tumors further outlining the potential effects of fibroblasts on cancer formation/progression (Kuperwasser, Chavarria et al. 2004). Inflammation and cancer is tightly linked together and as early as 1863 Rudolf Virchow reported that leukocytes were present in tumor tissues (rewieved in (Balkwill and Mantovani 2001)). Chronic inflammation creates a suitable microenvironment that promotes cancer progression with reciprocal interactions of tumor cells and inflammatory

cells (Lorusso and Ruegg 2008). Recruited macrophages release numerous growth factors that affect tumor growth including VEGF, bFGF, TNF, HGF, EGF and PDGF in addition to a wide variety of cytokines (Robinson and Coussens 2005; Benelli, Lorusso et al. 2006). Monocytes/macrophages are often present early on in cancer formation and in mice lacking CSF-1, monocyte recruitment is haltered and leads to reduced cancer progression and metastasis (Lin, Nguyen et al. 2001; Pollard 2004). In addition, mast cells and neutrophils have also been shown to increase tumor progression by the secretion of various growth factors, cytokines and proteases (Coussens, Tinkle et al. 2000; Ji, Houghton et al. 2006). It has become increasingly clear that inflammatory cells also play a role in tumor angiogenesis in conjunction with the tumor cells (Ruegg 2006; Shojaei, Zhong et al. 2008). Formation of new blood vessels is critical in cancer progression and the tumor mass is under tight control by the vasculature (Folkman 1998). Epithelial to mesenchymal transition (EMT) is believed to be an important part of the metastasis process and number of stromal cells, including fibroblasts, can induce the EMT process.

### 3.4 Epithelial to mesenchymal transition and cancer stem cells

Epithelial to mesenchymal transition is a dynamic process seen in normal development, wound healing, fibrosis and cancer progression. In development, the EMT process is crucial for the formation of the mesoderm and in neural crest delamination (Thiery, Acloque et al. 2009). In cancer, EMT is associated with increased aggressiveness, metastasis and adverse prognosis (Hugo, Ackland et al. 2007; De Wever, Pauwels et al. 2008). In cell culture, the EMT process leads to a mesenchymal phenotype including spindle shape morphology, increased expression of mesenchymal markers such as N-Cadherin, Vimentin, $\alpha$-smooth muscle actin and Fribronectin and loss or lowered expression of epithelial markers, including E-cadherin and most keratins (reviewed in (Moustakas and Heldin 2007; Peinado, Olmeda et al. 2007)). Expression of mesenchymal markers in EMT cells is associated with increased migratory and anti-apoptotic phenotype (Figure 5) (Thiery, Acloque et al. 2009).

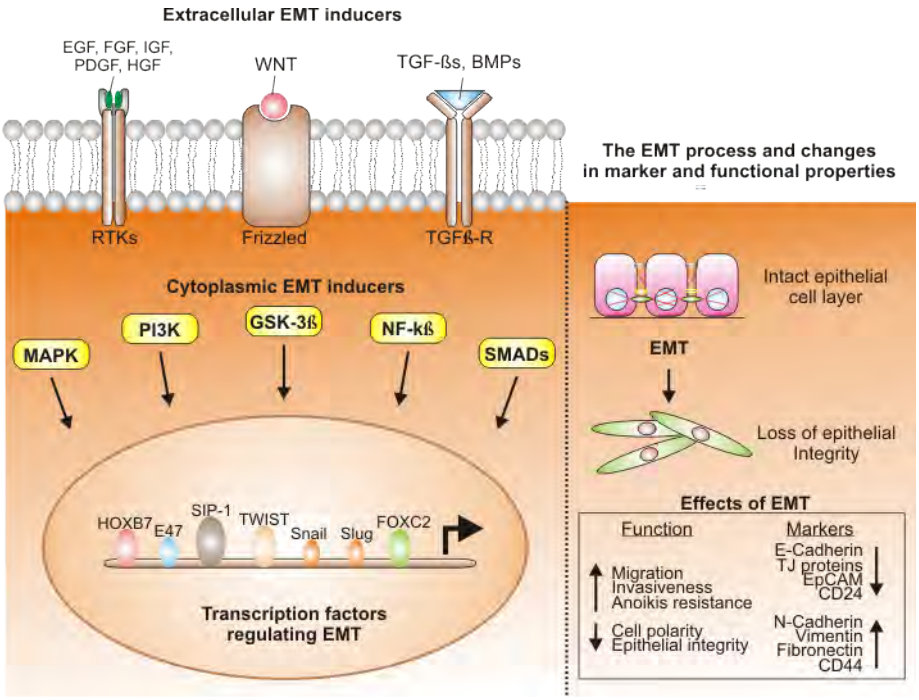


**Figure 5. Epithelial to mesencymal transition can be induced by multiple signals that result in changes in marker profile and functional properties.**
*Left.* The EMT process can be induced by many ligands of receptor tyrosine kinases (RTK), Wnt as well as TGFβs and BMPs. Extracellular signals are mediated through cytoplasmic inducers such as MAPK, PI3K, GSK-3β, NF-kβ and SMADs. The EMT is ultimately mediated through transcription factors like HOXB7, E47, SIP-1, TWIST, Snail, Slug and FOXC2 among others. *Right.* In general, EMT induces the transition from intact epithelial phenotype towards a mesenchymal phenotype. Downregulation of many epithelial markers such as E-cadherin, tight junction proteins (TJ), EpCAM and CD24 are seen and upregulation of mesenchymal markers such as N-cadherin, Vimentin and fibronection as well as increased expression of CD44. Functionally this results in migratory abilities, invasiveness and anoikis resistance and in the loss of epithelial integrity.

The molecular mechanisms of EMT has bee extensively explored in cell culture. Studies have highlighted signaling pathways induced by transforming growth factor-β (TGF-β) and receptor tyrosine kinase ligands such as EGF, PDGF, FGF and HGF in the EMT process (reviewed in (Moustakas and Heldin 2007; Yang and Weinberg 2008)). TGF-β and RTK signaling events eventually mediate their EMT effects through transcriptional regulatory factors such as Snail, Slug, Twist, ZEB1, ZEB2, FOXC1 and FOXC2 leading to increased and decreased expression of mesenchymal and epithelial markers, respectively (Yang and Weinberg 2008). In addition, miRNA changes have been linked to EMT, especially the downregulation of the miRNA-200 family (miRNA-200a, -200b, -200c, -141) but their presence is important for maintaining proper epithelial integrity (reviewed in (Brabletz and Brabletz 2010)). Recent studies have shown that members of the miRNA-200 family are crucial regulators of differentiation and epithelial characters in many tissues (Gregory,

Bracken et al. 2008). Interestingly, EMT is tightly linked to both the basal-like breast cancer subgroup and cancer stem cells (Mani, Guo et al. 2008; Morel, Lievre et al. 2008; Sarrio, Rodriguez-Pinilla et al. 2008). Analysis of tissue microarrays from different breast cancer subgroups show upregulation of EMT-associated markers and an overall reduction of epithelial markers, preferentially in poorly differentiated tumors within the basal-like group (Sarrio, Rodriguez-Pinilla et al. 2008). Cancer stem cells have been isolated from a diverse set of malignancies such as leukemia, brain tumor, colon cancer, and breast cancer (reviewed in (Gudjonsson and Magnusson 2005; Klonisch, Wiechec et al. 2008)). Al Haji et al. showed that breast cancer stem-like cells existed within breast tumors and they could be isolated (Al-Hajj, Wicha et al. 2003). These cells were originally characterized by the flow cytometry profile $CD44^{High}$ / $CD24^{Low}$ and their ability to initiate cancers from few cells, reflecting the original phenotype of tumors when inoculated in NOD/SCID mice (Al-Hajj, Wicha et al. 2003). Moreover, the connection between cancer stem cells and EMT is interesting. Breast epithelial cells gain stem-like properties such as the ability to grow as spheres in non-adherent cell culture (mammospheres) after EMT induction (Mani, Guo et al. 2008). Additionally, increased proportion of $CD44^{high}$/$CD24^{low}$ cells emerged that were able to initiate tumors in mice from limited number of cells (Mani, Guo et al. 2008; Morel, Lievre et al. 2008). Interestingly, EMT associated downregulation of miRNA-203 and -205 has been directly connected to this increased „stemness“ (Wellner, Schubert et al. 2009). Thus, EMT and the induction/origin of cancer stem cells seem to be tightly linked together. These observations raise the questions if basal-like breast cancers are more susceptible to EMT and/or if their cancer stem cell associated phenotype is the consequence of EMT. Defining the cellular and microenvironmental cues that trigger EMT during the progression of basal-like breast cancers is of high importance. In fact, studies have pointed out increased expression of EMT markers such as beta-catenin and snail at the tumor-stroma interface (Brabletz, Jung et al. 2001; Franci, Takkunen et al. 2006). Cancer cells are in close contact to fibroblasts and other stromal cells at the invading front which has been connected to EMT and the metastasis process (Brabletz, Jung et al. 2001; Franci, Takkunen et al. 2006). Fibroblasts, for example, can induce EMT in a hepatocellular carcinoma mouse model through PDGF and TGF-$\beta$ signaling (van Zijl, Mair et al. 2009). In prostate cancer, Giannoni et al. showed that there was a paracrine interaction between fibroblasts and prostate cancer

cells towards the induction of EMT (Giannoni, Bianchini et al. 2010). Fibroblasts isolated from breast carcinomas have also been implicated in EMT induction in coculture with breast cancer cells (Gao, Kim et al. 2010). Immune T cells have recently been implicated in mesenchymal transition where CD8 positive T cells were responsible for EMT through unidentified factors in a mouse model of mammary cancer (Santisteban, Reiman et al. 2009). Tumor associated macrophages have additionally been described as potent inducers of metastasis (thus likely EMT) through their interaction with breast cancer cells (Qian, Li et al. 2011). Recently, breast cancer stem cells have additionally been shown to be growth regulated and induced to undergo EMT by mesenchymal stem cells through IL-6 and CXCL7 (Liu, Ginestier et al. 2011). Endothelial cells have attracted attention as important regulators of organogenesis and stem cell maintenance but are not known inducers of EMT (Lammert, Cleaver et al. 2001; Matsumoto, Yoshitomi et al. 2001; Shen, Goderie et al. 2004).

## 4. Functional role of endothelial cells in tissue morphogenesis

Angiogenesis, the formation of new capillary blood vessels, is critical in embryonic development, wound healing and reproduction and has a dominating role in many pathological conditions, such as cancer (reviewed in (Folkman 2003)). Normal angiogenesis is highly regulated and all cells are dependent on being in the proximity of blood vessels for the continuous delivery of oxygen and nutrients (reviewed in (Carmeliet 2005)). The breast gland undergoes vast morphological changes from puberty to menopause thus the blood supply must be remodeled and adjusted (Djonov, Andres et al. 2001). One of the most powerful factors inducing endothelial cells to undergo angiogenesis is the vascular endothelial growth factor (VEGF) and its expression in tissues is correlated with neoangiogenesis (Carmeliet 2005). Using rats, Pepper et al. showed that VEGF was increased during pregnancy (5 fold) and lactation (15 fold) but decreased dramatically during involution (50%) (Pepper, Baetens et al. 2000). In fact, VEGF levels also increase in the normal breast gland during the luteal phase compared to the follicular phase of the menstrual cycle, indicating the creation of a pro-angiogenic environment (Dabrosin 2003). In contrast to normal angiogenesis, cancer associated vascularization leads to disorganized and

poorly functioning networks (Carmeliet and Jain 2011). Tumor vasculature promotes growth by delivering oxygen and nutrition to cancer cells so some have suggested that anti-angiogenesis therapy would be a feasible way to tackle cancer. Therapeutic agents such as Bevacizumab (VEGF neutralizing antibody) have been developed and shown effects in the treatment of metastatic colorectal cancer when administrated with chemotherapy (Carmeliet and Jain 2011). In contrast, little focus has been on paracrine effects of endothelial cells on cancer cells. Studies have suggested that endothelial cells are important in development before they participate in functional blood transport (reviewed in (Butler, Kobayashi et al. 2010)). In fact, mouse studies of embryogenesis suggest that vessel development precedes organ specification and that endothelial cells have a direct role on organ development (Nikolova, Strilic et al. 2007). There seems to be a direct interaction between endothelial cells and tissue specific cells in supporting organogenesis in a number of organs such as the adrenal gland, kidney, liver and pancreas (Stoos, Carretero et al. 1992; Linas and Repine 1999; Rosolowsky, Hanke et al. 1999; Tufro, Norwood et al. 1999; Lammert, Cleaver et al. 2001; Matsumoto, Yoshitomi et al. 2001). Studies focusing on these paracrine signals may implicate additional signaling pathways that are also important for cancerous tissues (Reviewed in (Butler, Kobayashi et al. 2010)). In fact, endothelial derived factors have been suggested to promote tumor progression in prostate cancer and Shekhar et al. showed that endothelial cells can mediate hyperplasia of preneoplastic breast epithelial cells in vitro (Pirtskhalaishvili and Nelson 2000) (Shekhar, Werdell et al. 2000). Another study focusing on head and neck carcinoma cell lines showed that endothelial cells produced IL-6, CXCL8 and EGF affecting migration and anoikis (cell death caused by loss of adherence) (Neiva, Zhang et al. 2009). In addition, studies have shown that endothelial cells play a part in self-renewal and neurogenesis in the subventricular stem cell niche of the brain (Shen, Goderie et al. 2004). Subsequently, other research groups were able to identify cancer stem cells residing in a similar perivascular niche in the brain that provides factors for their self-renewal (Calabrese, Poppleton et al. 2007; Veeravagu, Bababeygy et al. 2008). A similar endothelial dependent stem cell niche has been identified in the bone marrow where hematopoietic stem and progenitor cells are in close proximity to endothelial cells, thereby sustaining their proliferation and expansion (Butler, Nolan et al. 2010; Kobayashi, Butler et al. 2010). Angiogenesis can thus support normal development and cancer growth

by providing oxygen and nutrition but may also do this by paracrine signaling. However, angiogenesis and endothelial cells can also favor metastasis by providing access to the circulation and perhaps have an influence on the nature of metastasizing cells, for example through EMT inducing signals.

## 5. Modeling normal and cancerous morphogenesis in culture

To be able to understand the origin of breast cancer and breast cancer progression one must first define the critical factors that create normal breast morphogenesis. Mouse models have been instrumental in understanding how normal development of the mammary gland occurs and for identifying candidate mammary stem cells (reviewed in (Sternlicht, Kouros-Mehr et al. 2006; Stingl 2009). However, there are fundamental differences between the mouse mammary gland and the human breast gland where the difference in the combination and distribution of the stromal compartment are most striking (Cardiff and Wellings 1999). Better understanding of the signaling pathways that occur in human breast morphogenesis will rely on well characterized human primary cells and immortalized breast cell lines, and an appropriate cell culture assay that can capture critical aspects in breast structure and function. This is due to the obvious inability in the use of in vivo studies when working with primary human breast cells and cell lines. In the case of human breast stem cell biology, multipotent candidate cells have been defined by their ability to differentiate into luminal- and myoepithelial cells in cell culture (Kao, Nomata et al. 1995; Stingl, Eaves et al. 1998; Pechoux, Gudjonsson et al. 1999; Gudjonsson, Villadsen et al. 2002). Another assay, widely used to define these cells, is the mammosphere assay. Cells with stem cell characteristics grow in anchorage independent manner, on low adherence plates, forming spheres (Dontu, Jackson et al. 2004). The use of traditional monolayer cell culture has proven to be highly important to tackle many general questions in molecular- and cell biology. However, cells in monolayer loose many functional properties as a consequence of a lost three-dimensional (3D) tissue architecture (Weaver, Fischer et al. 1996). Monolayer culture is limited in its ability to ask questions about complex molecular processes that are often regulated spatially and temporally in vivo. In addition, the 3D

environment is essential for the maintenance of correct cellular function and development of physiologically relevant structures (Nelson and Bissell 2006). While cells in monolayer can respond to the mechanical nature of that culture system they have little capacity to manipulate the microenvironment that they reside in. In fact, cell lines cultured in the 3D setting exhibit different behavior, inhibiting their immense proliferation rate seen in monolayer culture, and enhancing abilities to form higher order structures (Fata, Werb et al. 2004). In these assays, cells form colonies in contrast to monolayers and this simulates better the in vivo conditions. In 3D cell culture assays it is common to use extracellular matrixes like laminin or collagen to form gels that cells are embedded into. The most widely used 3D cell culture assay consists of a laminin rich reconstituted basement membrane matrix (rBM), commercially known as Matrigel (Kleinman and Martin 2005). 3D cell cultures within rBM have proven to be very good in revealing the morphogenic potential of differentiated and stem-like breast epithelial cells and thus highly important in human breast stem cell biology (Gudjonsson, Villadsen et al. 2002; Villadsen, Fridriksdottir et al. 2007). For instance, breast luminal epithelial cells forms polarized acini with a central lumen when cultured within rBM (Barcellos-Hoff, Aggeler et al. 1989; Petersen, Ronnov-Jessen et al. 1992). Myoepithelial cells, on the other hand, form large clusters of cells without a central lumen (Petersen, Ronnov-Jessen et al. 1992). Gudjonsson et al. have shown that co-culture of primary luminal- and myoepithelial cells in collagen results in polarized, double layer acini, similar to the situation *in vivo* (Gudjonsson, Ronnov-Jessen et al. 2002). Furthermore, Gudjonsson et al. have shown that breast epithelial cells with stem-like properties can form TDLU-like branching morphogenesis when cultured in 3D rBM (Gudjonsson, Villadsen et al. 2002). In contrast to normal breast epithelial cells, cancer cells culture within 3D rBM show a complete loss of a morphogenic response, forming disorganized solid colonies or even colonies with spindle shaped cells reflecting their malignant nature (Petersen, Ronnov-Jessen et al. 1992; Kenny, Lee et al. 2007). Drug resistance is also higher in cancer cells that grow as colonies within 3D cell culture (Polo, Arnoni et al. 2010). Hence, the applications of 3D cell culture assays have provided valuable insights into breast morphogenesis, differentiation and into the exploration of cancer behavior. Another option is to grow human cells within immunosuppressed mice to explore their morphogenic potential. The problem is, however, that normal breast epithelial cells do

not generally grow when transplanted in mice in contrast to highly malignant cells and cell lines. However, as mentioned above, researchers have begun to use an in vivo xenograft model in mice where they are able to transplant normal breast epithelial cells of human origin into cleared mammary fat pads. To achieve this, the incorporation of human breast stromal cells (fibroblasts) was the determining factor facilitating the growth of normal epithelial cells (Kuperwasser, Chavarria et al. 2004). This opens up new options in culturing human breast epithelial cells in a physiologically relevant microenvironment although this assay is not very accessible because of its complexity.

# AIM OF THE THESIS

Cellular and molecular regulation of branching morphogenesis is a key question in developmental cell biology. In the breast gland, resident stem cells are responsible for tissue remodeling including branching morphogenesis. The branching breast epithelium undergoes continuous cycling of proliferation, differentiation and apoptosis and aberrant regulation of this is one of the hallmarks of breast cancer. Example of that is epithelial to mesenchymal transition, an important developmental process in branching morphogenesis. Branching is regulated by both extrinsic (stromal cells and extracellular matrix) and intrinsic (signaling pathways, transcription factors and microRNAs) factors. The general aim of this thesis was to study both extrinsic and intrinsic regulators of branching morphogenesis and how this highly regulated process changes during epithelial to mesenchymal transition. The breast stroma is highly vascularized but the impact of endothelial cells as providers of morphogenic signals has been to a great extent ignored and thus poorly understood. In this thesis I have explored the regulatory role of endothelial cells in branching morphogenesis using purified primary breast endothelial cells, primary breast epithelial cells, breast epithelial stem cell line -D492- and three dimensional cocultures. Finally, I have explored the molecular regulation of branching morphogenesis with focus on receptor tyrosine kinase signaling and their regulatory proteins, sprouty. The following four sub aims represent each article/manuscript enclosed with this thesis.

1) Isolation and propagation of primary breast endothelial cells. Paper #1 published in In Vitro Cell Biology-Animal.

2) To explore the effects of breast endothelial cells on the growth of primary breast epithelial cells and cell lines. Paper #2 published in BMC Research Notes.

3) To evaluate if breast endothelial cells in coculture with breast epithelial cells with stem cell properties influence morphogenic potential in 3D culture. Paper #3 published in PLoS One.

4) To determine if Sprouty-2 is an important regulator of branching morphogenesis and epithelial integrity in the human breast gland. Paper #4 manuscript.

# MATERIALS AND METHODS

**Cell culture**

The breast epithelial stem cell line D492 and daughter cell line D492-M were maintained in H14 medium (Briand, Petersen et al. 1987), consisting of DMEM/F12, 50 IU/ml penicillin, 50 µg/ml streptomycin (Invitrogen), 250 ng/ml insulin, 10 µg/ml transferrin, 2.6 ng/ml sodium selenite, 0.1 nM estradiol, 0.5 µg/ml hydrocortisone, 5 µg/ml prolactin (SIGMA) and 10 ng/ml EGF (Peprotech). D492 NS cells and D492 Spry2 KD cells were also maintained on this medium in monolayer. Luminal epithelial cell line D382 and MCF10A were also maintained on H14 medium. MDA-MB 231 was cultured on RPMI-1640 supplemented with 5% FBS. W2320 and MCF-7 on DMEM/F12 with 5% FBS. A549 lung carcinoma cell line and the MDCK cell line were maintained on DMEM/F12 with 10% FBS. Primary human BRENCs were isolated from breast reduction mammoplasties as described in details in the results and cultured on endothelial growth medium (EGM) (Lonza) containing 50 IU/ml penicillin, 50 µg/ml streptomycin, hydrocortisone, FGF, EGF, VEGF, R3-IGF-1, Ascorbic acid, Heparin, GA-1000 and supplemented with 5% FBS (EGM5). The MCF-7, MCF10A, A549, MDCK and MDA-MB-231 cell lines where purchased from ATCC and are routinely authenticated with genotype profiling according to ATCC guidelines. To further ensure cell line integrity D492, D492M and D382 cell lines were analyzed with the same method.

**Preparation of 3D rBM mono- and cocultures**

Co-culture experiments, in article #3, were carried out in 24 well culture plates (BD Falcon) with 500 D492 cells and 0 (monoculture), $5x10^4$, $1x10^4$ and $2x10^5$ BRENCs. The two cell types were mixed and suspended in 300µl rBM (Matrigel, BD) and cultured on EGM5 for 15 days. Cocultures of BRENCs with normal breast epithelial lines MCF-10A and D382, estrogen receptor positive breast cancer cell line MCF-7, basal-like / EMT breast cancer cell line MDA-MB-231 and primary metaplastic breast cancer cell line W2320 were done with 500 epithelial cells and $2x10^5$ BRENCs on EGM5 medium in the same setup. Transwell co-culture was conducted in a 24 well setup with a 0,4µm polyester membrane seperating the chambers (Costar). $5x10^4$ endothelial cells were seeded in the upper chamber as a

monolayer and 250 D492 cells in 100µl matrigel on the bottom of the lower chamber and cultured on EGM5. In article #4, 3D rBM monoculture and coculture were carried out in 96 well culture plates (BD, Falcon). In the 3D monoculture experiments, cells were seeded at a density of $1{,}3 \cdot 10^4$, $1 \cdot 10^4$ and $7 \cdot 10^3$ for D492 non silencing and Sprouty-2 knock down cells in 300µl of rBM. These 300µl of rBM were then split up to 3 portions and 100µl was added to each well in a 96 well plate. In the coculture setup $1 \cdot 10^3$ D492 NS and KD cells were mixed with $5 \cdot 10^4$ breast endothelial cells in 300µl rBM and split up in 3 x 100µl gels in a 96 well plate.

**Isolation of 3D coculture colonies, replating and secondary 3D coculture**

Branching, solid and spindle-like structures were isolated from 3D cocultures with gentle shaking on ice in PBS - EDTA (5mM) solution for 30 min. Single structures were placed in a 24 well plate and cultured on H14 medium. Several clones were isolated for each morphology of branching, solid and spindle-like colonies. Monolayer cultured cells from branching, solid and spindle like colonies were placed in 3D coculture of 500 cells with $5x10^4$ BRENCs in rBM. One of the spindle-like clones (D492M) was further propagated in monolayer culture for use in phenotypic and functional comparison to the motherline, D492.

**Retroviral insertional analysis**

The D492 cell line was initially established by transfection with a retroviral vector containing the E6 and E7 oncogenes and the neomycin resistance gene for selection (Gudjonsson et al., 2002b). To identify the genomic insertion site of the E6/E7 containing retrovirus we performed an inverse PCR (I-PCR) (Suzuki et al., 2002). 5 µg of cell line DNA was digested with 60 U of BamHI overnight. After heat inactivation, DNA was diluted, circularized by ligation with T4 DNA ligase at 16°C overnight, ethanol precipitated, and resuspended in Tris-EDTA. PCR was performed in 25 µl with 1 µl of the DNA template, 0.2 mM deoxynucleoside triphosphates, 10 pmol of each primer, 1.3 U of Expand Long Template Polymerase, and Expand Buffer 1 (Roche). Amplified products were cloned into the TOPO TA cloning vector

(Invitrogen) and clones selected and sequenced. With this method we mapped an insert to chromosome 20, 95 kb upstream of the PTPN1 gene.

**Immunohistochemistry of normal and malignant human breast tissue**

Formalin-fixed, parafin embedded normal and tumor tissue blocks were cut into 5µm serial sections and mounted on slides. Sections were deparafinized and rehydrated in xylene and ethanol. Antigen retrieval was done by boiling in citrate buffer for 15 min. The following primary antibodies and dilutions were used; CD-31 ( 1:50, M0823, DakoCytomation), Keratin 19 (1:100, ab7754, Abcam), Keratin 14 (1:50, NCL-LL002, NovoCastra), E-cadherin (1:100, #13-1700, Zymed), N-Cadherin (1:50, #610920, BD), Keratin 18 (1:50, M7010, DakoCytomation) and Sprouty-2 (1:100, #07-524, Millipore/Upstate). For double and triple labelling experiments we used fluorescence iso-type specific secondary antibodies (Invitrogen). Fluorescent nuclear counterstain, TO-PRO-3 (Invitrogen) was used in IF. Specimens were visualized on a Zeiss LSM 5 Pascal laser-scanning microscope (Carl Zeiss).

**Isolation of mouse mammary glands and immunohistochemistry.**

Mammary glands were isolated from C57BL/6 mice from 6 week old virgins, on day 15 of pregnancy and on day 2 of lactation. Mammary glands were snap frozen in liquid nitrogen and preserved at -80°C. The tissue was cryosectioned into 15µm sections and mounted on slides. The slides were formalin fixed and blocking was performed with PBS+10% goat serum. The following antibodies were incubated overnight at 4°C: p-EGFR (Tyr1068) (1:400, #3777, Cell Signaling), PCNA (1:50, ab29, Abcam) Sprouty-2 (1:100, #07-524, Millipore/Upstate). Fluorescence iso-type specific secondary antibodies were used in IF (Invitrogen). Fluorescent nuclear counterstain, TO-PRO-3 (Invitrogen) was used. Specimens were visualized on a Zeiss LSM 5 Pascal laser-scanning microscope (Carl Zeiss).

**Immunocytochemistry in 3D colonies**

Colonies were isolated by gently shaking the rBM in PBS-EDTA (5mM) buffer for 30 min on ice. Free colonies were spun down and mounted on slides and left to attach for 30 min. The blocking was done by incubation with PBS + 0,3% triton-x 100 and 10% goat serum for 1 h at

room temperature. Antibodies were diluted in PBS + 0,1% BSA + 0,3% triton-x 100 and incubated over night at 4°C. The following antibodies and dilutions were used: β4-Integrin (1:400, MAB1964, Chemicon), A488-phalloidin (1:50, #A12379, Invitrogen), EGFR (1:100, #4267, Cell Signaling), p-EGFR (Tyr1068) (1:400, #3777, Cell Signaling), ErbB2 (1:100, ab2428, Abcam) and Sprouty-2 (1:100-1:200, #07-524, Millipore/Upstate). Fluorescence isotype specific secondary antibodies were used in IF (Invitrogen). Fluorescent nuclear counterstain, TO-PRO-3 (Invitrogen) was used. Specimens were visualized on a Zeiss LSM 5 Pascal laser-scanning microscope (Carl Zeiss).

**Western blotting**

Equal amounts (3 μg) of proteins were separated on 10% NuPage Bis-Tris gels (Invitrogen) and transferred to a PVDF membrane (Invitrogen). Antibodies: E-Cadherin (1:500; Zymed), N-Cadherin (1:1000; BD), β-actin (1:5000; Abcam), GAPDH (1:5000; Abcam), K5/6 (1:1000; Zymed), K8 (1:1000; Abcam), K14 (1:1000; Abcam), α-SM-Actin (1:500; Dako) K17 (1:500; Dako), K19 (1:1000; abcam), Vimentin (1:1000; Dako), Sprouty-2 (1:2000; Millipore/Upstate) and FOXC2 (1:2000; Abcam) were used. Membranes were visualized with Supersignal (Thermo Scientific) after incubation with anti-mouse or rabbit secondary antibodies (1:20000; BD).

**Gene expression analysis**

RNA was isolated from D492 and D492-M at 50% and 90% confluency in monolayer culture using RNeasy mini kit (QIAGEN) for mRNA and with Trizol (Invitrogen) for the isolation of miRNA. Experiments were conducted in triplicate, on three different time points (36 samples) for mRNA and miRNA. RNA was analysed on NanoDrop ND-1000 spectrophotometer and run on Agilent 2100 Bioanalyzer chips. Microarray analysis was carried out using the Illumina BeadChip expression microarray (HumanWG-6 v3.0) platform. A bead chip expression array (Illumina) was also used for miRNAs. The data was contracted for background and normalized using cubic spline with all samples as a group using BeadStudio. Probes were quality filtered such that if p detect >0.01 then the intensity was replaced with a missing value. Probes with missing values for all 36 hybridizations were

omitted from future analysis. This left 16547 probes which had p_detect<=0.01 in at least one hybridization. To identify differentially expressed genes we used the MeV software (www.tm4.org) and the significance of microarrays (SAM) method. Genes that had detectable expression levels in 50% of the samples were used in the comparison.

**Migration, anchorage independence and mammosphere assays**

For migration experiments a total of $10^4$ and $2,5 \cdot 10^4$ starved cells were seeded in DMEM/F12 basic medium on collagen coated upper compartment of a transwell Boyden chamber (Corning) with an 8µm pore size. EGM5 medium was used as a chemoattractant in the lower chamber. After 12h incubation, the medium was removed and cells in the upper chamber scraped off with a cotton swab. Migrated cells on the bottom surface of the filter were fixed with formalin and stained with 0,1% crystal violet. Cells were counted in three representative fields in each transwell. Soft agar assay was performed by mixing $10^4$ cells to 1,5ml of 0,5% low melting agar (Invitrogen) and overlaid on 1% agar solution in a 6 well plate. The assay was performed in triplicate in H14 medium. After 20 days the colonies were stained with crystal violet and counted. Mammosphere assay was done in 24-well Ultra-Low attachment plates (Corning) where 500 single cell filtered cells were seeded and cultured on EGM5 medium. Number and size of spheres was evaluated after 8 days in culture.

**Proliferation assay and apoptosis resistance**

In the proliferation assay, $10^4$ cells were seeded per well in a 24 well plate (Falcon, BD). Cells were fixed with formalin and stained with 0,1% crystal violet (days 1-7), washed and left to dry. The crystal violet staining in each well was dissolved in acetic acid and measured at 570nm in a plate reader (Spectra max, Molecular devices). When apoptosis was induced, cells were seeded into 6 well culture plates (Falcon, BD) and grown to 70% confluency. 10µM of Camptothecin (Sigma) in EGM5 medium was used to induce apoptosis and cells were counted on culture days 0 - 4.

**Flow cytometry analysis**

Adherent cells were trypsinized and filtered through a 30nm nylon filter (Millipore). Cells were incubated for 20 minutes with fluorochrome-conjugated antibodies against CD44 (clone IM7, BD), CD24 (clone ML5, BD) or isotype-matched controls, subsequently washed and resuspended in PBS with 4% formaldehyde (cell-fix). Cells were collected ($2 \cdot 10^4$ events) on a FACS-Calibur (BD) and analysed using CellQuest (BD).

**Blocking experiments**

Direct coculture of 500 D492 cells with $2 \cdot 10^5$ BRENCs in 300µl of rBM were treated with 8µg/ml anti-HGF neutralizing antibody (#MAB294, R&D Systems) and with a IgG control in the rBM and in the medium. In transwell coculture HGF was also blocked with 8µg/ml anti-HGF in the rBM and in the medium in the lower transwell chamber. ALK5 kinase inhibitor (SB431542, Tocris Bioscience) was used to block signals through the ALK5 receptor. The inhibitor was diluted in the rBM (10µM) and in the medium (10µM) in coculture of 500 D492 cells and $5 \cdot 10^4$ BRENCs. We also blocked TGFβ1(8µg/ml) in 3D coculture with a neutralizing antibody (ab10517, Abcam) and with an IgG control in the rBM and in the medium.

**Microvessel density scoring**

Microvessel density was evaluated by immunohistochemistry of tumor vessels with an anti-CD31 antibody in whole tissue sections. Immunopositive cell or cluster of cells clearly separated from adjacent clusters, was considered an individual vessel. Microvessels were counted in three different areas of both low/medium and high N-cadherin expression, respectively, in three different biopsies in a 200x field.

**Q-RT-PCR analysis**

Total RNA was extracted with Trizol (Invitrogen), DNAase treated and reverse transcribed with Hexanucleotides using ReverAid (#K1622, Fermentas). Resulting cDNA was used for Q-RT-PCR, in master mix (Applied Biosystems) with primer pairs and probes for Spry2 (Hs00183386_m1, AB) and GAPDH (AB). Experiments were done in triplicate on a 7500 Real

Time PCR System (AB). Quantitations of Spry2 mRNA levels were normalized to GAPD and relative mRNA difference was calculated with the $2^{-\Delta Ct}$ Method.

**Sprouty-2 knock down by shRNA lentiviral transduction**

pGIPZ lentiviral shRNA constructs targeting Sprouty2 transcripts were purchased from Open Biosystems (RHS4430-101098640, RHS4430-101103852, RMM1766-96881511). A non-silencing construct (RHS4346) was used as a control. Viral particles were produced in HEK-293T cells using Arrest-In transfection reagent (ATR1740; Open Biosystems) according to instructions. Virus-containing supernatants were collected at 48 hours after transfection and target cells were infected in the presence of 8ug/ul polybrene. Stable, D492, Spry-2 KD cells were established by puromycin selection (3µg/µl) as well as selection for green fluorescent protein (GFP) expression.

**Statistical analysis**

Data is presented as mean + standard error of the mean from number of independent experiments as indicated. Statistical analysis was performed by two-tailed Students T-test using GraphPad. P values of <0,05 were considered to be statistically significant.

# RESULTS AND DISCUSSION

In this chapter I will discuss my data, both published (papers) and unpublished data (manuscript and additional data). My intention is to discuss my work critically in a retrospective way to give the reader a comprehensive overview of the thesis work. For detailed description of each article/manuscript I have attached them in supplement enclosed with this thesis.

## 1. Isolation and propagation of primary breast endothelial cells (Paper #1)

In this article I have characterized spatial location of blood and lymphatic vessels surrounding the TDLU structures in the human breast and established methods to purify and cultivate endothelial cells from human reduction mammoplasty specimens. Finally, I did an immunophenotypic characterization of isolated breast endothelia cells and compared them to the widely used human umbilical vein endothelial cells (HUVEC). Unlike epithelial cells that are relatively easy to isolate and culture *in vitro*, endothelial cells require more attention and optimized culture conditions. This is reflected in the fact that attempts to study organotypic endothelia-epithelial interactions in the human breast have been hampered by the lack of protocols for long term cultivation of breast endothelial cells (BRENCs) and a lack of immortalized breast-derived endothelial cell lines. The reason for these difficulties in culturing endothelial cells are largely unknown but could be partially explained by the fundamental difference between epithelial and endothelial cells. In the breast gland, the epithelial tissue is under constant remodeling compared to endothelial cells that are considered one of the most quiescent cell types in the human body. To gain better view of spatial interactions of breast epithelium and endothelium in the human breast I began by characterizing the location and distribution of endothelial cells in the human breast gland.

### 1.1 Characterization of breast endothelial cells in situ

Numerous markers have been used to identify endothelial cells in various tissues. Among the most commonly used are CD34, CD31, vWF and VE-cadherin (Uzzan, Nicolas et al. 2004). When I stained breast tissue for CD34 it was evident that this marker was not useable for either identification or isolation of pure endothelial cell populations (Figure 1 in Paper #1). In addition to endothelial cells, the CD34 antigen was also expressed on intralobular fibroblasts like others have shown in normal and cancerous tissue (Yamazaki and Eyden 1995; Barth, Ebrahimsade et al. 2002). Surprisingly, CD34 is still used to quantify the vasculature of the normal and malignant breast gland and is even preferred by some as it gives a more widespread staining (Naccarato, Viacava et al. 2003; Sullivan, Ghosh et al. 2009). In my studies staining for CD31, vWF and VE-Cadherin gave a better picture of BRENCs, showing that microvessels were a prominent feature of the intralobular breast tissue in close contact to the breast epithelium (Figure 1 in Paper #1). Dual immunofluorescence staining for VE-Cadherin and lymphatic vessel endothelial receptor-1 (LYVE-1) showed that blood and lymphatic vessels could be easily distinguished (Figure 1 in Paper #1). Numerous reports have shown that endothelial cells from different sources are both phenotypically and functionally different (reviewed in (Aird 2003)). Therefore I have invested considerable effort in improving methods for the isolation of breast-derived endothelial cells.

### 1.2 Isolation and propagation of breast endothelial cells

Tissue from breast reduction mammoplasties was used to refine preexisting protocols for the isolation of microvascular endothelial cells. Previous work by Hewett et al. demonstrated that it was possible to isolate endothelial cells from the human breast gland (Hewett, Murray et al. 1993). In their methodology they used collagenase digested breast adipose tissue with a subsequent incubation of CD31 antibody (dynabeads) to “fish” out microvascular fragments (Hewett, Murray et al. 1993). In my work I focused on two different sources for endothelial cell isolation, collagen rich stroma close to the epithelial compartment and breast adipose tissue that is not in contact with the epithelium. The tissue was initially separated, with scalpels, into collagen rich stroma and fat tissue and then

dissected by hand with scalpels and further disaggregated with collagenase I for 2h (fat tissue) and 24h (collagen rich stroma) to release the microvessel organoids. The difference in digestion time for fat tissue and collagen-rich stroma was due to the nature of these tissues. Fat tissue is a lose connective tissue that is easily disaggregated whereas collagen-rich stroma is a tightly compact tissue that needs more time for enzymatic digestion. Next, the two different sources were spun down and cleared of other stromal and epithelial cells (Figure 2 in Paper #1). Finally, microvascular organoids were isolated with CD31 antibody coated magnetic beads (Dynabeads). These two different sources for BRENCs resulted in large amounts of endothelial cells, although with different levels of purity. The adipose tissue usually gave a purer population but with fewer isolated microvascular organoids. In contrast, the collagen rich stroma gave substantially more microvascular organoids but with a higher risk of fibroblast contamination. Additionally, the collagen rich stroma could be use to isolate both epithelial and endothelial organoids from the same biopsy as well as fibroblasts. BRENCs grew out from organoids on endothelial specific medium, EGM with different amounts of fetal bovine serum (FBS) in the medium (Figure 2 in Paper #1). The endothelial specific medium is originally supplied with 2% FBS but in my experiments optimal expansion of BRENCs in culture was reached when 30% FBS was used. For the purpose of coculture experiments of BRENCs with other primary epithelial cells and cell lines, best result was reached when the endothelial growth medium was supplemented with 5% FBS. Since our publication, one article has been published describing previous existing methods in isolating endothelial cells from adipose tissue (Hewett 2009). However, to our knowledge (PubMed search, April 2011) our paper published in 2006 is still the latest entry into the literature describing isolation of endothelial cells from human breast tissue, emphasizing how laborious and difficult it is to isolate these cells. After isolation and propagation in culture I conducted phenotypic and functional characterization on the BRENCs and compared them to the commonly used human unbilical vein endothelial cells (HUVECs).

### 1.3 Characterization of isolated breast endothelial cells

BRENCs were compared to HUVECs because they are one of the most common sources of endothelial cells used in research. BRENCs retained critical endothelial traits, expressing CD31, vWF, VE-Cadherin, CD105, VEGFR2 and Vimentin, even at late passage (Table 1 in Paper #1). They showed uptake of low-density lipoprotein and expressed E-selectin upon treatment with TNF-alpha indicating functional activity (Figure. 5 in Paper #1). BRENCs show first sign of aging and senescence in passage 14 with chromosomal abnormalities including gain of trisomy at chromosome 11 (Figure. 6 in Paper #1). Abnormalities and signs of aging, including trisomy 11, in HUVEC cells were seen earlier, or in passage 8. Shorter lifespan of HUVECs in culture may not be surprising considering their origin and limited life span in the umbilical cord *in vivo*. At passage 18 BRENCs were growth arrested in senescence and started to show substantial chromosomal aberrations (Figure. 6 in Paper #1). Interestingly, Johnson et al. demonstrated that a trisomy of chromosome 11 was found in 11 out of 12 long term EC cultures examined indicating this trisomy in the aging of endothelial cells (Johnson, Umbenhauer et al. 1992). The access and isolation of endothelial cells from umbilical cords is relatively easy in comparison to BRENCs. However, these cell types have proven to be different in terms of morphology although they seem to express equally all of the common endothelial markers (Figure. 6 in Paper #1).

In this paper I have demonstrated that the breast microvasculature can be a reliable source for large-scale expansion of BRENCs. The BRENCs preserve their phenotypic and functional traits through extended cell culture *in vitro*. This work forms the basis for the following work where I explore endothelial-epithelial interactions in breast morphogenesis.

### 1.4 Supplementary material

Currently, there are no commercially available breast endothelial cell lines and to my best knowledge there is only one published article on an endothelial cell line from the human breast gland showing extended life span (O'Hare, Bond et al. 2001). Because of the restricted growth conditions (serum supplementation) and time consumption involved in working with primary breast endothelial cells I spent some effort in establishing an immortalized breast endothelial cell line. I transfected primary BRENCs with a retroviral

construct containing E6 and E7 oncogenes from HPV-16, creating the BRENC cell line V95. This cell line maintained all classical endothelial characteristics described above in addition to extended life span in culture (data not shown). I was, however, not successful in making a completely immortalized cell line as they entered senescence in passage 23 evidenced by cessation of proliferation and expression of beta-galactosidases which is an indicator of senescence. On the positive side, the V95 cell line was not as heavily dependent on serum supplementation compared to primary BRENCs. This makes V95 a good choice for future coculture studies with epithelial cells where the use of serum in the media must be minimal or not present at all. Having established protocols for isolation and long-term culture of BRENCs I wanted to explore the interaction between endothelial and epithelial cells. In the initial experiments I tested coculture of BRENCs with primary LEP and MEP and number of normal and cancerous breast epithelial cell lines.

## 2. Coculture of breast endothelial cells with normal and cancerous breast epithelial cells (Paper #2)

In this work I designed a 3 dimensional endothelial-epithelial coculture assay inside a reconstituted basement membrane (3D rBM). This was done by mixing BRENCs with epithelial cells and subsequently embedding them in a single cell suspension into 3D rBM. This setup optimizes colony formation and other underlying morphogenic potentials of epithelial cells. Several technical problems arise when culturing different cell types in the same assay. One aspect is to be able to recognize each cell type within the coculture. In traditional cell culture this problem is clearly present as most cells in monolayer look similar, although with differences in size and shape. Epithelial cell lines within 3D rBM do not present this problem as they usually grow as distinguishable colonies. Another aspect is the medium that must support the growth / survival of both cell types in addition to preserve their endo/epithelial phenotypic traits. The EGM5 medium (Endothelial Growth Medium + 5% FBS) turned out to be a necessary for breast endothelial cells and sufficient for breast epithelial cells so this medium is used in all cocultures described below.

### 2.1 Endothelial cells stay quiescent but metabolically active when cultured within 3D rBM

When BRENCs are cultured on top of a layer of rBM they form a capillary-like network, a trait that is widely used to characterize endothelial cells and in angiogenesis research in general (reviewed in (Arnaoutova, George et al. 2009)). However, this endothelial network is mostly dissolved within 48 hours that renders this setup unusable for long-term (>14 days) co-culture experiments (Figure 1 in Paper #2). In contrast, when BRENCs are embedded within the 3D rBM they remain as single non-proliferative cells (Figure 1 in Paper #2). Although endothelial cells appear as single non proliferative cells throughout a culture period of 16 days in rBM they survive and are metabolically active as seen by their uptake of fluorescently labeled AC-LDL (Figure 1 in Paper #2). The reason why endothelial cells do not form capillary network when cultured within the rBM is unknown. Possible explanations could be the stiffness of the rBM or composition of the matrix. Most coculture studies of endothelial cells with various cell types are most often focusing on angiogenesis capabilities of endothelial cells and the effects of other cells on that process (Arnaoutova, George et al. 2009). In some of these assays the endothelial cells are induced to form capillary-like networks within matrix substances like fibrin gels (Ruger, Breuss et al. 2008; Zhou, Rowe et al. 2008). Although, fibrin gels have been shown to be effective tool in coculture studies it is likely that colony formation and branching morphogenesis of epithelial cells is not optimal within these gels (Xiong, Austin et al. 2008). In contrast, by embedding the endothelial cells within the rBM gel together with epithelial cells allows for exploration of unidirectional effects of endothelial cells on breast morphogenesis although it may be argued that its not as physiologically relevant. To develop the coculture assay further it might be interesting to combine fibrin gels and rBM in a ratio that would both stimulate capillary-like formation of BRENCs in addition to support epithelial morphogenesis. To further tackle the uncertainty of our assay I have supplemented the experiments with cocultures in a transwell setup. In this assay the endothelial cells are cultured as a monolayer in the upper chamber and epithelial cells within rBM at the bottom as described in more detail in the methods section. The two chambers are separated with a membrane allowing free flow of soluble factors between the two different cellular compartments. The transwell coculture combines a more viable and physiological state to grow the endothelial cells in with the chance to induce morphogenic effects of epithelial cells.

### 2.2 Coculture of BRENCs with primary myo- and luminal breast epithelial cells

Initial work in this paper was to test if BRENCs affected growth and morphogenesis of primary cells. Our laboratory has previously succeeded in purifying primary Luminal epithelial- (LEP) and myoepithelial (MEP) cells from the human breast gland (Pechoux, Gudjonsson et al. 1999; Gudjonsson, Villadsen et al. 2002). Cells from these two epithelial compartments were isolated from breast reduction tissue with specific surface markers for LEP and MEP cells. When LEP cells are cultured within a 3D rBM they form small polarized colonies with a central lumen reminiscent of in vivo breast acini. MEP cells, however, form disorganized, non polarized, solid colonies (Petersen, Ronnov-Jessen et al. 1992). Coculture with BRENCs resulted in increased colony size of both LEP and MEP derived colonies. Furthermore, increased lumen size was seen in some LEP derived colonies (Figure 6) (Figure 2 in Paper #2).

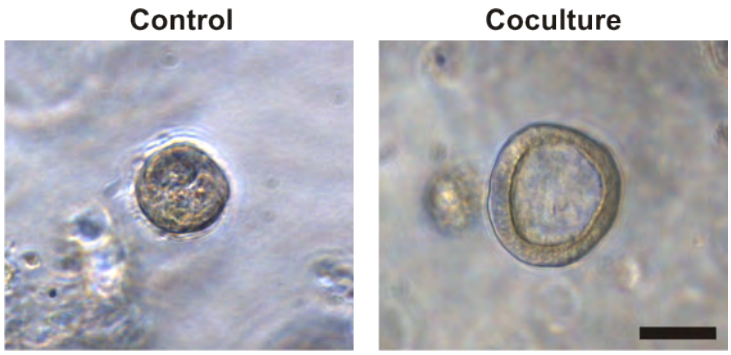


**Figure 6. Coculture of breast endothelial cells with primary luminal epithelial cells creates colonies with large lumens.**
*Left.* Inside rBM primary LEP cells form compact, polarized colonies with a small central lumen. *Right.* In coculture primary LEP cells form significantly larger colonies with a central lumen. Bar=100μm.

Acinus-like LEP colonies displayed basal polarization as evidenced with basal beta4-integrin staining and apical polarization seen when staining for the tight junction protein ZO-1 (Figure 2D in Paper #2). To further explore the morphogenic and proliferative induction potential of BRENCs we also cocultured them with several normal derived and cancerous breast epithelial cell lines.

### 2.3 Coculture of BRENCs with normal and cancerous breast epithelial cell lines

To expand our knowledge regarding proliferative and morphogenic effects of BRENCs on different breast epithelial cells we established cocultures of BRENCs and five normal and cancerous breast epithelial cell lines. When normal-derived cell lines D382 (luminal epithelial cell line) and MCF10A (Basal/mixed phenotype) were cultured alone at clonal dilution (500 cells/300µl rBM) there was no visible growth. However, in coculture with BRENCs a dramatic increase in number and size of colonies was seen. The same observation was seen in cocultures with the estrogen receptor positive cancer cell lines MCF7 and T47D and the basal-like cancer line MDA-MB231 with a dramatic increase in the number and size of colonies (Figure 3A in Paper #2). The phenotype of colonies from different cell lines differed from multiacinar MCF10A colonies, solid round D382, MCF7 and T47D colonies to a more mesenchymal phenotype in the coculture with MDA-MB231. Immunostaining for ki-67, a marker of active cell cycle, showed high proliferation rate in the cocultures (Figure 3B in Paper #2). Cloning efficiency of epithelial cells in coculture increased substantially with increasing amounts of endothelial cells seeded. In the control cultures cloning efficiency was under 5% for all cell lines but increased to 9% for T47D and up to 41% for MCF10A (Figure 4A-B in Paper #2).

### 2.4 BRENCs mediate their effects through soluble factors

To answer the question whether the growth inducing effects of endothelial cells were mediated through cell-cell contact or soluble factors a coculture was carried out in a transwell assay. In this assay the endothelial cells were cultured as a monolayer on top of a porous membrane (0,4µm) in the upper well allowing only soluble factors to be released and diffused into the medium of the lower well. On the bottom of the lower well the epithelial cells were cultured inside a rBM (Figure 5A in Paper #2). Colony size was significantly increased in transwell cocultures of BRENCs with MCF10A, D382 and MCF7 but no growth effect was on T47D and MDA-MB231 (Figure 5B-C in Paper #2). Cloning efficiency was increased in transwell cocultures for the MCF10A and D382 but not for the cancerous MCF7, T47D and MDA-MB231 (Figure 5D in Paper #2). This discrepancy might be explained by lower needs of growth stimuli of the malignant cell lines in comparison to the normal-like

cell lines in this assay in comparison to direct coculture. These results strongly suggest that endothelial cells may mediate their growth promoting effects through soluble factors.

In this paper, I could show in a coculture model that BRENCs mediate proliferation effects of normal and malignant breast epithelial cells and cell lines. By using clonal dilution of epithelial cells in coculture we optimized their morphogenic potential, for example, in a subpopulation of LEP cells showing increased lumen size. These effects of endothelial cells are novel and are likely mediated through soluble factor/s. Future research aims at using this coculture model to study branching morphogenesis from clonally cultured cells within rBM in addition to identify specific factor/s secreted by BRENCs in coculture that mediate proliferation and morphogenic effects.

**2.5 Supplementary material**

As mentioned above the culture conditions of BRENCs inside rBM might not be optimal although it is probably the best option to study epithelial-endothelial interactions in a 3D culture context. Another factor that I have considered is the amount of cells and ratio between different cell types in co-culture experiments. In the cocultures between BRENCs and primary LEP and MEP cells I tried many different ratios until we decided to use the ratio 1:20 (LEP/MEP: BRENCs). It might be criticized that this excess of endothelial cells is not truly describing what would be the case *in vivo*. However, when I optimized the assay I also tried ratios of 1:1 and 1:2 and they also showed these proliferation inducing effects of BRENCs (Not shown). We also see these effects at low BRENCs concentration on MCF10A cells, although they substantially increase colony size at higher concentrations of BRENCS. Furthermore, this setup serves the purpose of clonal derivation of colonies forming in the coculture assay. Another aspect that is important to consider is that the number of BRENCs is not increasing throughout the culture period while there is a large increase in the number of epithelial cells. This means that in later stages of coculture the number of epithelial cells far exceeds the number of BRENCs. Taken together, I conclude that skewed ratio or number of cells in the coculture should not be a factor affecting the result.

## 3. Endothelial cells in coculture with breast epithelial cells with stem cell properties induce epithelial to mesenchymal transition (Paper #3).

The work in Paper #3 and Paper #4 below have occupied a large part of my thesis work so I will use more effort in discussing this on the following pages. In Paper #3 I explored the interactions between BRENCs and the breast epithelial stem cell line D492. This cell line has stem cell and branching morphogenic potentials within 3D rBM gels. In light of our observations with primary breast epithelial cells and cell lines (Paper #2) and data from diverse organs showing the importance of endothelial cells in tissue remodeling, organogenesis and stem cell maintenance I wanted to test if BRENCs might impact breast morphogenesis.

### 3.1 D492 – a human breast epithelial cell line with stem cell properties generate TDLU-like structures in 3D rBM matrix

D492 breast epithelial stem cell line was isolated originally from the suprabasal niche of normal human breast gland and subsequently immortalized using retroviral construct containing E6 and E7 from HPV-16. This cell line has maintained its original phenotype through prolonged cell culture and can differentiate into LEP and MEP cells in monolayer and 3D culture (Gudjonsson, Villadsen et al. 2002; Villadsen, Fridriksdottir et al. 2007). Interestingly, D492 is one of few cell lines isolated from the human breast gland that can produce elaborate branching ductal-lobular-like structures when cultured in 3D rBM similar to the *in vivo* TDLUs (Figure 2). Optimal culture conditions for D492 cells in 3D culture are reached, traditionally, when $7 \cdot 10^3$ to $10^4$ cells are seeded in 300µl of rBM matrix. Due to the morphogenic capacity of D492 I wanted to explore if BRENCs could impact branching morphogenesis.

### 3.2 Coculture of BRENCs and D492 resulted in increased branching morphogenesis and the formation of an EMT-like phenotype

I began by coculturing D492 in clonal cell density of 500 cells with increasing concentrations of BRENCs (0, $5 \cdot 10^4$, $1 \cdot 10^5$ and $2 \cdot 10^5$) within 300µl of rBM. The initial result was clear,

increased overall colony formation and clonal derivation of colonies in the coculture similar to what I had seen before in paper #2. Increased cloning efficiency and proliferation seemed to be dose dependant on the amount of BRENCs in the coculture (Figure 1A in Paper #3). To my surprise, D492 did not exclusively produce branching and solid round colonies as seen previously (Gudjonsson, Villadsen et al. 2002). In the coculture setup they also produced disorganized colonies compact with spindle-like cells (Figure 7) (Figure 1B in Paper #3).

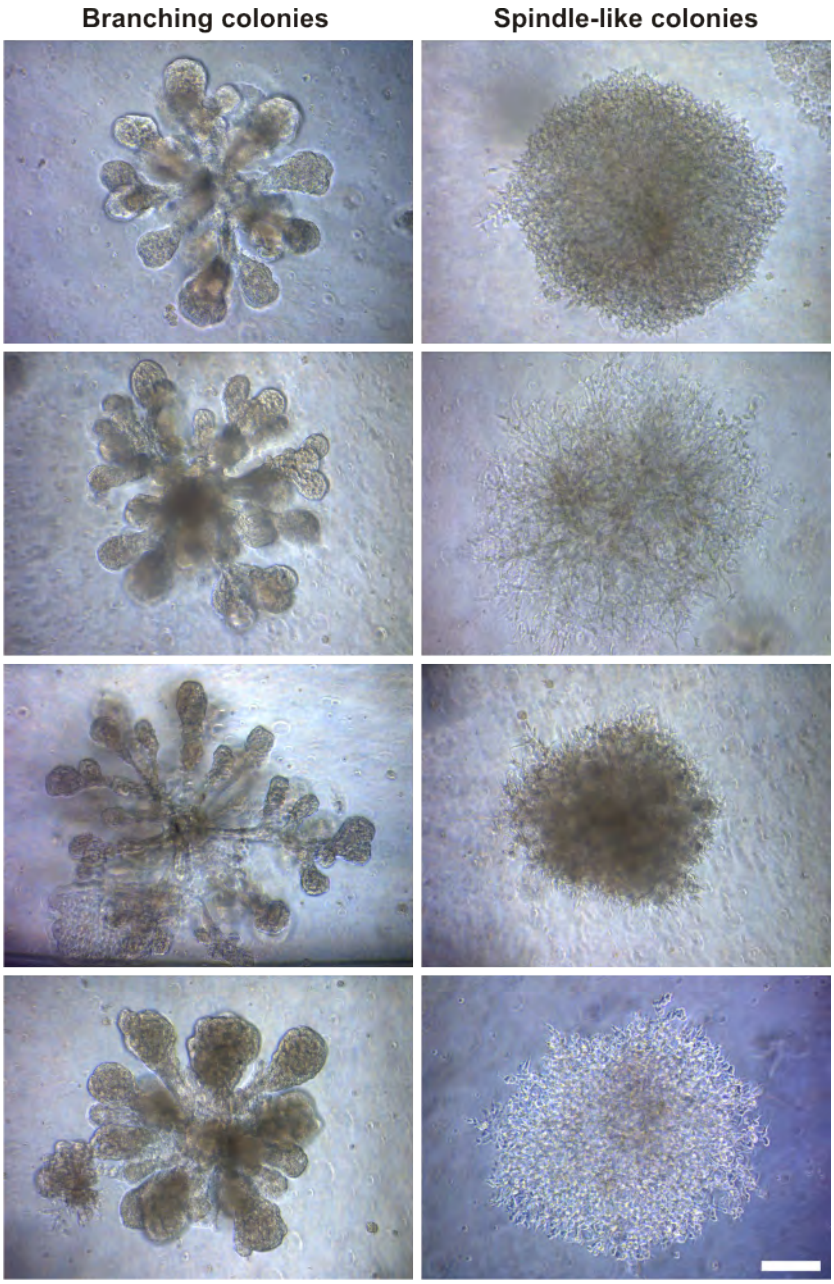


**Figure 7. Coculture of breast endothelial cells with D492 breast epithelial cell line creates branching and spindle-like colonies.**
*Left.* When D492 cells are seeded at low density in rBM coculture with BRENCs they form complex branching colonies similar to TDLUs. *Right.* In addition to branching, spindle-like colonies emerge in coculture with BRENCs . Bar=100μm.

When I quantified the amount of each colony type, branching-, solid- and spindle-like, the only doze dependent increase of BRENCs in coculture could be explained by more spindle-like colonies emerging in the culture (Figure S2 in Paper #3). In contrast, the branching-like colonies seemed to reach a plateau with the lowest amount of BRENCs while the amount of solid-like colonies decreased (Figure S2 in Paper #3). So the original coculture idea worked by increasing the amount of clonally derived branching colonies. However, the emergence of spindle-like colonies from D492 in 3D rBM seemed to be dependent on the presence of BRENCs in this assay in a dose dependent manner. The presence of colonies with spindle-like cells could also mean that cells with a mesenchymal phenotype were already present within the original D492 cell line and that they were induced to grow by signals from BRENCs. The other possibility was that the D492 cells had undergone a epithelial to mesenchymal transition (EMT) induced by the BRENCs. To resolve this we explored the morphogenic potential of the three colony types in secondary 3D rBM cultures.

### 3.3 Morphogenic potential of branching and spindle-like colonies in secondary 3D rBM culture

I began by looking for cells within D492 that had a pure mesenchymal-like program in traditional monolayer culture using immunofluorescence double staining. I had difficulties associating mesenchymal markers like vimentin or Thy-1 to specific mesenchymal cells within D492. This is due to the fact that these markers are promiscuous as they are also expressed on MEP-like cells. I could, however, identify cells that were expressing N-cadherin, a typical marker found on mesenchymal cells (but not MEP), but these cells were also expressing classical epithelial markers like E-cadherin, K14 and K19 (Figure 8A) Indeed, in my observations I have found cells that are N-cadherin positive within the normal human breast gland, often coexpressing markers of luminal epithelial cells (not shown). By flow cytometry on D492 I could identify a small portion of cells that were positive for N-cad and negative for epithelial CD24, similar to true mesenchymal cells (Figure 8B). So there was a small possibility that BRENCs were merely cloning up a preexisting mesenchymal phenotype in the cocultures. To explore this further I focused on the morphogenic potential of branching-like and spindle-like colonies in secondary rBM cultures.

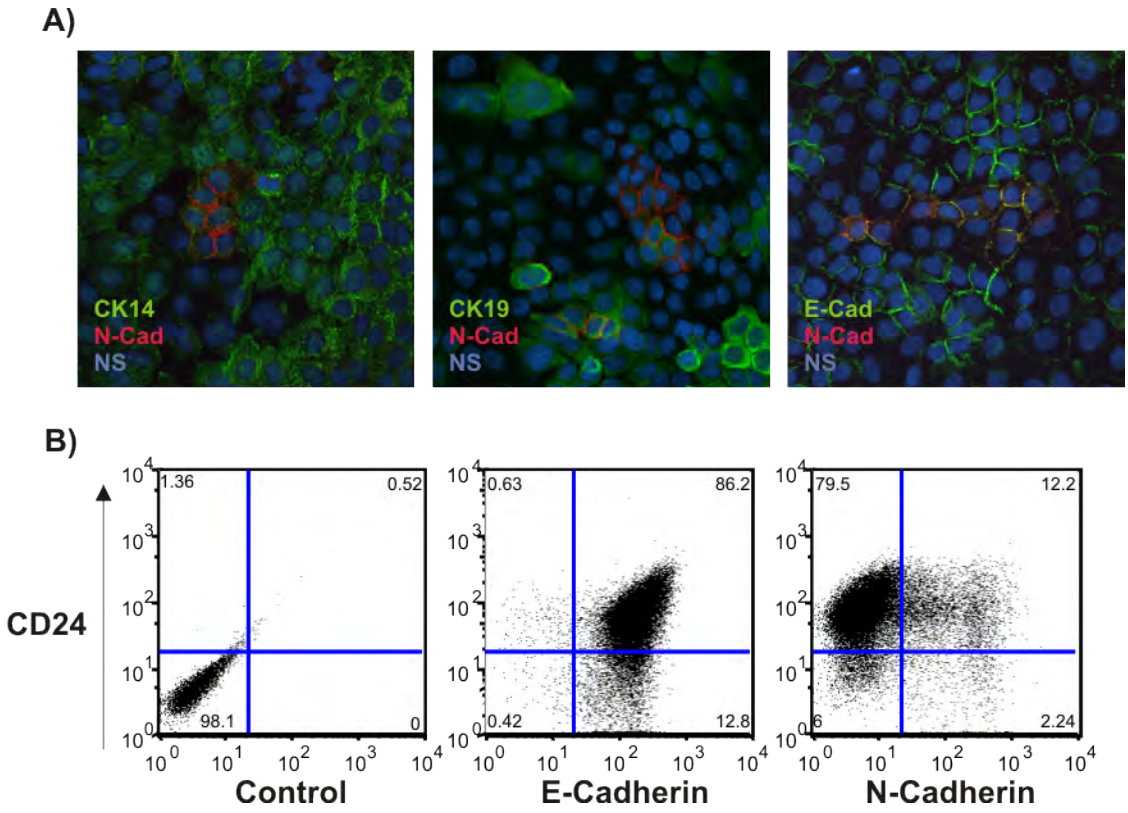


**Figure 8. A small portion of the D492 cell line show expression of mesechymal marker N-cadherin.**
*A.* D492 cells in monolayer were stained for CK14, CK19, E-cadherin (green) and N-cadherin (red). N-cadherin expressing cells also expressed E-cadherin, CK14 and most of the also CK19. *B.* Flow cytometry indicated that a small fraction (2,2%) of D492 cells expressed N-cadherin without CD24 and thus a possible EMT phenotype however they were also shown to express E-cadherin.

This was done by isolating branching and spindle colonies from cocultures of D492 and BRENCs. Cells derived from branching colonies had a typical epithelial cubodial phenotype in monolayer culture while cells from spindle colonies showed elongated spindle shaped cells. In secondary coculture, cells from spindle colonies were fixed in making more spindle-like colonies while cells from branching colonies could produce branching and spindle-like colonies (Figure 2 in Paper #3). Cells from one of the isolated spindle-like colonies were cultured further as the subline D492M (Mesenchymal). In addition to D492M several additional clones were isolated. In conclusion, these experiments confirmed that the original stem-like cells within D492 were undergoing EMT under stimuli from the cocultured BRENCs explaining the emergence of spindle-like colonies in the coculture. To further confirm these observations I compared the phenotype and function of D492 cells to the D492M subline.

### 3.4 Phenotypic traits of D492 and D492M

When I compared the expression of D492 to D492M for epithelial and mesenchymal markers I was able to confirm that the D492M cells had a true EMT phenotype. D492M cells showed a downregulation of epithelial specific markers E-Cadherin, K5/6, K8, K14, K19 and an upregulation N-Cadherin, alpha smooth muscle actin and Vimentin (Figure 9) (Figure 3A-B in Paper #3).

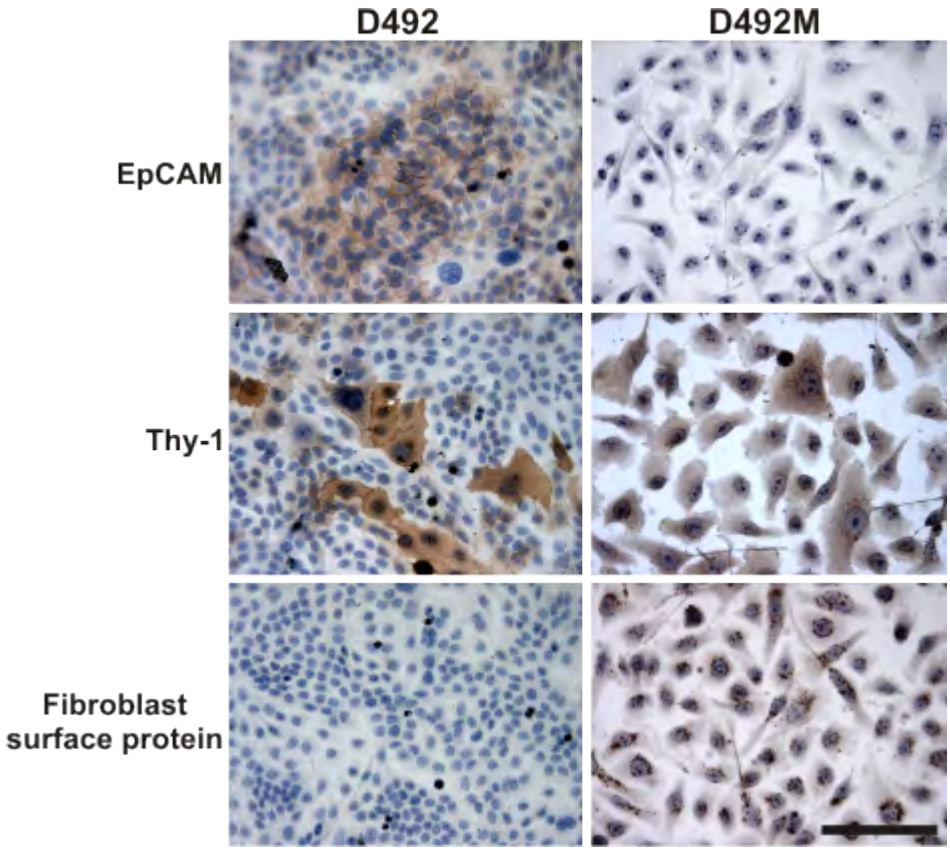


**Figure 9. The EMT subline D492M shows spindle-like morphology and a mesenchymal marker profile.** D492M cells have lost the epithelial marker EpCAM. D492 expresses the myoepithelial marker Thy-1 in a subpopulation of cells while D492M shows expression in all cells. Additionally, D492M cells express the fibroblast surface protein in contrast to D492. Immunoreactivity seen as brown. Counterstained with hematoxylin (blue). Bar=100μm.

I also isolated RNA from D492 and D492M and compared the gene expression on a microarray. The array showed a vast difference in the expressional profiles of the two cell lines, with a difference in over 6 thousand genes (Figure S5 in Paper #3). Among upregulated genes were important EMT linked transcriptional factors FOXC1 and FOXC2. Upregulation of FOXC2 was confirmed by WB (Figure 3C-D in Paper #3). Functionally the D492M cells also showed their transition towards a mesenchymal phenotype with up to 7

fold increase in migration (Figure 4A in Paper #3). The D492M cells showed signs of malignant transformation by their growth in soft agar assay and by decreased growth inhibition in monolayer culture (Figure 4B in Paper #3). The flow cytometry profile $CD44^{High}$ / $CD24^{Low}$ has been used extensively in breast cancer studies to identify cancer stem cells and recently this profile has been linked to EMT (Al-Hajj, Wicha et al. 2003; Mani, Guo et al. 2008). When D492 and D492M are compared with this profile around 20% of D492 cells reside within the cancer stem cell gate while almost all D492M cells are $CD44^{High}$ / $CD24^{Low}$ (Figure 4C in Paper #3). The ability to form colonies in a mammosphere assay has, furthermore, been linked to stem cells and cancer stem cells. In this assay, cells are seeded at low density and form floating spheres in a nonadherant plate. It was not surprising that D492, being a stem cell line, could form spheres in this assay but D492M showed a significant increase in sphere formation and formed larger spheres (Figure 4D in Paper #3). Another hallmark of cancer stem cells and EMT is apoptosis resistance. D492M cells showed increased resistance to chemically induced apoptosis compared to D492 (Figure 4E in Paper #3). In conclusion, my analysis of D492M confirms that these cells have acquired a mesenchymal phenotype as a consequence of the EMT process. Furthermore, these cells bare resemblance to breast cancer stem cells. It is important to mention that the above mentioned assays are not restricted to stem cells and cancer stem cells, rather they are characteristics associated with the stem cell phenotype among many others. To test if these EMT inducing effects of BRENCs in coculture were restricted to the D492 cell line we tested additional normal and malignant cell lines.

**3.5 BRENCs induce EMT in poorly differentiated cell lines of different origin.**

Because the $CD44^{High}$ / $CD24^{Low}$ profile has been connected to EMT I wanted to know if these markers could distinguish between cell lines that would be susceptible to BRENCs induced EMT. However, the risk is that this profile is also identifying cells that have already undergone the EMT process and show a spindle-like phenotype. The differentiated breast epithelial cell lines D382 and MCF10A both show a typical epithelial, $CD44^{High}$ / $CD24^{High}$, populations with only a minor population (<1%) of MCF10A containing $CD24^{low}$ cells. In contrast, the highly malignant basal-like breast cancer cell line MDA-MB-231 is almost

exclusively (99%) CD44$^{high}$,CD24$^{low}$ and is thus likely a post-EMT cell line (Figure 10). When these cell lines were cocultured with BRENCs in rBM the MDA-MB-231 continued to generate mesenchymal colonies while the CD44$^{High}$,CD24$^{High}$ cell lines, D382 and MCF10A, generated only round epithelial-like colonies (Figure S3 in Paper #3).

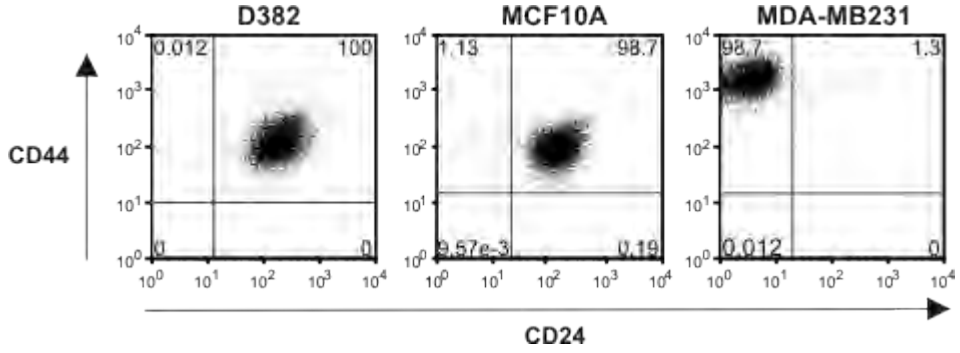


**Figure 10. The flow cytometry profile CD44$^{High}$/CD24$^{Low}$ is a marker of EMT and compromised epithelial integrity.**
The normal-like cell lines D382 and MCF10A only have a minor portion of cells within the CD44$^{High}$/CD24$^{Low}$ gate. In contrast, the EMT-like, MDA-MB231 almost exclusively resides within this gate.

Furthermore, MCF-7, an estrogen receptor positive breast cancer cell line generated only large solid round colonies in coculture with BRENCs (Figure S3 in Paper #3). It is interesting to note that in the literature the MCF-7 cell line is generally described with a minor population (<5%) of CD44$^{high}$,CD24$^{Low}$ cells (Gong, Yao et al. 2010; Sun, Liao et al. 2010). Because the population of basal-like breast cancers has been described to have EMT-associated markers, I also tested the W2320 metaplastic breast cancer cell line. W2320 cells have previously been shown to generate mesenchymal like cells in culture (Petersen, Nielsen et al. 2003). The W2320 cell line generated solid epithelial-like colonies when cultured alone in 3D rBM but when they were cocultured with BRENCs there was a dramatic increase in total colony formation and in the number of spindle-like colonies (Figure 1C in Paper #3). To test if this only applied to cell lines of breast origin we included the lung adenocarcinoma cell line, A549, which has previously been shown to have some of the characteristics associated with cancer stem cells (Meng, Li et al. 2009). A549 could also be induced to form spindle-like colonies in coculture with BRENCs (Figure 11). To further support that this might be a general EMT inducing factor I used the EMT susceptible Madin-

Darby canine Kidney epithelial cell line (MDCK). These cells have the abilities to form polarized spheres in 3D culture in addition to form EMT and scattering when induced with a powerful EMT-factor like TGF-beta (Zhang, Dong et al. 2006). In line with our coculture data MDCK cells produced disorganized, EMT-like colonies, in coculture with BRENCs (not shown).

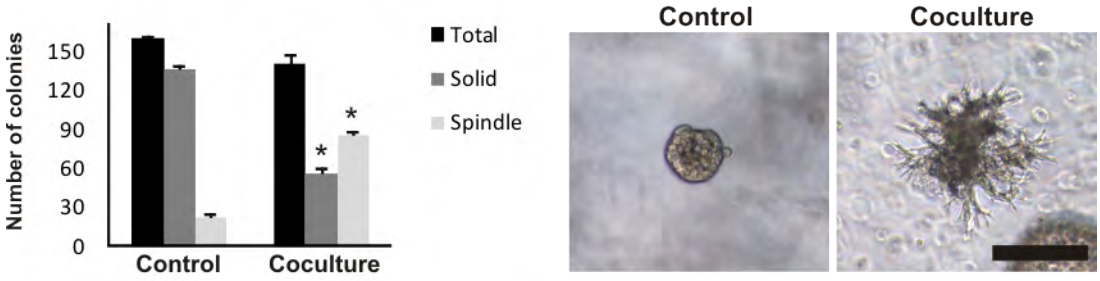


**Figure 11. Breast endothelial cells can induce the emergence of spindle-like colonies in coculture with A549 lung cancer cell line.**
*Left.* In control cultures of A549 inside rBM the majority of the colonies have a solid round phenotype. In contrast, the spindle-like phenotype is significantly more common in coculture with endothelial cells indicating EMT inducing effect of BRENCs on A549. *Right.* Representative figures of a solid colony in the control and a spindle-like colony in the coculture. * $p<0.05$. Bar=100μm.

In conclusion, this data indicates that breast cell lines with stem-like (D492) and basal-like/metaplastic (W2320) characteristics have the plasticity for mesenchymal conversion in coculture with endothelial cells. Other cell lines, more differentiated (D382, MCF10A and MCF7), are unable to undertake this transition by BRENCs induction although they are capable of undergoing EMT with other methods. Furthermore, my data is in line with recent publications showing that breast cancer cells and cell lines show varying degrees of mesenchymal phenotype and propensity to show or undergo EMT (Kenny, Lee et al. 2007; Sarrio, Rodriguez-Pinilla et al. 2008). In addition, the effects BRENCs are not bound to cells of breast or human origin because A549 cells and MDCK cells were also induced by BRENCs to make spindle-like colonies reminiscent of EMT. This data suggests that BRENC derived factor/s inducing EMT must be common factors known to be involved in EMT in different systems. In that regard, I turned my focus to the TFG-beta signaling pathway that is one of the best-known and most effective pathways to induce EMT in cells of different origin and species (Moustakas, Pardali et al. 2002).

### 3.6 Effects of TGF-beta signaling on branching and EMT in D492

TGF-β is a powerful inducer of EMT in various cell culture models and is one of the most studied factor that mediates epithelial stromal interaction (reviewed in (Zavadil and Bottinger 2005)). In order to identify endothelial-derived factors responsible for facilitating EMT I first focused on TGF-β1. When D492 cells were treated with TGF-β1 (10ng/ml) in monolayer for 15 days the cells showed a more elongated phenotype and increased their number of $CD44^{high}$, $CD24^{low}$ cells, indicating EMT. However, this effect was reversible when TGF-β1 was removed from the medium (Figure 12A). Immunocytochemical characterization showed that TGF-β induces a partial EMT phenotype, as evidenced by down regulation of E-cadherin, EpCAM and K14 and a partial induction of N-cadherin (Figure 12B).

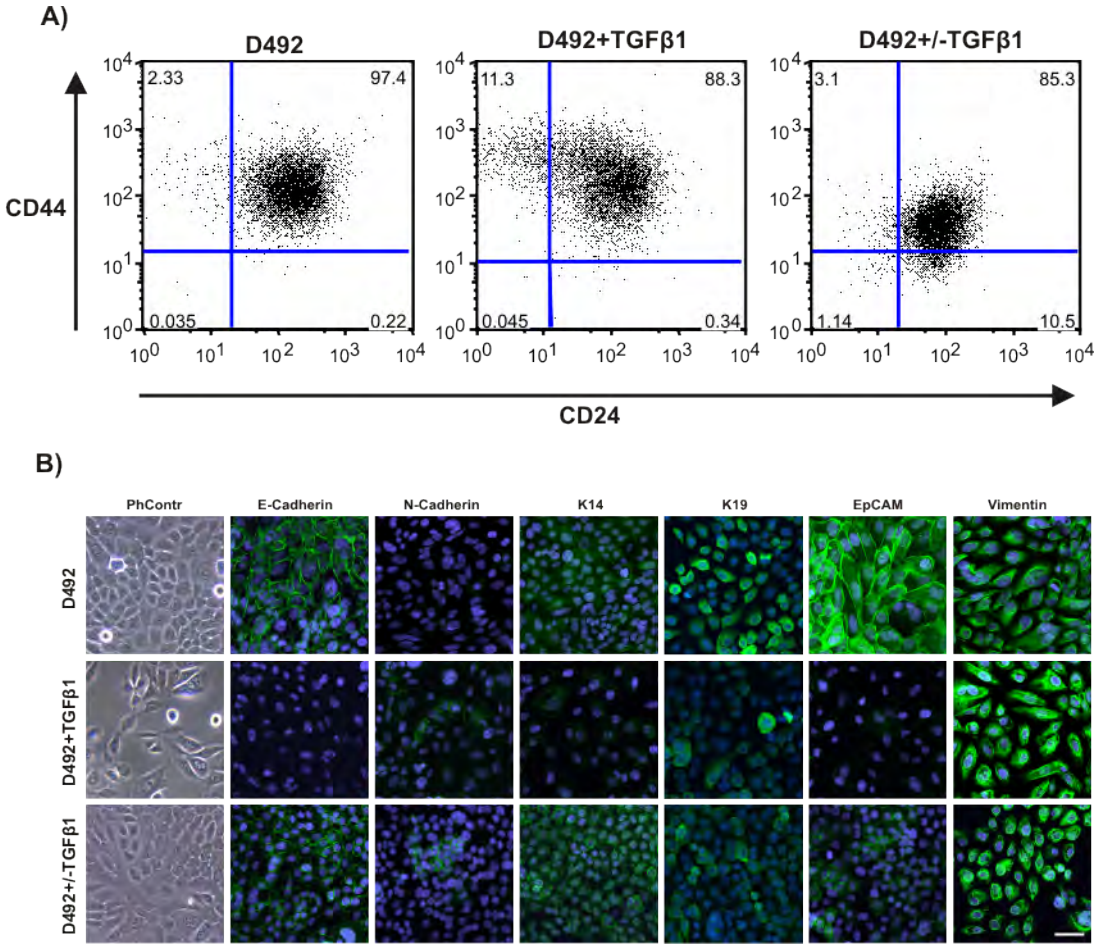


**Figure 12. TGF-β1 induces a partial, reversible EMT in monolayer culture of D492 cells.**
*A.* Flow cytometry for the $CD44^{High}/CD24^{Low}$ profile was used to evaluate the effects of TGF-β1 treatment. When D492 cells were treated with TGF-β1 a slight increase (from 2% to 11%) was seen in cells in the $CD44^{High}/CD24^{Low}$ gate that was reverted back when TGF-β1 was removed (D492+/-TGF-β1). *B.* When staining for epithelial and mesenchymal markers in TGF-β1 treated cells a similar result was seen by a general decrease in epithelial markers E-cadherin, K14, K19 and EpCAM and an increase in mesenchymal N-cadherin and Vimentin that was mostly reverted back when TGF-β1 was removed from the culture medium. Immunoreactivity seen as green. Counterstain TOPRO3 (Blue). Bar= 50μm.

TGF-β1 withdrawal from D492 resulted in recovery of the epithelial phenotype (Figure 12B). When D492 cells were exposed to TGF-β1 in 3D rBM culture we did not observe any spindle-like colonies. When TGF-β1 was added to the culture medium at day 0 no colony growth was observed indicating growth inhibiting effects of TGF-β1. Interestingly, if TGF-β1 was added at day 7, before branching normally starts a dramatic increase in branching morphogenesis was seen indicating different effects in early and late culture periods (Figure 13A).

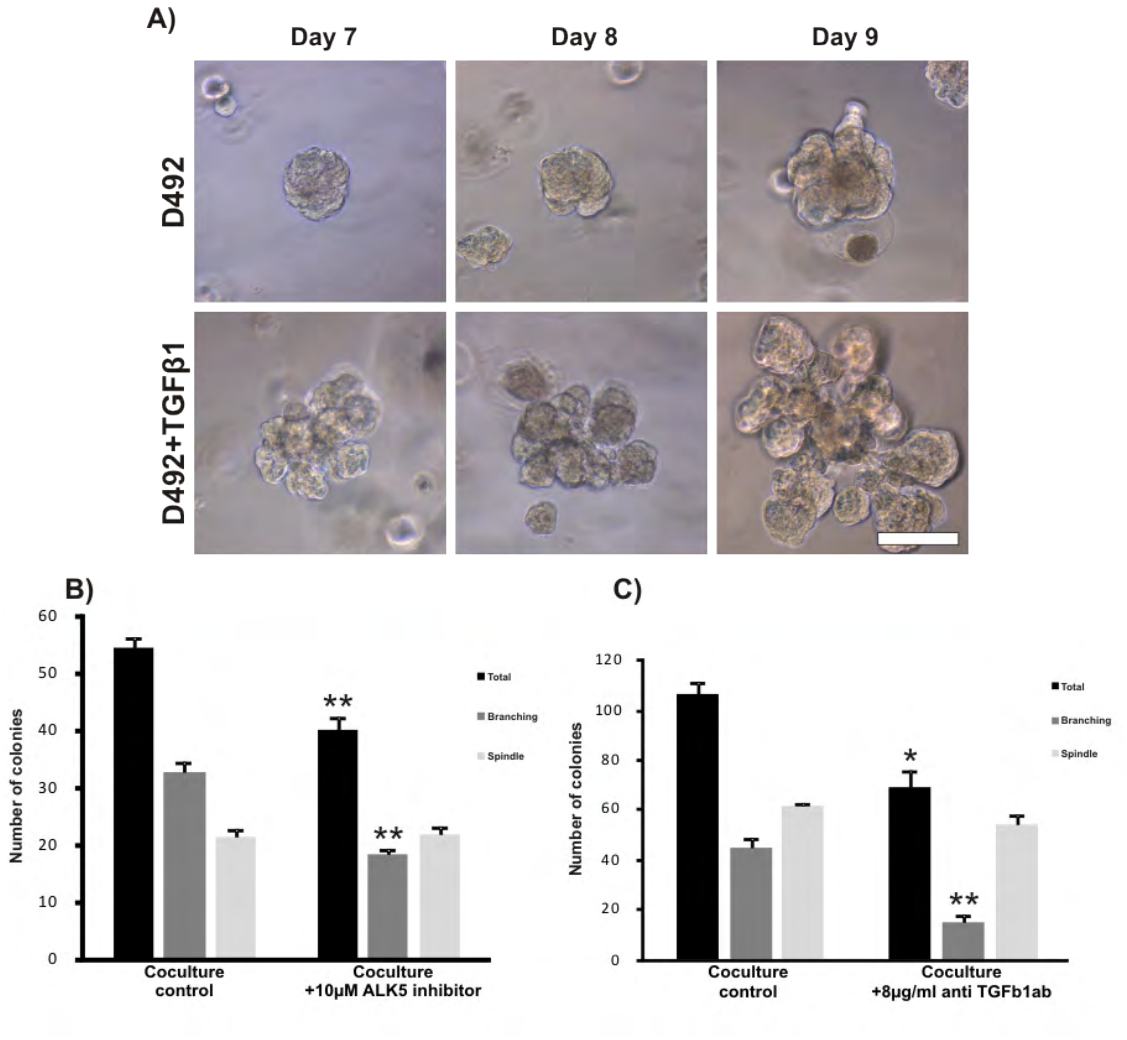


**Figure 13. TGF-β1 induces branching morphogenesis in 3D rBM culture of D492.**
*A.* D492 was treated with TGF-β1 from Day 6 in 3D culture resulting in early branching but not spindle-like morphology. *B.* When cocultures of D492 and BRENCs were treated with an ALK5 inhibitor a significant decrease in branching colonies was seen with no effects on spindle-like colonies. *C.* The same effect was seen with a neutralizing anti-TGF-β1 antibody, a decrease of branching colonies but no change in spindle-like colonies. * $p<0.05$; ** $p<0.01$. Bar= 50μm.

To further test if TGF-β1 mediates some of the endothelial effects on D492 we treated co-cultures with a small molecule inhibitor against TGF-β receptor-1 (ALK5) and a TGF-β neutralizing antibody. At 10μm concentration of ALK5 kinase inhibitor, we observed a dramatic reduction in branching colony formation whereas no changes were seen in the number of spindle colonies (Figure 13B). Similar effects were seen when TGF-β1 was blocked with anti- TGF-β1 antibody (8μg/ml), a marked reduction of branching colonies but no change in the number of spindle colonies (Figure 13C). This is in line with my data that TGF-β1 stimulates formation of branching- but not spindle colonies from D492 when cultured alone in 3D rBM. Importantly, this indicates that TGF-β1 and signals through the TGFbeta pathway are crucial for proper branching morphogenesis of D492 in 3D rBM cultures. Data from others, using mouse models, have in fact shown that TGF-β1 and its downstream pathway are crucial for proper branching of the mammary gland (Ingman and Robertson 2008; Jahchan, You et al. 2010; Pavlovich, Boghaert et al. 2011). This also demonstrates a novel phenomena in that EMT can have different consequences in 2D and 3D culture microenvironments because TGF-β1 induction in 2D produced partial EMT but not in 3D rBM culture. It should, however, be noted that branching morphogenesis shares some characteristics with EMT. Thus, TGF-β1 induced branching in my 3D assay could be a consequence of a partial EMT. The link between branching and EMT is emerging in the literature and in light of that I need to explore this further in our assay (Micalizzi, Farabaugh et al. 2010). There are numerous other factors that can elicit EMT in culture and I decided to focus my attention to other known EMT inducers.

### 3.7 EMT inducing effects of BRENCs are mediated through soluble factors and can be partially blocked by anti-HGF

The EGM5 medium that all coculture experiments were conducted in has powerful ligands for tyrosine kinase receptors like EGF, FGF and VEGF which have previously been linked to EMT (Moustakas and Heldin 2007; Yang and Weinberg 2008). However, monocultured D492 cells in this medium do not show signs of EMT so I focused on hepatocyte growth factor (HGF) that is a known inducer of branching morphogenesis and EMT in breast epithelial cells (Niranjan, Buluwela et al. 1995; Grotegut, von Schweinitz et al. 2006). HGF is a stromal

derived factor that is expressed in endothelial cells, among other stromal cells in the breast (Sonnenberg, Meyer et al. 1993; Pollard 2001; Haslam and Woodward 2003). Increased expression of the RTK receptor for HGF, c-MET, has been shown to be upregulated in breast cancer and has especially been associated with the basal like breast cancer subtype (Tuck, Park et al. 1996; Yao, Jin et al. 1996; Camp, Rimm et al. 1999; Edakuni, Sasatomi et al. 2001; Charafe-Jauffret, Ginestier et al. 2006). I could show that BRENCs are producing and secreting HGF inside 3D rBM (Figure S7 in Paper #3). When I treated cocultures of D492 and BRENCs with a neutralizing antibody against HGF (8μg/ml) it had no effect on total colony formation but a significant decreasing effects (~50%) on the formation of spindle-like colonies. In contrast, there was a significant increase in the portion of branching colonies (Figure 5D in Paper #3). To verify that the EMT inducing effects of BRENCs were mediated through soluble factor/s I set up a transwell coculture as described above (Figure 5A in Paper #3). In this assay BRENCs induces EMT of D492 cells in a more effective way, with over 70% of all colonies showing the spindle-like phenotype (Figure 5B-C in Paper #3). When the neutralizing antibody against HGF was added there was a significant decrease (~25%) in the spindle-like phenotype with no significant effects on branching colonies or total colony formation (Figure 5E in Paper #3). Collectively, this shows that BRENCs-derived HGF mediates EMT induction in the 3D coculture model. This data also suggests that altering HGF signaling can modulate the balance in the formation of branching- and spindle colonies formed by D492. However, the neutralization of HGF did not completely block the formation of spindle-like colonies. There might be additional factors needed and in support of that I have done numerous studies where I tried to induce monocultures of D492 cells in 3D rBM to form spindle-like EMT colonies by simply adding HGF. This was done in different concentrations of HGF, various cell concentrations of D492 and in combinations to different culture medium but these experiments have failed so far (not shown). It is also noteworthy that in transwell coculture a few spindle-like colonies emerged in the control. I have never seen this in the monoculture controls for the direct coculture studies and this might be explained by the different experimental setup in the transwell coculture assay. In light of my data, HGF seems to be a necessary BRENCs derived component to induce EMT in our coculture but not sufficient alone. This may also demonstrate the redundant nature of the EMT process where you can have number of different components that can induce EMT to

certain degree in different cells types and settings. The D492 cell line has a basal and stem cell-like phenotype corresponding to their in vivo counterparts. Interestingly, evidence suggests that basal-like breast cancers might originate in stem or progenitor cells with a similar profile. Furthermore, studies have shown that these tumors are highly vascularized and rich in EMT associated markers (Sarrio, Rodriguez-Pinilla et al. 2008; Linderholm, Hellborg et al. 2009; Miyashita, Ishida et al. 2011). In light of my coculture data of D492 and BRENCs in 3D rBM I wanted to explore further the connection between vascularization and EMT within these tumors.

### 3.8 Basal-like breast cancers show increased EMT phenotype close to highly vascularized areas

Recently, the basal-like breast cancers were shown, by tissue microarray, to have an EMT phenotype (Sarrio, Rodriguez-Pinilla et al. 2008). In addition, some studies suggest that the microvascular density (MVD) may be higher within ER- / PR- breast cancers and within basal-like breast cancers (Linderholm, Hellborg et al. 2009; Miyashita, Ishida et al. 2011). To explore the connection between vascularization and EMT I used four breast cancers that were positive for the estrogen receptor and nine cancers defined as triple negative ($ER^-PR^-$ $ErbB2^-$). The basal-like phenotype of the triple negative breast cancers was further verified by immunostaining for K5/6, K14, K19 and Vimentin in accordance to commonly used markers to classify these tumors (Figure 14). I also stained the basal-like tumors with antibodies against E-cadherin, N-cadherin and CD31. In the $ER^+$ cancers there was little or no expression of N-cadherin in contrast to all the basal-like cancers that were positive, suggesting a mesenchymal / EMT phenotype (Figure 6A in Paper #3). The expression level of N-cadherin within the basal-like cancers varied but was often strongest close to vascular rich areas (Figure 6B-C in Paper #3). Interestingly, microvascular density (MVD) was higher in areas with strong expression of N-cadherin compared to areas of low N-cadherin expression (Figure 6B and S8 in Paper #3). Low expression of E-cadherin was seen in all of the basal-like tumors, further confirming their mesenchymal / EMT phenotype (Figure 6D in Paper #3). In conclusion, the cellular context in basal-like breast cancer reveals an interesting pattern of cancer cells showing an EMT phenotype closely associated with vascular rich components.

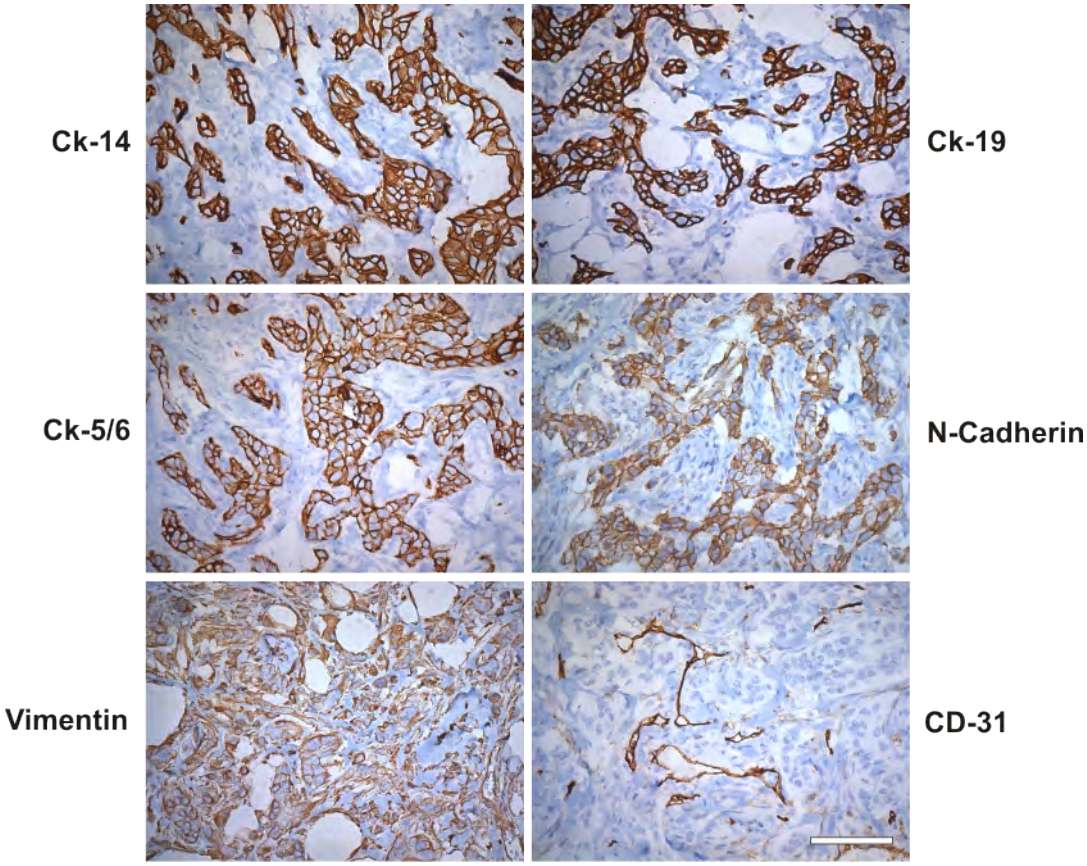


**Figure 14. Phenotypic characterization of basal-like breast cancers.**
In addition to the triple negative profile (ER-, PR-, ErbB2-), the basal-like breast cancers express CK14, CK19, CK5/6, N-cadherin (varying within cancers) and Vimentin. The cancers were also stained for CD-31 to visualize the vasculature. Immunoreactivity seen as brown. Counterstained with hematoxylin (blue). Bar= 100μm.

This data together with the *in vitro* coculture experiments support the hypothesis that the endothelial compartment might influence the EMT phenotype often found within basal-like breast cancers. As mentioned above, the D492 cell line was originally immortalized by inactivating p53 and RB, making the cell line vulnerable against further insults. It is interesting that P53 and RB are frequently downregulated or mutated in basal-like breast cancers (Sorlie, Perou et al. 2001; Hu, Stern et al. 2009; Jiang, Jones et al. 2011). This further demonstrates that the D492-D492M cell lines are optimal in capturing genetic- and phenotypic traits in basal-like breast cancer. Hellner et al. have recently demonstrated that E6 and E7 transfected into human foreskin keratinocytes modulate expression of genes involved in EMT, including the cadherin switch from E- to N-cadherin (Hellner, Mar et al. 2009). This is not seen in D492 as it retains all major epithelial characteristics. However, an

inactive p53 and RB together with stem cell properties may predispose D492 cells towards EMT conversion facilitated by the endothelial-rich 3D environment.

### 3.9 Supplementary material

Recent papers have shown that micro RNAs (miRNA) play a crucial role in regulating EMT. Gregory et al. showed that the miRNA-200 family and miRNA-205 is repressed during TGF-β induced EMT (Gregory, Bracken et al. 2008). Furthermore, enforced expression of these miRNA´s prevented TGF-β induced EMT and they were shown to regulate expression of E-cadherin through their silencing of ZEB1 and ZEB2 (Gregory, Bracken et al. 2008). Likewise, Shimano et al showed that mirRNA-200c is downreglated in human breast cancer stem cells (Shimono, Zabala et al. 2009). I wanted to explore if the expression of miRNAs had changed in D492M. MiRNA was isolated from D492 and D492M at 50% and 90% confluency in monolayer culture. Using Illumina BeadChip microarray I screened the expression pattern of the two cells lines (Figure 15). Interestingly, of the top eight miRNA genes downregulated in D492M half belonged to the miR-200 family (miR141, 200c, 200a, 200b). As mentioned above, these miRNAs are emerging as major regulators of epithelial integrity in human and murine cells (Gregory, Bracken et al. 2008; Gibbons, Lin et al. 2009). MiRNA-205 and -203 were also in the top downregulated miRNAs. These miRNAs have been described as inhibitors of “stemness” and thus important in maintaining epithelial integrity, but they also link the EMT process to the emergence of cancer stem cells (Gregory, Bert et al. 2008; Wellner, Schubert et al. 2009). In fact, Mani et al. and Morel et al. have recently suggested that cells can acquire properties of cancer stem cells by undergoing EMT (Mani, Guo et al. 2008; Morel, Lievre et al. 2008). It is interesting to see that the D492 breast stem cell line expresses miRNA-203 and -205 that are downregulated in D492M. Thus, downregulation of miRNA-203 and -205 might have more relevance in the creation of cancer stem cells by EMT. MiRNAs interfere / inhibit the expression of genes so it is easier to relate to the effects that downregulated miRNAs have in the EMT process.

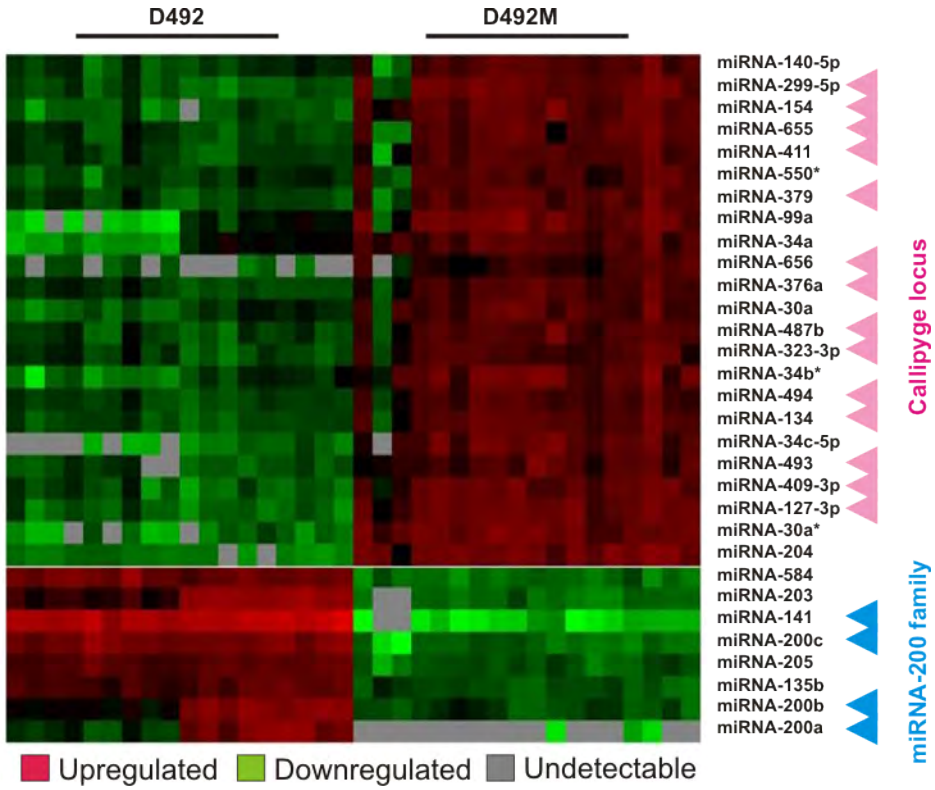


**Figure 15. Specific changes in miRNA expression are involved in the EMT process.**
Heatmap showing 31 most differentially expressed miRNAs in D492 and D492M. The miRNA-200 family (miRNA-200a, -200b, -200c and -141) is downregulated in D492M cells (blue arrows). Of the 23 most upregulated miRNAs, 14 belong to the callipyge locus (pink arrows). Upregulated seen as red, downregulated seen as green and undetectable seen as grey.

However, multiple miRNAs were upregulated in the D492M cells. Most interestingly, there was a marked up-regulation of many members of a miRNA-cluster on chromosome 14q32 (Callipyge locus). Of the 23 most highly upregulated genes 14 belong to this cluster. This miRNA-cluster has not been previously connected with EMT in humans or any other model system. A recent paper, however, has shown that in mice the Dlk1-Gtl2 imprinted cluster, a homolog to callipyge locus in humans, is upregulated in metastasis indicating the importance of this locus in tumor progression (Olson, Lu et al. 2009). Stadtfeld et al. have also shown that a long non coding RNA from this cluster, MEG3, is important for pluripotency of mouse embryonic stem cells (Stadtfeld, Apostolou et al. 2010). Notably, the MEG3 gene is upregulated 3 fold in D492M (not shown). However, the MEG3 gene has also been suggested to be a tumor suppressor gene through interactions with p53 (Zhou, Zhong et al. 2007; Benetatos, Vartholomatos et al. 2011). In the case of D492-D492M they don't have an active p53 nor do over 80% of basal-like breast cancers. Thus, the Callipyge locus,

both miRNAs and MEG3, might be important in the progression of basal-like breast cancer impacting EMT and metastasis. Further studies on this are currently ongoing in our laboratory.

## 4. Sprouty-2 is an important regulator of branching morphogenesis and epithelial integrity in the human breast gland (Paper #4)

In this work I cultured D492 in 3D rBM to explore the molecular mechanism of branching morphogenesis in the breast gland with special focus on the functional role of Sprouty-2 (Spry-2). Branching morphogenesis is a relatively conserved process seen between organs and different species (Davies 2002). Signals through receptor tyrosine kinases (RTKs) play an important role in cell proliferation, differentiation but also in branching (Lu, Sternlicht et al. 2006). Proper signals through the EGFR and FGFR RTK families are essential for the correct development of the breast gland both at puberty and in adult branching associated with pregnancy (Dillon, Spencer-Dene et al. 2004; Zahnow 2006). Sprouty proteins are thought to work in a negative feedback loop, dampening signals through the RTKs by binding Grb, Sos and Raf (Figure 3) (reviewed in (Cabrita and Christofori 2008)). Of the human Sprouty proteins, Spry-2 has the highest and most widespread expression within epithelial tissues (Guy, Jackson et al. 2009). In the breast gland, one study noted higher levels of Spry-2 expression during pregnancy and a lowered expression in breast cancers suggesting a tumor suppressive role (Lo, Yusoff et al. 2004). However, detailed analysis into the function of Spry-2 in branching morphogenesis of the human breast gland has not been done. In this study I first explored the expressional pattern of Spry-2 in the normal human breast gland.

### 4.1 Sprouty-2 is highly expressed in the luminal epithelial cells of the human breast gland

To explore the expression of Spry-2 in the human breast gland I utilized tissue from reduction mammoplasties. I stained paraffin embedded tissues with a Spry-2 antibody and explored the expression of Spry-2 in isolated LEP and MEP cells with q-rt-PCR. In general, the expression of Spry-2 was high within the epithelial compartment compared to the

surrounding stroma. Spry-2 was detectable by immunohistochemistry in all epithelial cells of large ducts and in TDLUs (Figure 1A in Paper #4). Within the stroma, Spry-2 was detected with limited distribution, most likely in endothelial cells (Figure 1A in Paper #4). To verify the integrity of our Spry-2 antibody we also did an in situ hybridization that showed a similar pattern of expression in breast epithelial cells (Figure S1 in Paper #4). By using linage-restricted markers for LEP (K18) and MEP (K14) cells in immunofluorescence costaining I could show that Spry-2 was highly expressed within the luminal compartment in ducts and TDLUs (Figure 1A in Paper #4). To support this observation I isolated primary LEP and MEP cells from three different reduction mammoplasties and performed a quantitative real-time PCR. LEP cells showed 15-58 fold higher Spry-2 expression compared to the lowest expressing MEP cells (Figure 1B in Paper #4). Taken together, Spry-2 expression can be found in epithelial and stromal cells of the normal human breast gland but is predominantly expressed within luminal epithelial cells. However, the expressional pattern within reduction mammoplasties does not show how Spry-2 is expressed in conjunction with branching. For obvious reasons breast tissue from pregnant and lactating women is not readily available. To explore Spry-2 at critical points in breast branching morphogenesis I used mammary glands from virgin, pregnant and lactating mice.

### 4.2 Sprouty-2 expression is increased during pregnancy and lactation in the mouse mammary gland

As described earlier Spry-2 is known to modulate signals through the EGFR RTK. To analyze EGFR signaling and its negative modulator, Spry-2 during critical periods of branching morphogenesis I isolated mammary glands from virgin, pregnant and lactating mice. Expression of phosphorylated EGFR (p-EGFR) was generally low in the virgin mammary gland but increased focally at branching end buds in the pregnant gland. Furthermore, a dramatic increase in p-EGFR was seen in end buds during lactation (Figure 2A in Paper #4). Increased Spry-2 expression was seen during pregnancy that reached its highest level during lactation associated with p-EGFR expression (Figure 2A in Paper #4). During lactation, p-EGFR and Spry-2 expression was inversely correlated with cell proliferation as evidenced by dramatic reduction in cell proliferation (PCNA staining) (Figure 2A in Paper #4). Spry-2 expression was

also accompanied with increased signals through EGFR downstream signaling pathway measured by total- and pospho-ERK, especially during the lactational period (Figure 2B in Paper #4). Thus, increased Spry-2 expression was associated with activation of EGFR signaling and the downstream Erk/MEK pathway. These data suggest that Spry-2 and pEGFR/ERK/MEK signaling may work together to maintain maximal differentiation state during mouse mammary lactation. Interestingly, this also points to a classical negative feedback in the expressional pattern of Spry-2 during pregnancy / lactation. Signals through the EGFR pathway seem to be essential on branching end buds during pregnancy, associated with high proliferation index. When the mammary gland has reached its full differentiation potential during lactation, expression of Spry-2 is increased over 38 fold compared to the virginal state (Figure 2B in Paper #4). In contrast, little or no proliferation is seen during lactation. Signals through the EGFR pathway seem to be at the highest level in lactation so one of the roles of increased Spry-2 expression at this level might be to block further proliferation. In light of this data, expressional pattern of Spry-2 during pregnancy and lactation points to two different roles. Increased expression during pregnancy connected to branching morphogenesis and in the terminal differentiation of end buds during lactation. Next, I wanted to explore the expression of Spry-2 in D492 branching morphogenesis in 3D culture.

### 4.3 Expression of Sprouty-2 correlates with critical points in branching morphogenesis of D492 cells in 3D rBM culture

As previously mentioned, D492 has stem cell properties and through branching morphogenesis it forms TDLU-like colonies when cultured within a 3D rBM (Gudjonsson, Villadsen et al. 2002). D492 cells form branching colonies over a period of 16 days in 3D culture (Figure 16) (Figure 3A in Paper #4). In the beginning D492 cells grow as solid round colonies (up to day 8-9) followed by a branching period during days 8-16. After primary branching, ductal structures elongate and secondary branching appears with bifurcation or trifurcation on the lobular-like ends (Figure 3A in Paper #4).

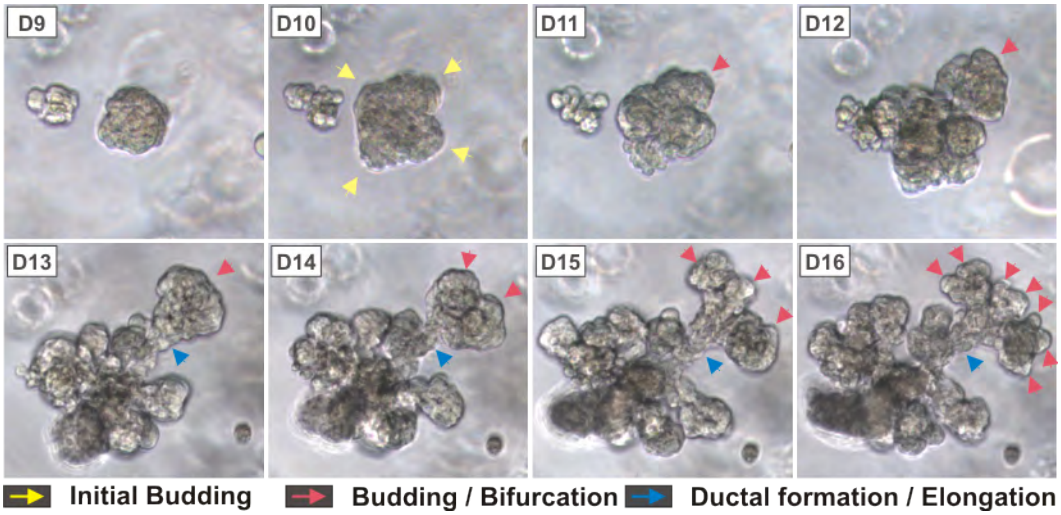


**Figure 16. Branching morphogenesis of D492 occurs in a period of 16 days in 3D rBM culture.**
Until day 8 in 3D culture, colonies grow as solid spheres. On days 8-10 initial budding starts followed by more budding, ductal formation and elongation. On the ends of branching ducts further bifurcation and trifurcation occurs resulting in a TDLU-like colony.

Spry-2 expression was analyzed by qRT-PCR on 3D culture days, 8, 10, 12, 14 and 16. Pre-branching, round colonies showed moderate expression of Spry-2 (Figure 3B in Paper #4). Interestingly, during the formation of primary branching (days 10-12) the expression of Spry-2 decreases. At day 16 TDLU-like structures have formed and the expression of Spry-2 was increased to more than 4fold compared to day 10 (Figure 3B in Paper #4). Spry-2 expression was also confirmed with a western blot on D13, D16 and D19 showing an increase in Spry-2 from D13 to D16 and constant high expression on D19. Interestingly, immunoblotting also showed that p-EGFR is increased from D13 to D16 but is lowered on D19 when further branching has stopped (Figure 3C in Paper #4). This expression pattern suggests that Spry-2 has a regulatory role during the temporal formation of branching structures Furthermore, this pattern is similar to my data with the mouse mammary gland. In support of this, Immunofluorescence staining of branching colonies at day 16 showed that Spry-2 expression was mainly concentrated at the branching, lobular-like tips but lowered at sites of cleft formation (Figure 17) (Figure 3D in Paper #4).

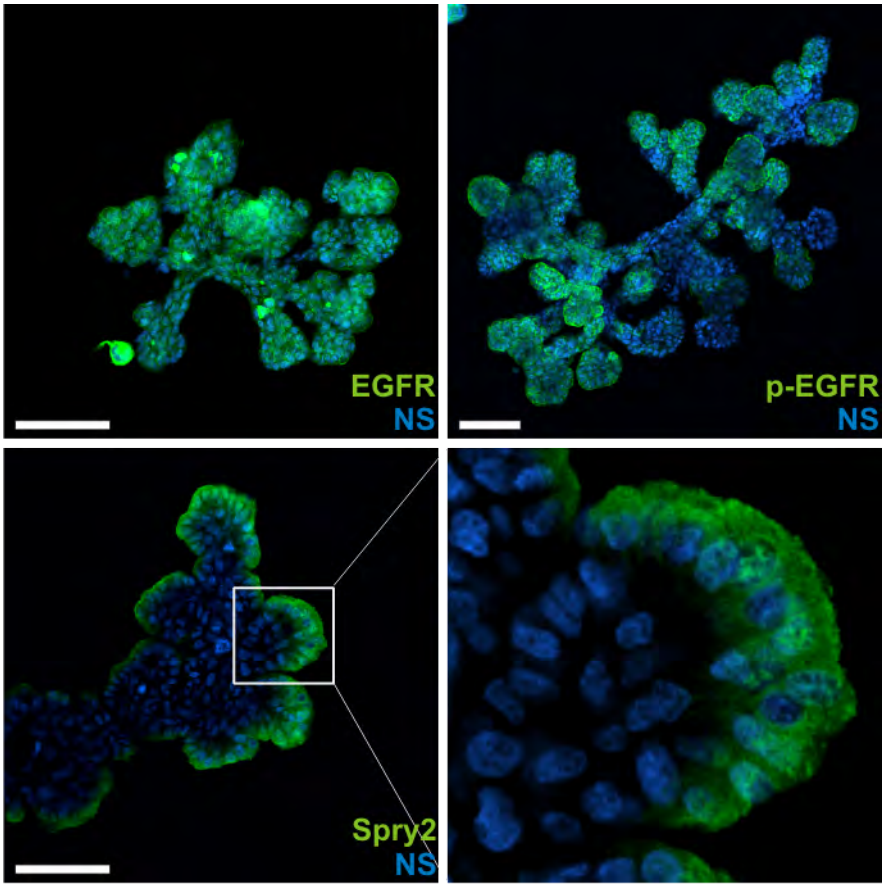


**Figure 17. Sprouty-2 is most prominently expressed on branching end buds in 3D rBM culture of D492.** Immunofluorescence staining of branching D492 colonies at day 16 in 3D culture shows a general distribution of total EGFR in most cells. In contrast, p-EGFR is mostly located at the branching end buds where active branching is still present. A similar location is seen for Sprouty-2 at the branching end buds although not in clefts. Immunoreactivity seen as green. Counterstained with TOPRO3 (blue). Bar=100μm.

The location of Spry-2 at day16 was similar to the expression of p-EGFR at branching tips while staining for total EGFR showed a more general distribution in the branching colonies (Figure 17) (Figure 3D in Paper #4). Staining for F-actin gives a general outline of a branching structure and β4-integrin the connection to the surrounding basement membrane (Figure 3D in Paper #4). In conclusion, the spatial and temporal expressional pattern of Spry-2 during in vitro branching morphogenesis points to a specific role in the branching process. The highest expression is seen at late branching localized to peripheral branching buds and significantly reduced in clefts at the same location. This expressional pattern is similar to what I saw in the pregnant and lactating mouse mammary gland. In conclusion, this data strongly indicates that the D492 cell line can generate branching TDLU-like colonies that

capture critical pathways of in vivo branching morphogenesis. This involves signals through the EGFR pathway and the negative feedback of Spry-2. To further explore the effects of Spry-2 on branching morphogenesis I wanted to manipulate the expression of Spry-2 in the D492 cell line.

### 4.4 Knocking down Sprouty-2 in D492 cells affects migration but not proliferation

In light of the interesting expressional pattern of Spry-2 during branching I wanted to explore the functional role of Spry-2 by knocking it down (KD) in D492 cells. In the effort to produce stable Spry-2 KD cell lines I used a lentiviral approach. I transfected D492 with a non-silencing control (GFP) and 3 different shRNA constructs (Spry-$2^{KD-1}$, Spry-$2^{KD-2}$ and Spry-$2^{KD-3}$) targeting Spry-2. The Spry-$2^{KD-3}$ construct was most effective, decreasing Spry-2 levels 4 fold (Figure 4A-B in Paper #4). Continuing work focused on this knock down line and a single cell subclone called Spry-$2^{KD-3a}$. No morphological difference was seen between NS cells and KD cells when visualized in monolayer (Figure 4C in Paper #4) but D492$^{Spry-2-KD3}$ and D492$^{Spry-2-KD3a}$ both showed increased migration compared to D492$^{NS}$ cells (Figure 4D in Paper #4). However, there was no significant difference in the proliferation of D492$^{Spry-2-KD3}$ and D492$^{Spry-2-KD3a}$ compared to D492$^{NS}$ cells (Figure 4E in Paper #4). Having established the D492 Spry-2 KD cells I wanted to explore the morphogenic potential of these cells in 3D rBM cultures.

### 4.5 D492-Sprouty-2 KD cells show increased branching morphogenesis in 3D rBM culture

To analyze the effects of Spry-2 KD on branching morphogenesis I compared the cell lines in 3D cell culture. D492$^{Spry-2-NS}$ generated *in vivo*-like 3D branching colonies, similar to the original D492 cells, while D492$^{Spry-2-KD3}$ and D492$^{Spry-2-KD3a}$ showed an overall increased branching potential (Figure 18) (Figure 5A in Paper #4). The effect of Spry-2 KD was quantified by counting colonies of complex/late branching, simple/early branching and other (mostly solid colonies) morphology (Figure 5B in Paper #4). In a setup with $1{\cdot}10^4$ cells both Spry-2 KD cell lines formed significantly more branching colonies in total and substantially more colonies that showed the complex branching phenotype in comparison

to NS cells (Figure 5C in Paper #4). Large complex colonies, >250µm, increased twofold in both Spry-2 KDs cell lines compared to NS cells (Figure 5D in Paper #4). $D492^{NS}$ and $D492^{Spry\text{-}2\text{-}KD3}$ Cells were also tested in three different cell concentrations $1.3{\cdot}10^4$, $1{\cdot}10^4$ and $7{\cdot}10^3$ cells. This was done because the degree of branching changes with different number of cells seeded in the 3D rBM.

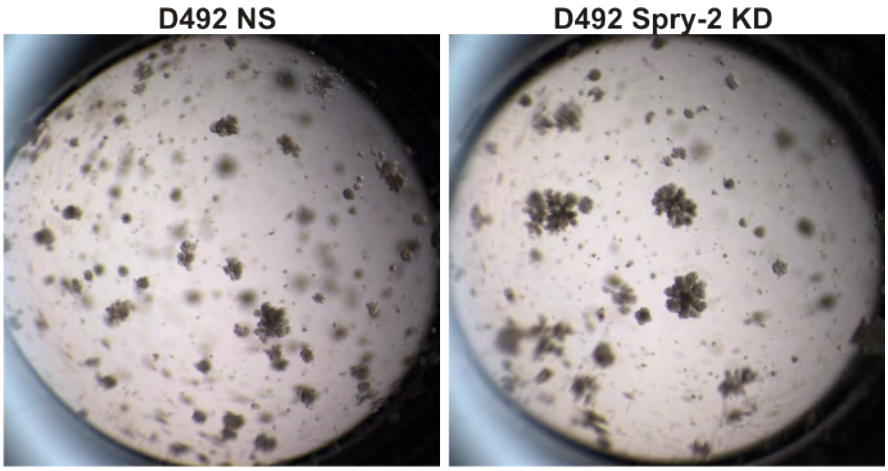


**Figure 18. Sprouty-2 knock down results in large colonies with a complex branching phenotype.**
*Left.* In a setup of $10^4$ cells in 300µl of rBM D492 NS cells form a moderate amount of colonies showing branching morphogenesis. *Right.* When D492 Spry-2 KD cells are used in the same setup an increase in branching is observed, especially in the formation of large complex colonies.

This is most likely caused by geometric /spatial inhibition of adjacent branching colonies so if the KD cells form larger colonies they might have inhibiting effects on other colonies. Less branching was seen in cultures with higher cell density, however, the $D492^{Spry\text{-}2\text{-}KD3}$ cells formed more branching colonies in all setups (Figure 5E in Paper #4). When I looked at the expression of Spry-2 at D16 in $D492^{NS}$ and $D492^{Spry\text{-}2\text{-}KD3}$ cells I could see that the NS cells showed normal expression of Spry-2 at lobular-like tips while the KD cells showed little or no expression at the same location (Figure 5F in Paper #4). Thus, by suppressing Spry-2 in a cell line that has unique abilities to generate branching structures, their morphogenesis potential increases substantially. To further explore the role of Spry-2 in branching morphogenesis and EMT I cocultured D492 Spry-2KD cells with BRENCs.

### 4.6 D492 Sprouty-2 KD cells show propensities towards EMT-like colony formation

When endothelial cells are cocultured with epithelial cells they support morphogenesis and improve clonal efficiency of epithelial cells as described above (Paper #2) (Franzdottir, Axelsson et al. 2010). Furthemore, I have seen that in coculture, breast endothelial cells (BRENCs) induce breast epithelial cells (D492) to undergo EMT (Paper #3). In addition, when D492 cells are cocultured with BRENCs I see a marked stimulation in branching morphogenesis. Branching TDLU-like structures in coculture can be generated from as few as 100 D492 cells in 300 µl rBM compared to the usual amount of $7x10^3$ – $10^4$ cells used in monoculture in this assay (Figure 6 in Paper #4). Thus, D492 cells have increased clonal production of branching colonies in addition to higher percentage of colonies showing the complex branching phenotype in coculture with BRENCs. However they also have a tendency to undergo epithelial to mesenchymal transition (Paper #3) (Figure 6B in Paper #4). I seeded our D492 NS and D492 Spry-2 KD cells in 3D coculture with BRENCs. Similar to what I have seen before $D492^{NS}$ cells formed 40% EMT-like colonies and 26% branching colonies. In contrast, $D492^{Spry\text{-}2\text{-}KD3}$ cells formed over 53% EMT-like colonies and only 7% of the total colonies showed branching in coculture (Figure 6C in Paper #4). The $D492^{Spry\text{-}2\text{-}KD3a}$ clone, which produced larger and higher number of branching colonies in the monoculture, was also used in coculture. Interestingly, they almost exclusively produced EMT-like colonies (>93%) (Figure 6C in Paper #4). This data suggests that Spry-2 KD cells have a compromised epithelial integrity and when put under pressure as in the coculture they are even more predisposed to undergo EMT.

# GENERAL DISCUSSION AND CONCLUSION

Organ development as well as tissue maintenance is under precise cellular and molecular regulation. This control depends on heterotypic interaction between tissue-specific cells and the surrounding stromal compartment. In the breast gland, these interactions are instrumental for correct histology, functional differentiation and homeostasis. Endothelial cells are emerging as important players in organ development and as regulators of stem cell niches (Butler, Kobayashi et al. 2010). Their role in development and maintenance of the breast gland is, however, relatively unknown.

In the present thesis I have explored the effects of breast endothelial cells (BRENCs) on breast morphogenesis using organotypic 3D cultures. In addition, I have also studied the role of Sprouty-2 in branching morphogenesis. My initial efforts focused on isolation and propagation of human breast endothelial cells and exploration of their role in heterotypic signaling to normal and malignant breast epithelial cells from the human breast gland. In 3D coculture, I was able to show that BRENCs are potent inducers of breast epithelial proliferation and morphogenesis of normal and malignant cells. In transwell coculture I was able to show that these effects were mediated through soluble factors. Using a human breast epithelial cell line (D492) with basal and stem cell properties, I showed that BRENCs induced formation of single cell derived, TDLU-like, branching structures. Furthermore, BRENCs were able to induce D492 cells to undergo non-reversible epithelial to mesenchymal transition (EMT). EMT derived D492 cells (D492M) acquired cancer stem-like characteristics with increased growth in non-adherent cell culture, $CD44^{High}$ /$CD24^{Low}$ profile and apoptosis resistance. HGF signaling from BRENCs was, at least partially, responsible for these EMT inducing effects. I also showed that in proximity to endothelial rich areas in basal-like breast cancer tumor cells have increased expression of the EMT associated marker N-cadherin and reduced epithelial characteristics. This means that BRENCs/ endothelial cells are possible effectors in the metastasis process through paracrine signaling. In attempts to explore the intrinsic regulation of branching and EMT I studied Sprouty-2 (Spry-2), a RTK negative regulator. I showed that Spry-2 was instrumental in the control of branching morphogenesis and in the maintenance of epithelial integrity. Spry-2 was highly expressed during critical points in branching morphogenesis and when it was

knocked down, hyperplastic-like phenotype and abnormal branching was observed. In coculture with BRENCs D492 Spry-2 KD cells showed increased abilities to undergo EMT, indicating that Spry-2 may be an important regulator of epithelial integrity in the mammary gland.

**Epithelial to mesenchymal transition**

My initial aim was to study the effects of BRENCs on breast morphogenesis. To my surprise I also observed that BRENCs are active facilitators of the EMT process in D492. This suggests that endothelial cells may provide a favorable environment for the emergence of the EMT phenotype. In support of this, recent reports have linked EMT to the basal-like subtype of breast cancer, demonstrated by upregulation of EMT markers (vimentin, smooth-muscle-actin, Fibronectin and N-Cad) together with reduction of characteristic epithelial markers (E-Cad and keratins) (Mahler-Araujo, Savage et al. 2008; Sarrio, Rodriguez-Pinilla et al. 2008) (Brabletz, Bajdak et al. 2011). This is in line with my data that basal-like breast cancers contain a characteristic EMT phenotype. However, my results that the EMT phenotype is strongest close to vascular rich areas is novel. This directly connects endothelial cells in the emergence of the EMT phenotype and thus in the metastasis process in a new way.

In general, Intratumoral angiogenesis, assessed by microvessel density, has been proposed to identify patients at high risk of recurrence, especially in node-negative breast cancer (Uzzan, Nicolas et al. 2004). Interestingly, recent studies have shown that microvessel density might be a major risk factor in triple negative breast cancer and levels of vascular endothelial growth factor (VEGF), a marker of angiogenesis, has also been shown to be significantly higher in this subclass(Linderholm, Hellborg et al. 2009; Miyashita, Ishida et al. 2011). In the same way, higher MVD has also been associated with breast tumors with a predominant $CD44^{high}/CD24^{low}$ cancer stem cell profile (Lopes, Sousa et al. 2009; Giatromanolaki, Sivridis et al. 2010). In addition, Niu et al. have showed that hepatocellular carcinomas expressing Twist, have significantly higher MVD (Niu, Zhang et al. 2007). However, Twist and Snail overexpressing EMT cells have also been shown to mediate higher angiogenesis activity in mouse xenografts (Peinado, Marin et al. 2004; Mironchik, Winnard et al. 2005). In this respect, one might conclude that cancer cells with an EMT phenotype

induce angiogenesis, explaining the apparent higher MVD associated with the EMT phenotype. On the other hand, endothelial cells are key components of stem cell niches in the brain and the bone marrow mediating self-renewal, differentiation and normal homeostasis in addition to their involvement in developmental organogenesis (reviewed in (Butler, Kobayashi et al. 2010)). Therefore, in my opinion, it is likely that this is a heterotypic crosstalk between tumor cells and endothelial cells leading to increased EMT and angiogenesis thus facilitating the metastasis process. In conclusion, the EMT phenotype observed in basal-like breast cancers in connection to my data and others shows an interesting relationship between EMT and angiogenesis. This brings breast endothelial cells into the spotlight and makes anti-angiogenesis therapy more relevant in the basal-like breast cancer subtype where limited clinical options are available

Defining the cellular and microenvironmental cues that trigger EMT during cancer progression is important. Studies have shown increased expression of EMT markers at the tumor-stroma interface and stromal cells are now recognized as major players in cancer progression (Brabletz, Jung et al. 2001; Franci, Takkunen et al. 2006; Ronnov-Jessen and Bissell 2009). Most studies on EMT have been conducted on cultured cells due to difficulties in identifying these cells in situ. There are numerous factors that can elicit EMT in cell culture. Of these, TGF-β1 and receptor tyrosine kinase ligands such as EGFs, FGFs, VEGFs, PDGFs and HGF have received much attention (Sabbah, Emami et al. 2008). In this thesis I show that inducing D492 with TGF-β1 or inhibiting TGF-β1 or the ALK5 receptor did not affect the formation of spindle-like colonies in 3D cocultures. This suggests that TGF-β1 is not necessarily involved in the endothelial induced EMT in the 3D-context. Moreover, the inhibition of TGF-β1 signaling resulted in restricting the branching morphogenesis process. These results are in contrast to the partial EMT induction that I observed after TGF-β1 treatment on D492 in monolayer cell culture. The fact that TGF-β1 signaling has differential effects in 2D and 3D culture environment demonstrate the importance to explore EMT in a physiologically relevant setting like the 3D rBM cultures. By inhibiting the c-MET ligand, HGF, I was able to reduce the emergence of spindle-like, EMT, colonies significantly in the 3D cocultures. I could also confirm that the BRENCs were secreting HGF in 3D culture so HGF was, at least partially, responsible for EMT in the coculture model. Interestingly, high levels of HGF and c-MET have been associated with invasive human breast cancer, metastasis and

more recently to the basal-like subtype (Elliott, Hung et al. 2002; van Zijl, Mair et al. 2009). Furthermore, Mostov et al have reported that HGF induces partial EMT in MDCK cells cultured in 3D collagen gels (Leroy and Mostov 2007). In this regard, I have seen that BRENCs can also induce an EMT phenotype in MDCK cells. Similar to my results with BRENCs, endothelial cells have previously been shown to express and secrete HGF in vitro and in vivo (Nakamura, Morishita et al. 1995). Furthermore, LeCouter et al. showed that endothelial derived HGF can stimulate hepatocyte proliferation in coculture and that VEGF stimulated this process (LeCouter et al 2003). Interestingly, there might also be a connection to hypoxia in our cocultures as it is likely that oxygen tension is somewhat lower within 3D cell culture. In support of these speculations, cell culture within 3D collagen hydrogels results in intracellular hypoxia of MDA-MB231 breast cancer cells with upregulation of HIF-1$\alpha$ and VEGF in contrast to monolayer culture (Szot, Buchanan et al. 2011). Hypoxic responses are mediated mainly through HIF-1$\alpha$ and there are hypoxia response elements within the promoter region of the VEGF gene (Forsythe, Jiang et al. 1996). Increased VEGF production is thought to induce angiogenesis in response to hypoxia (reviewed in (Cassavaugh and Lounsbury 2011)). However, hypoxia has also been shown to increase the stability of HGF mRNA and the expression of c-Met, leading to increased sensitivity to HGF (Pennacchietti, Michieli et al. 2003; Chu, Feng et al. 2009). However, the coculture model described in this thesis is limited in the way that it only allows for the exploration of phenotypic traits of the epithelial cells used in coculture while the endothelia cells do not show angiogenesis activity. Nevertheless, my findings suggest a novel role for endothelial cells and angiogenesis in cancer progression in addition to the more classical role of oxygen and nutritional delivery. In addition, HGF /c-MET signaling might be an interesting therapeutic target within basal-like breast cancer

Although EMT can easily be recognized in monolayer culture of cells, recognizing these cells *in situ* is more troublesome, due to its transient nature. In contrast to monolayer cultures, 3D culture models capture more closely the *in vivo* situation (Lee, Kenny et al. 2007). Studies have shown the importance of 3D cultures to elucidate the functional role of the stroma as an instructive factor in normal breast morphogenesis and cancer progression (Weaver, Fischer et al. 1996; Gudjonsson, Ronnov-Jessen et al. 2002; Lee, Kenny et al. 2007; Ronnov-Jessen and Bissell 2009). Numerous cell lines, such as MCF10A and MCF-7, have been

reported to be susceptible to EMT in traditional monolayer culture (Blick, Widodo et al. 2008; Blick, Hugo et al. 2010). My results, however, show that in 3D culture EMT induction by BRENCs is only achieved in some breast epithelial cell lines, i.e. those harboring stem/progenitor characteristics (D492). I also show that primary metaplastic breast cancer cells, W2330, can be facilitated to undergo EMT in 3D coculture with BRENCs. In contrast, the luminal epithelial cell line D382, the basal-like cell line MCF10A and the ER positive cancer cell line MCF-7 show no signs of EMT in coculture with BRENCs. In light of my results, response to endothelial derived HGF seems to be important for the emergence of the EMT phenotype. In addition, the EMT phenotype seems to be enriched within basal-like breast cancer and breast cancer cell lines with a basal phenotype (Charafe-Jauffret, Ginestier et al. 2006; Sarrio, Rodriguez-Pinilla et al. 2008). In light of that it is not surprising to see that MCF-7 cells show no signs of EMT although they clearly respond to growth stimuli from the BRENCs (Article #2). Even though MCF10A has been shown to have a basal-like phenotype and to readily undergo EMT after TGF-β stimulation, they lack fundamental stem cell properties that D492 has, such as branching morphogenesis within 3D rBM (Blick, Hugo et al. 2010). This may explain why MCF10A cells are non-responsive to endothelial induced EMT in the 3D cocultures.

Recent studies have shown that induction of EMT in immortalized human breast epithelial cells was associated with acquisition of cancer stem cell associated properties (Mani, Guo et al. 2008; Morel, Lievre et al. 2008). In these studies, immortalized breast epithelial cells (HMECs) were induced to undergo EMT in 2D culture conditions with TGF-β1 or transfected with potent inducers of EMT. These studies are in line with my data where D492M shows cancer stem cell and tumorigenic phenotype as evidenced by an increased ratio of $CD44^{high}/CD24^{low}$ cells, ability to form mammospheres, increased motility, anchorage independent growth and resistance to chemically induced apoptosis. There are numerous transcription factors (TFs) that influence the EMT phenotype such as Twist, Snail, ZEB1 and 2, FOXC1 and 2 (Thiery, Acloque et al. 2009). The only EMT associated TFs that I could indentify as significantly upregulated by microarray were FOXC1 and FOXC2. These factors have proven to be potent inducers of the mesenchymal phenotype of EMT cells and their expression in breast cancer correlates to the basal-like subtype (Mani, Yang et al. 2007; Taube, Herschkowitz et al. 2010). The other TFs such as Twist, Snail and ZEBs have been

shown to be important in the downregulation of epithelial characteristics such as suppression of E-cadherin expression (reviewed in (Yang and Weinberg 2008)). The most likely reason why I don't see an upregulation of these TFs is because they are transiently expressed during the switch in phenotype. However, the expression array was done on D492M cells after EMT conversion. Increased expression of EMT associated TFs also leads to the transition of cells towards the flow cytometry profile $CD44^{high}/CD24^{low}$. At first sight it is not very clear how this profile relates to cancer stem cells and EMT. In breast epithelial cells, CD24 can be used as a measurement of epithelial integrity, which is obviously lacking in EMT cells but also in breast cancer stem cells (Al-Hajj, Wicha et al. 2003; Sleeman, Kendrick et al. 2006; Mani, Guo et al. 2008). In contrast, increased CD44 expression in cancer cells has been connected to increased adhesion, motility, proliferation and cell survival (Marhaba and Zoller 2004; Afify, Purnell et al. 2009). These features are all connected to cancer progression so high expression of CD44 is thought to be important in cancer stem cells and in facilitating the metastatic cascade (Giatromanolaki, Sivridis et al. 2010). In my coculture studies, endothelial derived HGF is partly responsible for the EMT phenotype of D492M cells. Interestingly, CD44 isoforms bearing heparin sulfate chains have been described as functional co-receptors by binding HGF and facilitating its presentation to c-MET (van der Voort, Taher et al. 1999; Wielenga, van der Voort et al. 2000). Other studies have also noted that HGF stimulation can both upregulate CD44 and the c-MET receptor (Recio and Merlino 2003). In this respect, my findings are even more relevant where endothelial derived HGF results in the $CD44^{high}/CD24^{low}$ profile and increased EMT phenotype. This also suggests that cancer cells within the basal like subtype may be more prone to undergo EMT due to their origin in stem / progenitor cells of the breast having a similar marker expression as the D492 cell line. Interestingly, in my study D492M appears to lose it's normal epithelial "stemness" such as the ability to generate differentiated luminal and myoepithelial cells and to form branching TDLU-like structures in 3D rBM culture. However, after EMT induction they acquire a phenotype associated with breast cancer stem cells. This suggests a fundamental difference between the properties of breast epithelial stem cells and cancer stem cells that is not so obvious in regular monolayer cell culture but can be identified by the use of 3D rBM cultures.

**Branching morphogenesis and Sprouty-2**

Sprouty proteins have been identified as novel antagonists of RTKS such as FGFR, c-Met and EGFR in lungs, kidneys and vasculogenesis but their expression and role in morphogenesis of the human breast gland has not been analyzed in detail. My initial work focused on the expression of Spry-2 in the normal human breast gland. I could show that luminal epithelial cells in ducts and lobules express higher levels of Spry-2 compared to myoepithelial cells. Lo et al. have showed previously that Spry-1 and Spry-2 genes are suppressed in breast cancers suggesting a tumor suppressor role and that Spry-2 is expressed in the normal breast gland (Lo, Yusoff et al. 2004). Using the mouse mammary gland I show that the levels of Spry-2 are low in virgin mice, increase in pregnancy but reach their highest point during lactation. Lo et al. have also explored the expression of Spry-2 in virgin, pregnant and lactating mammary glands by in situ hybridization. Their results showed increased Spry-2 expression during pregnancy and reduced expression in lactation (Lo, Yusoff et al. 2004). The reason for the difference between my data and the paper published by Lo et al. is unknown. Possible explanation could be the different time points where the expression is measured (in my case day 2 of lactation), different methodology (In situ vs Immunocytochemistry) or different mouse strains. However, as mentioned earlier, I have validated the specificity of the sprouty-2 antibody with in situ hybridization and this method gives similar results. It is possible that at day 2 of lactatition branching morphogenesis is still active explaining the Spry-2 expression or that Spry-2 is important in stopping further branching to occur. Spry-2 might also be important in the differentiation of luminal epithelial cells into fully differentiated, lactating cells. However, we need to do further experiments to explore these possible roles of Spry-2.

To explore Spry-2 expression in branching morphogenesis I used the D492 cell line and 3D cell culture. Spry-2 was analyzed by qRT-PCR on critical time point in the branching morphogenesis process (Day 8 – Day 16). Pre-branching, round colonies showed moderate expression of Spry-2 but during the formation of primary branching (days 10-12) the expression of Spry-2 decreases. At day 16 elaborated TDLU-like structures have formed and the expression of Spry-2 reaches its highest point. This expression pattern suggests that Spry-2 may have a regulatory role during the temporal formation of branching structures and the formation of lobular units at the ductal ends. Furthermore, this pattern is similar to

my observations in the mouse mammary gland where a constant high expression of Spry-2 is seen when branching morphogenesis has completed in the lactating gland. In support of this, immunofluorescence staining of branching colonies at day 16 shows that Spry-2 expression is mainly concentrated at the branching, lobular-like tips but lowered at sites of cleft formation. The location of Spry-2 at day16 is similar to that of p-EGFR at branching tips while staining for total EGFR has a more general distribution in the branching colonies. Interestingly, this same expressional pattern of Spry-2 at the ends of branching tips has also been seen in the development of the mouse and chicken lung (Warburton, El-Hashash et al. 2010; Moura, Coutinho-Borges et al. 2011). The expression of Spry-2 seems to be at the highest level on the same location as activated EGFR signaling, seen by staining for p-EGFR. Interestingly, through D16-D19, when further branching has stopped, p-EGFR levels decrease but the expression of Spry-2 is still high, possibly hindering further branching.

In light of the expressional pattern of Spry-2 during branching I wanted to explore the functional role of Spry-2 by knocking it down (KD) in D492 cells. As the classical role of Spry-2 is to dampen signals downstream of RTKs one would expect that the Spry-2 KD cells would show increased proliferation. Spry-2 KD in D492 had no effects on cell proliferation in monolayer. This might be explained by the method in which the D492 cell line was established originally, a retroviral vector containing E6 and E7 viral oncogenes. By knocking out functional p53 and RB proteins the D492 cells are immortal and have an unlimited cell proliferation potential with limited checkpoint control. So the inhibiting effects of Spry-2 on proliferation in monolayer culture could be outweighed by the proliferation potential of D492. Another possibility is that anti-proliferative effects of Spry-2 are dependent on intact p53 and/or RB. Saifudeen et al. have actually seen that p53 deficiency releases inhibition of migration and proliferation signals in the developing uteric bud resulting in abnormal branching although they did not see a change in sprouty expression in that context (Saifudeen, Dipp et al. 2009). In contrast to unchanged proliferation, I saw significantly increased migration abilities in Spry-2 KD cells. Others have actually shown that Spry-2 can effect migration of cells in culture (Lee, Putnam et al. 2004). Yigzaw et al. demonstrated that anti-migrational effects of Spry-2 were mediated through the protein tyrosine phosphatase 1B (PTP1B) (Yigzaw, Poppleton et al. 2003). However, my initial results were obtained in monolayer cell culture. When D492 Spry-2 KD cells were cultured in 3D rBM I saw an

increased branching morphogenesis potential. In fact, this resulted in hyperplastic like, uncontrolled branching morphogenesis. This abnormal branching is in line with other studies focusing on Sprouty proteins and branching morphogenesis. The first paper on drosophila Sprouty and branching by Hachohen et al. showed that when it was missing excess tracheal branching occurred (Hacohen, Kramer et al. 1998). Since then, numerous studies in mouse models have confirmed the role of Spry-2 and other sprouty proteins as important regulators of branching morphogenesis in the lung, uteric bud and in the vascular system as described earlier in this thesis (reviewed in (Guy, Jackson et al. 2009)).

As Lo et al. described Spry-2 as a possible tumor suppressor in breast cancer I wanted to know if Spry-2 KD would affect EMT. To that means I used the coculture model described in Paper #3 that gives the opportunity to explore if Spry-2 KD has an impact on epithelial integrity. Indeed, D492 Spry-2 KD cells showed a substantial increase in the formation of spindle-like EMT colonies in 3D coculture with BRENCs, suggesting a compromised epithelial integrity. Additionally, I had already seen hints of this in monolayer culture where D492 Spry-2 KD cells showed increased migration. It is not unlikely that this is due to the loss of Spry-2 induced negative feedback on signals through RTKs. In Paper #3 I have shown that endothelial derived HGF is one of the factors that can influence the emergence of EMT-like colonies in coculture. Interestingly, Lee et al. showed that Spry-2 has the same negative modulation effects on HGF receptor c-MET and its downstream signaling (Lee, Putnam et al. 2004). C-MET is a powerful RTK which is increasingly being recognized as an important player in triple negative and basal-like breast cancers (reviewed in (Gastaldi, Comoglio et al. 2010). In fact, branching morphogenesis and EMT are not as far from each other as one might think. Epithelial cells need to be able to transiently activate critical EMT associated transcription factors and mesenchymal properties to be able to invade the surrounding matrix in branching morphogenesis (Lee, Gjorevski et al. 2011). It is likely that successful branching is dependent on a partial EMT process under tight control from morphogenic signals, intrinsic factors such as Spry-2 and the surrounding microenvironment.

**The role of EMT in branching morphogenesis**

Remodeling and flexibility in the epithelial nature seems to be important during branching morphogenesis. Cells located at the leading edge of an elongating epithelial tube show loss of polarity, increased cell protrusion and migration. This process is accompanied by acquisition of transient but reversible EMT phenotype (Andrew and Ewald 2010). Additionally, most of the growth factors that are known to be potent EMT inducers such as HGF, FGFs, EGFs are also potent inducers of branching (Fata, Werb et al. 2004). Furthermore, microarray analysis of the branching mouse mammary gland indicate that EMT transcription factors such as Snail and Twist, are upregulated in the branching end buds compared to ducts (Kouros-Mehr and Werb 2006). EMT regulators have been implicated in branching morphogenesis of different organs such as the mammary gland and the salivary gland and in 3D experimental models of branching (Kouros-Mehr and Werb 2006; Leroy and Mostov 2007; Onodera, Sakai et al. 2010). Recently, Lee et al showed that EMT transcription factors Snail1 and Snail2 are upregulated at branch sites, mediating partial-EMT, in an experimental model of mammary branching (Lee, Gjorevski et al. 2011). Experiments have also shown that a localized repression or redistribution of E-cadherin is a feature of partial-EMT seen at cleft sites of the branching salivary gland (Sakai, Larsen et al. 2003; Onodera, Sakai et al. 2010). This repression or dislocalization of E-cadherin has also been seen in the mammary gland and in the prostate during branching(Xue, Smedts et al. 2001; Lee, Gjorevski et al. 2011). Additionally, Leroy et al. showed that the distribution of E-cadherin changes in branching MDCK cells without a decrease in the total amount (Leroy and Mostov 2007). Alternatively, a complete loss of E-cadherin disrupts epithelial integrity and promotes the individual migration of cells leading to disorganized structures such as in full EMT. In addition to partial-EMT, epithelial cell plasticity has also been described as a process called collective migration. This describes the movement of epithelial cells as a group while they are still somewhat connected and has been shown to occur in mammary gland morphogenesis (Ewald, Brenot et al. 2008). Although cell-cell junctions connect epithelial cells undergoing collective migration this process has many similarities to EMT such as alteration of polarity, modification of the extracellular matrix and the acquisition of a motile phenotype (Ewald, Brenot et al. 2008). These similarities suggest that the partial-EMT and collective migration are not totally independent processes, instead they represent

different stages in the spectrum of epithelial-mesenchymal properties. The data on partial-EMT and collective migration fits nicely to my observations with Spry-2 in branching and EMT. Interestingly, when I knocked down Sprouty-2 in D492 cells they showed increased abilities to undergo EMT. In that respect, Sprouty-2 seems to be an important part of both the branching process and in maintaining epithelial integrity. Additionally, these observations further support a role for partial-EMT in the branching process as I could influence the balance of branching and EMT by Spry-2 KD and thus a loss in important negative feedback control.

**Concluding remarks and future perspcetives**

In this thesis I have explored the impact of breast endothelial cells on breast morphogenesis including EMT. I have also analyzed the expression pattern of Sprouty-2 in human and mouse mammary gland and studied how downregulation of this protein affects breast morphogenesis. I have shown that BRENCs stimulate proliferation and morphogenesis of normal and cancerous breast epithelial cells in 3D rBM culture. Coculture of BRENCs and D492, a breast epithelial stem cell line, resulted in increased branching morphogenesis and in the transition towards a mesenchymal phenotype. My data indicate that BRENCs may play an important role in the stem cell niche in the human breast gland. The EMT inducing effects were, at least partially, mediated through endothelial derived HGF. *In situ* studies showed that basal-like breast cancers contain areas with an EMT phenotype at the invading front close to microvascular-rich areas. To explore expressional changes of Sprouty-2 at critical points of branching morphogenesis I used mammary glands of virgin, pregnant and lactating mice. Sprouty-2 expression was upregulated during pregnancy but reaching its highest level during lactation and this expression was accompanied by expression of pEGFR/Erk/MEK pathway. Interestingly, similar effects were seen in D492-dervied branching structures in 3D rBM culture. When Sprouty-2 was knocked down in D492, marked increase was seen in branching in 3D culture further demonstrating the regulatory role of sprouty. Interestingly, I could show that Sprouty-2 KD cells had a compromised epithelial integrity as they were more prone to undergo epithelial to mesenchymal transition in coculture with endothelial cells.

My data indicates that anti-angiogenesis and anti HGF/c-MET therapy might be a relevant choice for future treatment within basal-like breast cancers. However, additional endothelial-derived factors might be involved in the coculture so a more comprehensive analysis must be conducted in vitro and in vivo in the near future. Additionally, Sprouty-2 seems to be critical for breast epithelial integrity as well as in the control of branching. Effects of the other human Sprouty proteins (Sprouty-1-3-4) have not been analyzed in this thesis but they might also be involved. It would be of interest to look closer at the expression levels of Sprouty-2 within the basal-like breast cancers and see if their absence is a marker for poor prognosis. Finally, my preliminary observation that miRNA expression is different between D492 and D492M suggest their role in epithelial integrity and needs to be studied further, especially the Callipyge locus with its associated miRNA cluster are of particular interest. The functional role of the Calipyge locus is now ongoing at the laboratory.

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# ORIGINAL PAPERS

# Paper #1

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# HUMAN BREAST MICROVASCULAR ENDOTHELIAL CELLS RETAIN PHENOTYPIC TRAITS IN LONG-TERM FINITE LIFE SPAN CULTURE

VALGARDUR SIGURDSSON, AGLA J. R. FRIDRIKSDOTTIR, JENS KJARTANSSON, JON G. JONASSON, MARGRET STEINARSDOTTIR, OLE WILLIAM PETERSEN, HELGA M. OGMUNDSDOTTIR, AND THORARINN GUDJONSSON[1]

*Faculty of Medicine, University of Iceland (V. S., H. M. O., T. G.), Molecular and Cell Biology Research Laboratory, Icelandic Cancer Society (V. S., A. J. R. F., H. M. O., T. G.), Reykjavik, Iceland, Structural Cell Biology Unit, Department of Medical Anatomy-A, The Panum Institute, University of Copenhagen, Denmark (A. J. R. F., O. W. P.), St. Josefs Hospital, Iceland (J. K.), Department of Pathology, University Hospital of Iceland (J. G. J.), Chromosome Laboratory, Department of Genetics and Molecular Medicine, University Hospital of Iceland (M. S.)*

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## SUMMARY

Attempts to study endothelial–epithelial interactions in the human breast have been hampered by lack of protocols for long-term cultivation of breast endothelial cells (BRENCs). The aim of this study was to establish long-term cultures of BRENCs and to compare their phenotypic traits with the tissue of origin. Microvasculature was localized in situ by immunohistochemistry in breast samples. From this tissue, collagen-rich stroma and adipose tissue were dissected mechanically and further disaggregated to release microvessel organoids. BRENCs were cultured from these organoids in endothelial specific medium and characterized by staining for endothelial markers. Microvessels were a prominent feature of intralobular tissue as evidenced by immunostaining against endothelial specific markers such as CD31, VE-cadherin, and von Willebrand factor (VWF). Double staining against VE-cadherin and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) showed that blood and lymphatic vessels could be distinguished. An antibody against CD31 was used to refine protocols for isolation of microvasculature from reduction mammoplasties. BRENCs retained critical traits even at high passage, including uptake of low-density lipoprotein, and had E-selectin induced upon treatment with tumor necrosis factor-α. The first signs of senescence in passage 14 were accompanied by gain of trisomy 11. At passage 18 cells showed chromosomal aberrations and growth arrest as revealed by β-galactosidase staining. We demonstrate here that breast microvasculature may serve as a large-scale source for expansion of BRENCs with molecular and functional traits preserved. These cells will form the basis for studies on the role of endothelial cells in breast morphogenesis.

*Key words:* breast endothelial cells; isolation; cultivation; characterization

## INTRODUCTION

Blood vessels perfuse all tissues in the body and mediate metabolic exchanges between tissues and blood. Furthermore, recent data have demonstrated that blood vessels participate in embryo development and tissue morphogenesis (Shekhar et al., 2000; Lammert et al., 2001, 2003; Cleaver and Melton, 2003; Shen et al., 2004). Although endothelial cells from different organs share many morphological and functional features, subtleties at both levels have been shown to be organ specific (McCarthy et al., 1991; Jackson and Nguyen, 1997; Belloni and Nicolson, 1988; Bachetti and Morbidelli, 2000; Bouis et al., 2001; Chi et al., 2003). In particular the microenvironment seems to determine form and function as most convincingly revealed in the blood–brain barrier (Wagner and Risau, 1994; Abbott, 2002).

The human breast gland is a good example of a dynamic organ composed of a branching epithelium surrounded by vascularized stroma. Indeed, the stroma accounts for more than 80% of the resting breast volume (Rønnov-Jessen et al., 1996) and collectively is composed of cellular components such as fibroblasts, immune cells, and fat cells in addition to vascular-derived endothelial cells and smooth muscle cells embedded in a collagen-rich extracellular matrix. While blood vessels and particularly angiogenesis have received considerable attention in relation to breast cancer (Gasparini and Harris, 1995; Boudreau and Myers, 2003) the structure and function of the vasculature in the normal human breast are poorly understood. Recently, Naccarato et al. (2003) demonstrated that morphological differences exist between intralobular and extralobular vasculature. Whereas ducts were surrounded by many small capillaries, lobules showed fewer but larger microvessels with a sinusoidal phenotype. A similar pattern has been found by other (Rønnov-Jessen et al., 1996).

Most of the information on human endothelial cells in culture is derived from experiments with human umbilical vein endothelial cells (HUVECs) since these are readily accessible (Bouis et al., 2001). In general, HUVECs have been regarded as representative also of microvascular endothelial cells (Manconi et al., 2000). While this may be safely assumed for a number of investigations, when studying cellular interactions at the level of individual organs the tissue specificity must be considered. This was elegantly demonstrated by the recent gene expression profiling of endothelial cells

[1] To whom correspondence should be addressed.

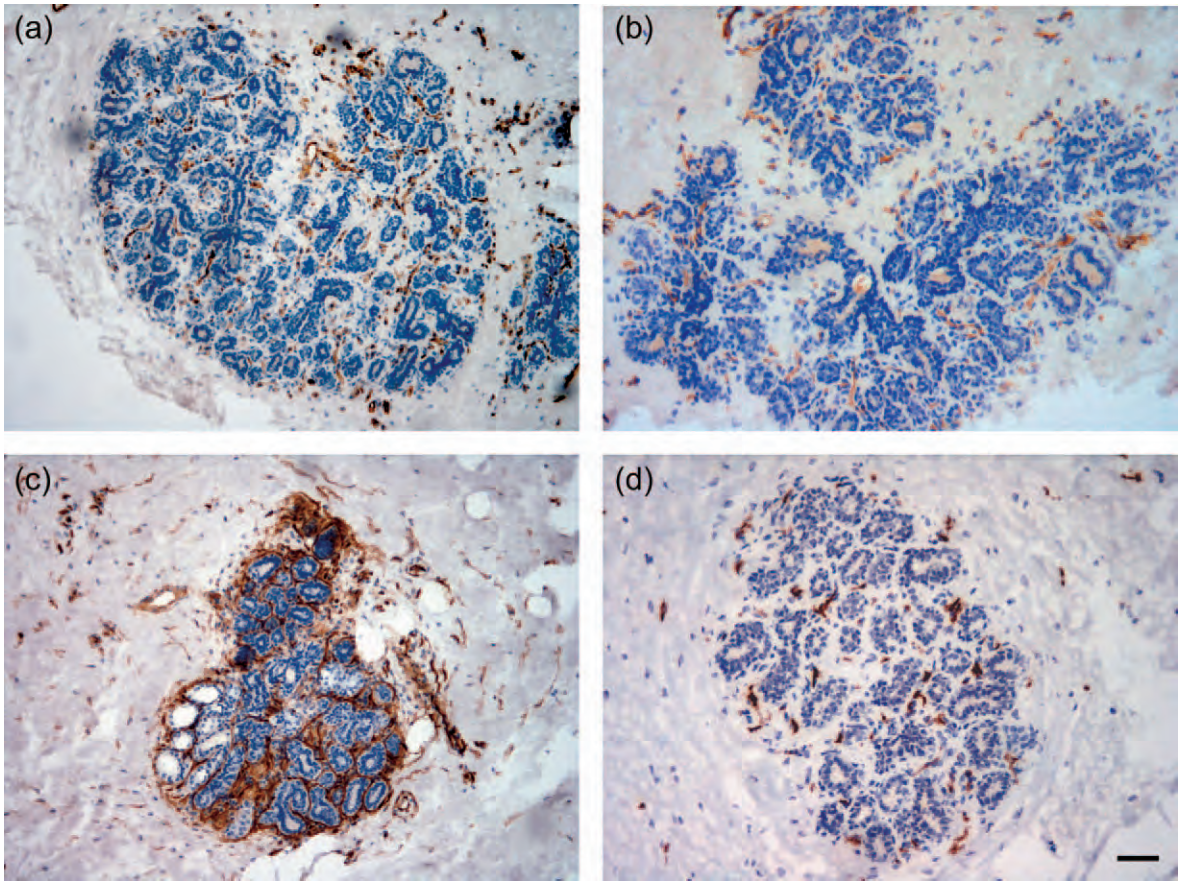


FIG. 1. Microvessels are a prominent feature of TDLU in the human breast. Cryostat sections of normal human breast tissue stained with (*A*) CD31, (*B*) VE-cadherin, (*C*) CD34, (*D*) LYVE-1. Note intralobular staining with CD31, VE-cadherin, and LYVE-1. CD34 stain stromal cells broadly. Sections were counterstained with hematoxylin. *Bar* = 50 μm. Figure is published in color online at http://inva.allenpress.com/invaonline/?request=index.html.

of different tissue origins (Pasqualini et al., 2002; Pasqualini and Arap, 2002; Chi et al., 2003).

There are only few published reports of in vitro studies of normal BRENCs (Hewett et al., 1992; Hewett and Murray, 1993b; O'Hare et al., 2001), mainly because of limited access to tissue material as well as the lack of protocols for isolation and cultivation. Methods for the isolation and culture of BRENCs have been regarded as extremely laborious and time consuming. Hewett et al. (1993) established a method for the isolation of BRENCs by sequential digestion of the breast fat tissue with collagenase and trypsin followed by specific selection of microvessel fragments with *Ulex europaeus* agglutinin-1 (UEA)–coated magnetic beads. A modification of this method included replacement of UEA with CD31 (PECAM) (Hewett and Murray, 1993b). Rønnov-Jessen and Petersen (1993) established a method for the isolation of different cellular compartments of the human breast including microvessel organoids. The enriched microvessel compartment is mostly derived from collagen-rich stroma and provides an alternative source to adipose tissue for further purification of BRENCs. With regard to increasing data from other organs demonstrating that endothelial cells play an active role in organogenesis and cell differentiation (Lammert et al., 2001, 2003; Matsumoto et al., 2001; Shen et al., 2004), it is important to improve conditions for both isolation and long-term culture of BRENCs and to study them in the normal context of breast morphogenesis. The aim of this study was to establish long-term cultures of BRENCs and to compare the endothelial phenotype in culture with their counterpart in situ.

## MATERIALS AND METHODS

*Materials.* Breast tissue specimens where obtained from reduction mammoplasties after informed consent. The study was approved by the Data Protection Commission and the National Bioethics Committee in Iceland (permission number 99/111). Culture dishes were from Nunc (Roskilde, Denmark) and Chamber Slides from BD (Bedford, MA). Vitrogen, was from Cohesion Technologies (Palo Alto, CA). Growth factor–reduced matrigel was obtained from Becton Dickinson (Bedford, MA). Collagenase IA was obtained from Sigma, Brondby, Denmark (C-9891). Cell culture medium, Dulbecco modified Eagle medium (DMEM)/F12, and fetal calf serum was obtained from GIBCO (GIBCO BRL, Life Technologies, Grand Island, NY) and endothelial growth medium, EGM-2 from Cambrex (Cambrex Bio Science, Walkersville, MD). Anti-CD31 Dynabeads where obtained from Dynal Biotech (Dynal Biotech ASA, Oslo, Norway). MACS columns and microbeads where purchased from Miltenyi (Miltenyi Biotec GmbH, Gladbach, Germany).

*Primary cell culture and isolation.* Primary breast endothelial cells were isolated from interstitial stroma or adipose tissue from breast biopsies as described in more detail in the result section. Briefly, epithelial tissue and collagen-rich interstitial stroma were separated from adipose tissue and minced into small pieces and digested in DMEM/F12 medium supplemented with collagenase-1A (900 U/ml) overnight at 37° C on a rotary shaker (60

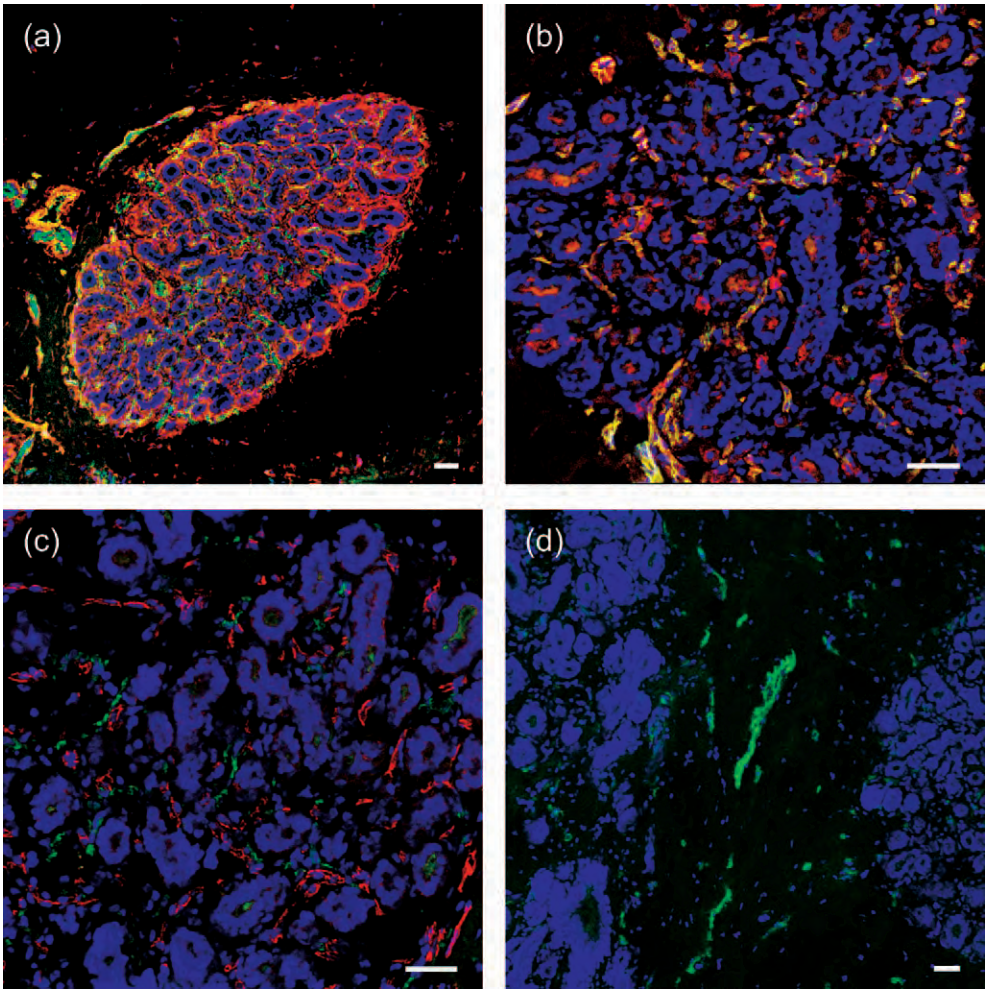


FIG. 2. Blood and lymph endothelial cells stain differently in TDLUs of normal human breast tissue. (*A*) Immunofluorescence double staining with VE-cadherin (green) and Thy-1 (red). Blood vessels were clearly distinguishable from fibroblasts and epithelial cells by expression of VE-cadherin. Fibroblasts and myoepithelial cells were recognized by their expression of Thy-1. (*B*) Double staining against VE-cadherin (green) and CD31 (red) in endothelial cells shows colocalization. (*C*) Blood and lymphatic endothelial cells were identified by expression of VE-cadherin (red) and LYVE-1 (green), respectively. (*D*) Extralobular expression of LYVE-1 (green). Nuclear staining (blue). *Bar* = 50 μm. Figure is published in color online at http://inva.allenpress.com/invaonline/?request=index.html.

rpm). After enzymatic disaggregation the digest was differentially centrifugated as described by others (Rønnov-Jessen and Petersen, 1993). This resulted in four different components enriched in acini and ducts, large vessels, microvessels, and fibroblasts. The microvessel pellet was washed twice with 5 ml incubation buffer (phosphate-buffered saline [PBS]/0.1% bovine serum albumin [BSA]/2 m*M* ethylenediaminetetraacetic acid [EDTA]) and incubated with anti-CD31 Dynabeads for 20 min at 4° C. Microvessel organoids bound to the Dynabeads were isolated on a magnetic concentrator. Microvascular organoids from adipose tissue were isolated as described by Hewett et al. (1993). Briefly, adipose tissue was collagenase-treated for 2 h at 37° C. The crude digest was centrifuged and microvessel organoids were isolated using anti-CD31 Dynabeads. All breast endothelial organoids were seeded on vitrogen-coated T25 culture flasks and cultured in EGM-2 supplemented with 30% fetal bovine serum (FBS). Serum concentration could be reduced to 2–5% after the first passage for short-term culture. When needed the MACS cell sorting system was used for the selection of contaminating fibroblasts. Anti–Thy-1 antibody (ASO2, Dianova, Hamburg, Germany) was used to deplete fibroblasts.

*Immunocytochemistry and confocal microscopy.* Breast biopsies were frozen in n-hexan (Merck) and mounted in tissue freezing medium (Leica instruments) for sectioning. Frozen biopsies were sectioned at a 5-μm setting in a cryostat. The sections were dried for 15 min at room temperature and fixed in methanol as described previously (Petersen and van Deurs, 1988). Primary antibodies included anti-CD31 (JC70A, DakoCytomation, Denmark), anti-VE-cadherin (BV9, abcam, Cambridge, UK), anti-CD34 (QBEnd/10, Novocastra, Newcastle, UK), anti-LYVE-1 (ab10278, abcam), anti-Thy1 (ASO2, Dianova), anti-VWF (F8/86), anti-VEGFR2 (KDR/EIC, abcam), anti-CD105 (SN6h, NeoMarkers, Fremont, CA), anti-vimentin (V9, DakoCytomation), and anti-keratin K19 (BA17, DakoCytomation). Rabbit anti-mouse immunoglobulins (Z0259, DakoCytomation) were used as secondary antibodies and a peroxidase conjugated anti-peroxidase mouse mAb was used as a tertiary antibody (P850, DakoCytomation). The peroxidase reactions were performed using 0.5 mg/ml 3,3-diaminobenzidine (DakoCytomation) and 0.02% $H_2O_2$ (Merck) for 10 min. The cultures were counterstained with hematoxylin. For double-labelling experiments we used fluorescent isotype-specific secondary antibodies (Molecular Probes, Invitrogen). Antibody incubations were carried out for 30 min, and specimens were rinsed twice for 5 min each at room temperature. Fluorescent nuclear counterstaining was performed with TO-PRO-3 iodide (Molecular Probes, Invitrogen, Groco, Reykiacik, Iceland). After staining specimens were mounted with coverslips using Fluoromount-G (Southern Biotechnology). Immunofluorescence was visualized using a Zeiss LSM 5 Pascal laser-scanning microscope (Carl Zeiss).

*Functional assays.* Acetylated low-density lipoprotein (AcLDL) uptake assay was performed on semiconfluent breast endothelial cells cultured on four-

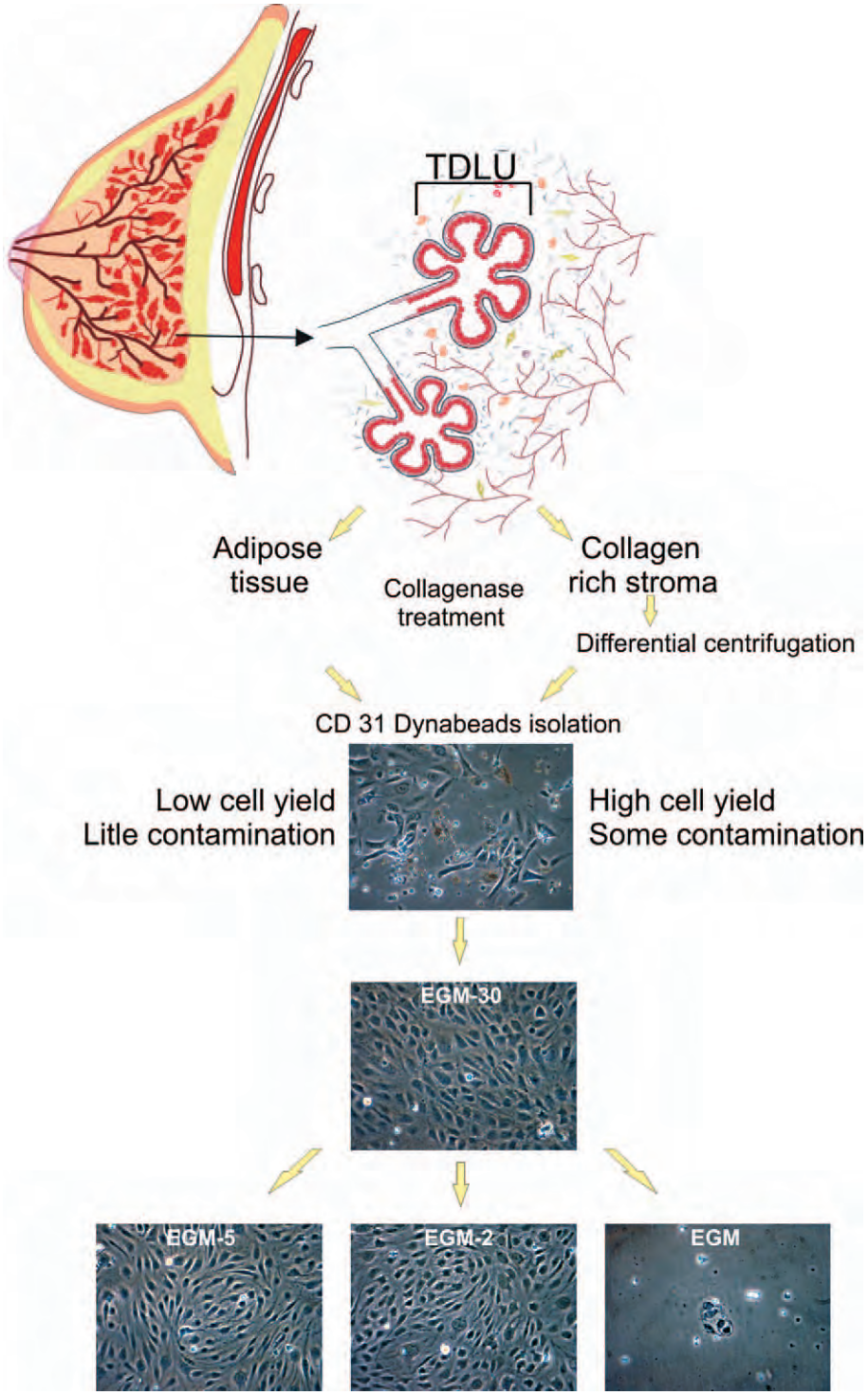


FIG. 3. Schematic presentation of the human breast including the TDLU and a protocol for the isolation of breast blood vessel endothelial cells. Tissue from breast reduction mammoplasties was minced and separated into an adipose component and an epithelial/stromal component. Each component was digested with collagenase for 4 and 24 h, respectively. Digestion of adipose tissue resulted in relatively pure microvessel organoids. In contrast, digestion of epithelial/stromal component resulted in a mixture of cells and organoids. This could be further purified into microvasculature by differential centrifugation. The microvasculature from either component was incubated with anti-CD31 Dynabeads and isolated on a magnetic concentrator. This was seeded into collagen-coated culture flasks and cultured in the EGM-2 plus serum. Maximum propagation was achieved when cells were cultured in 30% serum. For short-term culture serum concentration could be lowered to 2–5 %, however, BRENCs did not grow without serum supplement. Figure is published in color online at http://inva.allenpress.com/invaonline/?request=index.html.

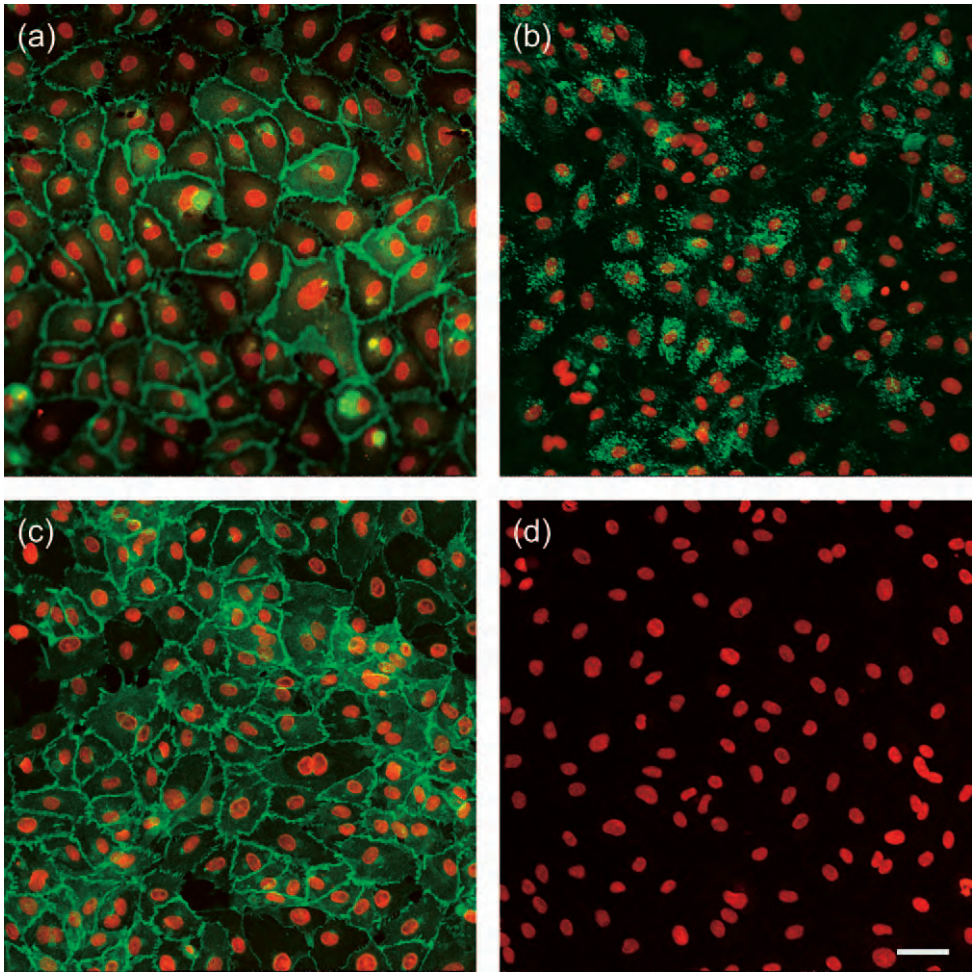


FIG. 4. Characterization of breast endothelial cells in culture. Primary cultures of human breast endothelial cells stained with (*A*) CD31 (green), (*B*) VWF (green), (*C*) VE-cadherin (green), (*D*) LYVE-1 (green). Breast endothelial cells in culture express classical endothelial markers but not the lymphatic endothelial marker. Nuclear staining (red). *Bar* = 50 μm. Figure is published in color online at http://inva.allenpress.com/invaonline/?request=index.html.

well chamber slides. Conjugated AcLDL-A488 was added at 10 μg/ml to the cells and the cells were then incubated for 4 h. The cultures were then washed twice with PBS and fixed with 1% formalin solution. Chamber slides were mounted with coverslips and AcLDL uptake visualized using a Zeiss laser-scanning microscope. E-selectin expression was induced with tumor necrosis growth factor-α (20 ng/ml) for 4 h on subconfluent breast endothelial cells cultured on four-well chamber slides. Subsequently, the slides were fixed with methanol and incubated with anti–E-selectin (ELAM) antibody (Research Diagnostic clone 1.2B6, Concord, MA) for 30 min at room temperature. Rabbit anti-mouse immunoglobulins were used as secondary antibodies as described above. Breast endothelial tube formation assay was performed on matrigel coated four-well chamber slides. Each well was coated with 50 μl matrigel and incubated at 37° C for 30 min. Endothelial cells were trypsinized and seeded into the wells and formation of capillary-like net was observed and photographed after 24 h of culture.

*Senescence assay.* Staining for senescence-associated β-galactosidase (SA-β-gal) was performed according to the manufacturers instructions (senescence β-galactosidase staining kit, Cell Signalling Technology, Danvers, MA). Briefly, cells were cultured on T25 cell culture flasks until semiconfluent. The cells were then fixed and incubated with the staining solution, which contained X-gal. Senescent cells showed β-galactosidase activity at pH 6, which was detected as a blue color. This procedure was used on primary breast endothelial cells from three different individuals in passage 6, 13, and 18. For comparison HUVECs from three different individuals were also stained with this procedure.

*Karyotype analysis.* Karyotype analysis was performed at the Chromosome Laboratory at the Landspitali University Hospital, using standard cytogenetic procedures. Briefly, cells were incubated with MAS (metaphase arresting solution, Genial Genetic Solutions, Ltd., Cheshire, UK) for 3 h, followed by hypotonic treatment (0.0075 *M* KCl) for 20 min at 37° C and fixed with methanol/acetic acid (1:3). Slides were aged for 2 d and G-banded with trypsin solution and Leishmans stain. From each culture 30 cells were analyzed and karyotypes described following International system for human cytogenetics (ISCN) recommendations (Mitelman, 1995).

## RESULTS

*Characterization of breast endothelial cells in situ.* We first mapped the spatial localization of the blood vasculature within the terminal duct lobular unit (TDLU) using classical endothelial-specific markers. These include CD31, VE-cadherin, and VWF, which are all expressed on different blood vessels. To discriminate between blood and lymphatic vessels we used an antibody against LYVE-1 that is highly specific for lymphatic vessels (Fig. 1). When stained with antibodies against CD31 and VE-cadherin, a prominent intralobular expression was seen (Fig. 1*A*,*B*) and it was evident that BRENCs were in close contact with the epithelial compartment.

TABLE 1

CHARACTERISTICS OF BREAST ENDOTHELIAL CELLS IN CULTURE

| Phenotype/passages[a] | BRENCs[b] 6 | BRENCs 13 | BRENCs 18 |
|---|---|---|---|
| CD31 | + | + | + |
| vWF | + | + | + |
| VE-cadherin | + | + | + |
| VEGFR2 | + | + | ND |
| CD105 | + | + | + |
| Vimentin | + | + | + |
| SA-β-gal | − | + | +/− |
| THY-1 | − | − | − |
| Ck-18 | − | − | − |
| LYVE-1 | − | − | − |
| | | − | |
| Function | | | |
| Tube formation on matrigel | + | + | −/+ |
| E-selectin | + | + | ND |
| AcLDL uptake | + | ND | ND |
| Karyotype | Normal | Normal | Abnormal |

[a] vWF, von Willebrand factor; SA-β-gal, senescence-associated β-galactosidase; LYVE-1, lymphatic vessel endothelial hyalvronan receptor; AcLDL, acetylated low-density lipoprotein; VEGFR2, vascular endothelial growth factor receptor 2; Ck-18, cytokeratin-18; ND, not done.

[b] BRENCs, breast endothelial cells.

CD34 has been extensively used as a marker for endothelial cells in situ. However, in our hands this marker was expressed on a wide variety of other cell types such as fibroblasts and myoepithelial cells (Fig. 1*C*, additional data not shown). Localization of lymphatic vessels was studied by staining for LYVE-1, which is exclusively expressed on lymphatic vessels. The observed expression indicated that intralobular lymphatic vessels were also a prominent feature of the TDLU (Fig. 1*D*). The intralobular organization of blood and lymphatic vessels was further demonstrated with immunofluorescence double labeling (Fig. 2). Double staining against VE-cadherin and Thy-1 revealed a distinction between the vascular and the fibroblast/epithelial compartment (Fig. 2*A*). Double staining of VE-cadherin with CD31 showed colocalization (Fig. 2*B*). To distinguish between blood and lymphatic vascular cells we performed double staining against VE-cadherin and LYVE-1 (Fig. 2*C*). Figure 2*D* shows extralobular expression of LYVE-1 in a large lymphatic vessel. In summary, the intimate relationship between the endothelial and epithelial compartments inside the TDLU suggests the possibility of a reciprocal cellular interaction across these compartments in the normal breast gland.

*Isolation and cultivation of breast endothelial cells.* Breast microvasculature was obtained by extraction from the collagen-rich interstitial stroma or the surrounding adipose tissue with a modification of methods published by Rønnov-Jessen et al. (1996), Rønnov-Jessen and Petersen (1993), and Hewett et al. (1993), also described in Material and Methods and outlined in Fig. 3. Modifications from previous methods included immunomagnetic purification in a CD31 (PECAM) retaining column followed by a flow-through in an anti–Thy-1 column in order to remove contaminating fibroblasts. Enriched microvessel organoids were plated onto collagen-coated tissue culture flasks and cultivated in the EGM-2 medium (Fig. 3, schematic figure). This medium was further supplemented with 30% fetal calf serum which was indispensable for long-term endothelial growth. Under these culture conditions primary BRENCs were kept up to 18 passages with a split ratio of 1:4 at each passage. For short-term culture purposes (4–6 passages) the serum level could be reduced to 2% (Fig. 3). The success rate in establishing long-term BRENCs cultures in terms of biopsies was 9 out of 35 (25.7%). This allowed for cryopreservation of several vials from each biopsy for future purposes. A high viability after cryopreservation was observed if cultures were provided with fresh medium before handling and 55% fetal calf serum was added as a cryopreservative.

*Characterization of cultured BRENCs.* BRENC cultures were characterized in passage 6, 13, and 18. As seen in Fig. 4 and Table 1 BRENCs stained brightly with the endothelial markers CD31, VWF, and VE-cadherin but were negative for the lymphatic marker LYVE-1, the fibroblast marker Thy-1, and the epithelial cytokeratin 18. The endothelial lineage of the cultured BRENCs was further evidenced by their ability to form capillary-like networks on top of matrigel (Fig. 5*A*,*B*). Furthermore, BRENCs showed uptake of the endothelial specific marker AcLDL when added to the culture medium, and addition of tumor necrosis factor-α resulted in the induction of another endothelial specific marker, E-selectin. Table 1 summarizes the marker expression and functional characteristics of cultured BRENCs.

*BRENCs remain karyotypically normal until the onset of senescence.* Karyotyping revealed that BRENCs were remarkably stable even after long-term culture. Generally, at passage 14 a subpopulation of BRENCs emerged exhibiting signs of senescence as evidenced by expression of SA-β-gal (data not shown). This was accompanied by chromosomal changes in terms of trisomy 11. For comparison with BRENCs we used HUVECs and these started to accumulate chromosomal changes already in the sixth passage with the same split ratio. Interestingly, the same chromosomal abnormalities, i.e., trisomy 11, appeared in BRENCs and HUVECs. Figure 6 shows the karyotype of BRENCs and HUVECs at passage 13. At passage 18 BRENCs expressed a widespread senescent phenotype as evidenced by the appearance of large cells with fragmented nuclei and expression of SA-β-gal. Nevertheless, BRENCs retained the ability to form tube-like structures even at the onset of senescence after passage 14 (data not shown) which indicates that this behavior is independent of cell growth.

## Discussion

We have characterized long-term cultures of human BRENCs and compared their phenotypic traits to the tissue of origin. Furthermore, we optimized protocols for isolation of BRENCs from reduction mammoplasty. When compared to endothelial cells in situ, cultured BRENCs clearly retained critical phenotypic traits until passage 14 when signs of senescence were seen concurrently with the appearance of chromosomal instability. BRENCs were completely senescent at passage 18.

The first successful isolation of human microvessel endothelial cells was reported in 1979 when endothelial cells from adrenal cortex, neonatal foreskin, and spleen were grown in vitro (Folkman et al., 1979). Since then, human microvessel endothelial cells have been isolated and cultured from many different organs such as the brain (Dorovini-Zis et al., 1991; Lamszus et al., 1999), dermis (Davison et al., 1983; Jackson et al., 1990; Richard et al., 1998), lung

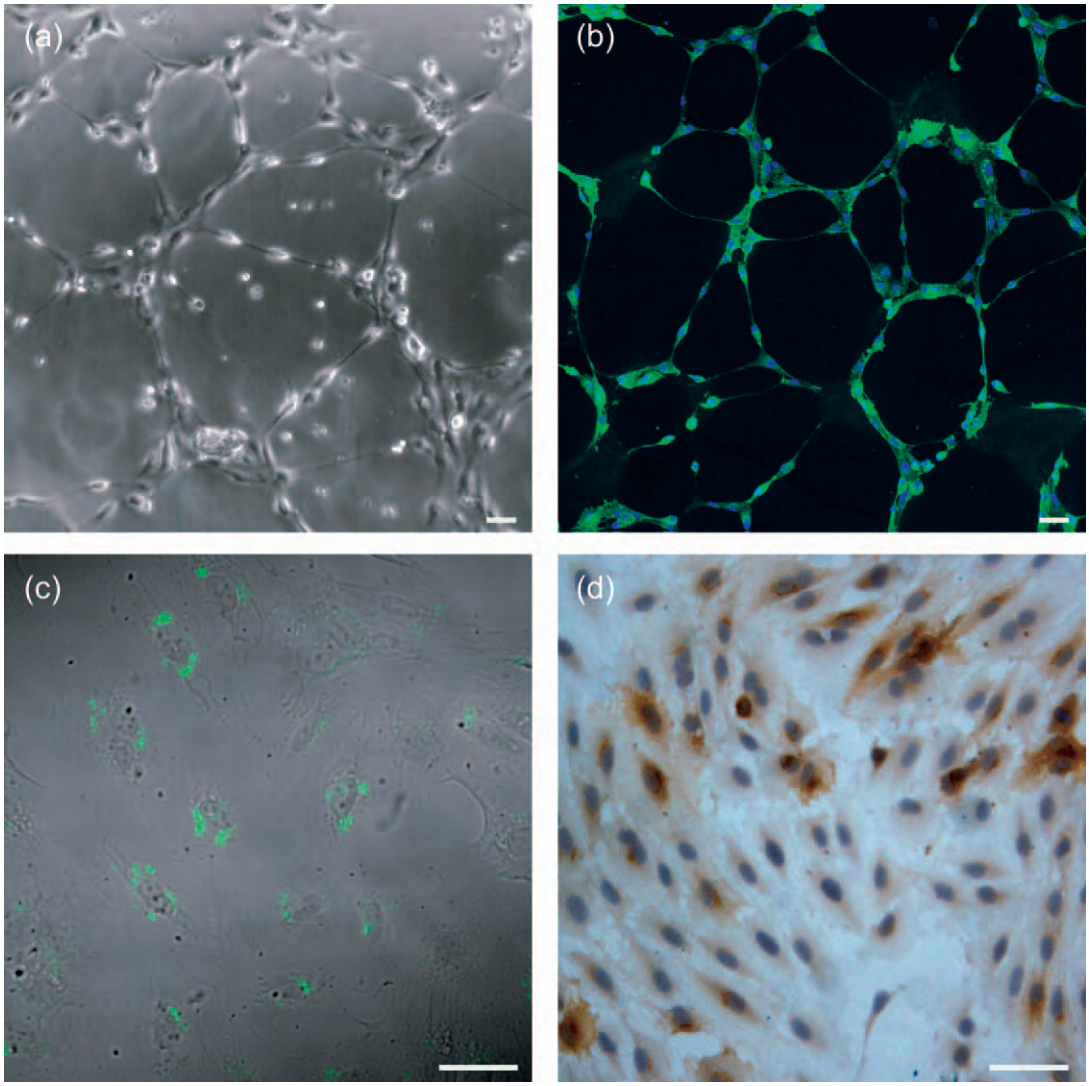


FIG. 5. Breast endothelial cells recapitulate a microvascular pattern on matrigel and retain functional properties in monolayer culture. (*A*) Phase contrast micrograph of BRENCs on matrigel. Note the formation of capillary-like structures. (*B*) Immunofluorescence staining of BRENCs in endothelial cells cultured on matrigel with CD31 (green). Nuclear staining (blue). (*C*) Monolayer culture of BRENCs incubated with fluorescein isothiocyanate (FITC)-labelled AcLDL (green). Note the endothelial specific uptake of AcLDL (*D*) Monolayer culture of BRENCs induced with tumor necrosis factor-α and stained with E-selectin immunoperoxidase. *Bar* = 50 μm. Figure is published in color online at http://inva.allenpress.com/invaonline/?request=index.html.

(Hewett and Murray, 1993a), bone marrow (Richard et al., 1998), intestine (Haraldsen et al., 1995), and breast adipose tissue (Hewett et al., 1993) However, most of these methods are time consuming and laborious thus limiting their application.

Long-term BRENCs cultures were successfully established from 26% of the biopsies. One reason for this relatively low success rate could be the phase of the menstrual cycle of each patient when undergoing the reduction mammoplasty. It has been shown that the vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) levels increase in the normal breast tissue in the luteal phase compared with the follicular phase of the menstrual cycle which may provide a pro-angiogenic environment in the luteal phase (Dabrosin, 2003a, 2003b).

The fact that we observe trisomy on chromosome 11 in passage 6 in HUVECs and passage 14 in BRENCs is in line with data from other groups (Nichols et al., 1987; Johnson et al., 1992). Nichols et al. (1987) serially subcultivated endothelial cells from adult human arteries and veins and compared them to endothelial cells from umbilical cord; polyploidy, including trisomy 11, was only seen in HUVECs. Johnson et al. (1992) demonstrated trisomy 11 in all but one of 12 adult EC cultures. Interestingly, a number of oncogenes are present on chromosome 11 including *Ha-ras* and the B cell lymphoma 1 (*bcl.1*) (Johnson et al., 1992). Because the development of polyploidy is a sign of senescence, it is possible that the early appearance of polyploidy and trisomy 11 in HUVECs is related to the fact that these cells are derived from a tissue that is at the end of its in vivo life span when studied. The ability of BRENCs to retain genotypic and phenotypic stability for up to 14 passages in culture will allow the use of these cells for studies on their role in normal development and morphogenesis of the human breast gland.

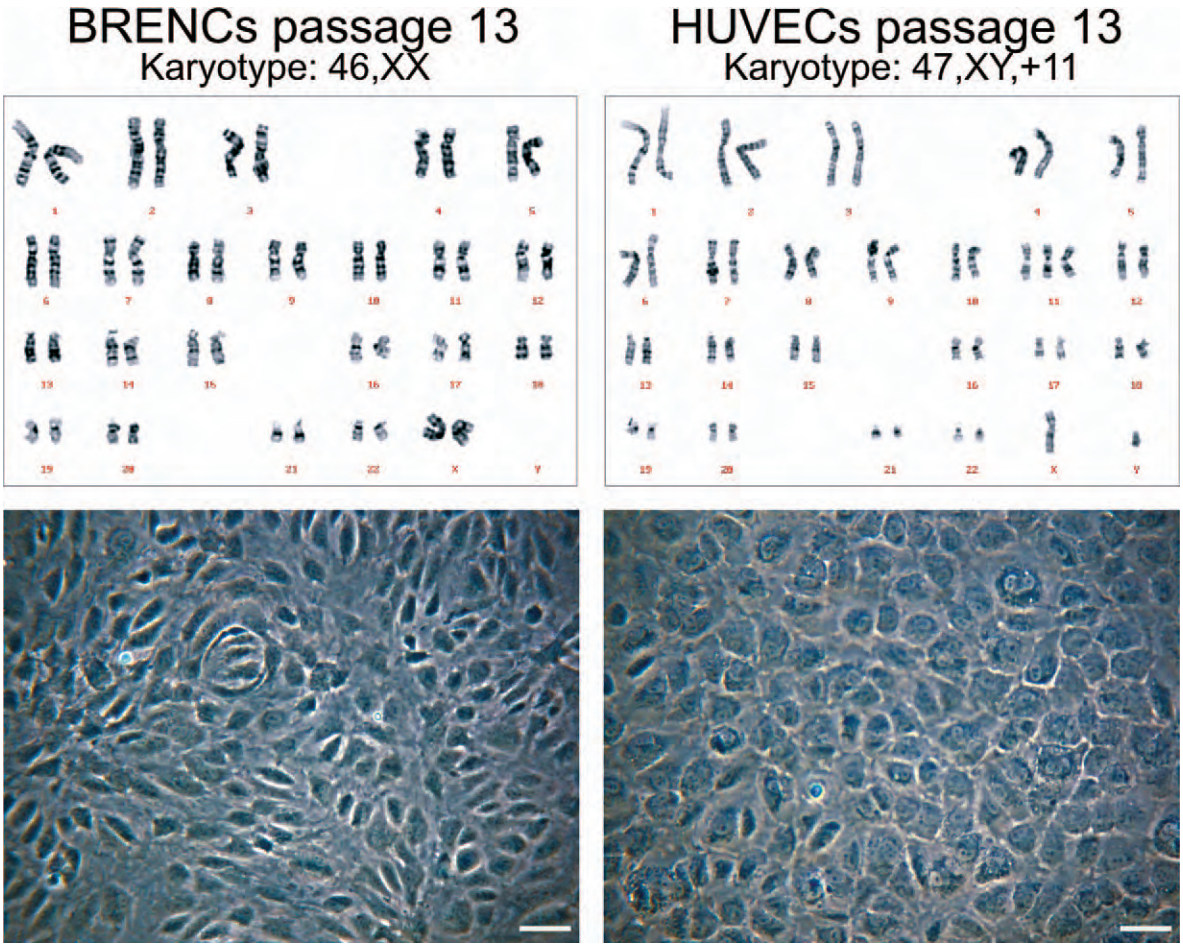


FIG. 6. Breast endothelial cells show chromosomal stability even after long-term propagation in culture. Karyotypes of BRENCs (upper left) and HUVECs (upper right) in passage 13. Trisomy 11 is seen in passage 13 in HUVECs but not in BRENCs. Phase contrast images of BRENCs (lower left) and HUVECs (lower right) in passage 13. *Bar* = 50 μm. Figure is published in color online at http://inva.allenpress.com/invaonline/?request=index.html.

## CONCLUSION

The data presented here show that BRENCs retain characteristic in situ markers in culture and that cultivation can be achieved for up to 13 passages without any significant phenotypical or chromosomal changes. The ability to culture BRENCs provides the possibility to create "designer" microenvironments in vitro, incorporating different cell types for the study of mutual interactions during normal morphogenesis (Bissell et al., 2002). Such studies are currently in progress in our laboratory.

*Competing interests.* The authors declare that they have no competing interests.

*Authors' contributions.* V. S. and A. F. performed cell isolation, cell cultures and immunohistochemistry, J. K. and J. G. J. provided access to tissue material and were consultants on this project including proofreading of the article. M. S. carried out karyotype analysis, H. M. O. and O. W. P. contributed by critical reading of the article and interpretation of data. T. G. coordinated the study and was responsible for supervision of the laboratory work and writing the article. All authors read and approved the final manuscript.

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# Paper #2

BMC Research Notes

RESEARCH ARTICLE Open Access

# Endothelial cells stimulate growth of normal and cancerous breast epithelial cells in 3D culture

Saevar Ingthorsson[1], Valgardur Sigurdsson[1], Agla JR Fridriksdottir[2], Jon G Jonasson[3], Jens Kjartansson[4], Magnus K Magnusson[1,5] and Thorarinn Gudjonsson*[1]

## Abstract

**Background:** Epithelial-stromal interaction provides regulatory signals that maintain correct histoarchitecture and homeostasis in the normal breast and facilitates tumor progression in breast cancer. However, research on the regulatory role of the endothelial component in the normal and malignant breast gland has largely been neglected. The aim of the study was to investigate the effects of endothelial cells on growth and differentiation of human breast epithelial cells in a three-dimensional (3D) co-culture assay.

**Methods:** Breast luminal and myoepithelial cells and endothelial cells were isolated from reduction mammoplasties. Primary cells and established normal and malignant breast cell lines were embedded in reconstituted basement membrane in direct co-culture with endothelial cells and by separation of Transwell filters. Morphogenic and phenotypic profiles of co-cultures was evaluated by phase contrast microscopy, immunostaining and confocal microscopy.

**Results:** In co-culture, endothelial cells stimulate proliferation of both luminal- and myoepithelial cells. Furthermore, endothelial cells induce a subpopulation of luminal epithelial cells to form large acini/ducts with a large and clear lumen. Endothelial cells also stimulate growth and cloning efficiency of normal and malignant breast epithelial cell lines. Transwell and gradient co-culture studies show that endothelial derived effects are mediated - at least partially - by soluble factors.

**Conclusion:** Breast endothelial cells - beside their role in transporting nutrients and oxygen to tissues - are vital component of the epithelial microenvironment in the breast and provide proliferative signals to the normal and malignant breast epithelium. These growth promoting effects of endothelial cells should be taken into consideration in breast cancer biology.

## Background

The human breast gland is composed of two main cellular compartments, the branching epithelium, commonly referred to as the terminal duct lobular units (TDLUs) and the surrounding stroma. The TDLUs consist of an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells separated from the surrounding vascular rich stroma by a basement membrane [1,2]. The breast stroma is composed of cellular components such as fibroblasts, immune cells and endothelial cells and the extracellular matrix (ECM) as well as entrapped growth factors within the ECM. Breast stroma accounts for roughly 80% of the total tissue volume and exerts a dominant effect on tissue morphogenesis in both the normal and malignant breast gland [3,4]. Recent studies have underscored the dominant role of breast stroma during epithelial morphogenesis (reviewed in [4]). Previous studies have shown that normal and malignant breast epithelium can mimic certain aspects of the breast gland histoarchitecture - such as lumen formation and branching morphogenesis - when cultured alone or in co-culture with fibroblasts in three-dimensional matrix [5-7]. The importance of the stroma in the normal and cancerous breast is becoming increasingly appreciated. Boulanger *et al.* demonstrated that spermatogonial cells underwent a breast epithelial differentiation program upon interaction

* Correspondence: tgudjons@hi.is
[1] Stem cell research unit, Department of anatomy, Faculty of medicine, University of Iceland and Department of laboratory hematology, Landspitali, university hospital, (Vatnsmýrarvegur 16), Reykjavík, (101), Iceland
Full list of author information is available at the end of the article

with the mammary gland microenvironment [8]. Furthermore, Booth *et al.* showed that breast stroma can redirect neural progenitor cells to produce cellular progeny committed to breast epithelial differentiation [9]. While the functional role of fibroblasts and various extracellular matrix components in breast morphogenesis has been extensively studied [10-12], much less is known of the role of the vascular endothelium in the process. Previously, the role of endothelial cells has been seen as a passive conducting system, transporting oxygen and nutrients to tissues. In recent years however, studies in organogenesis and stem cell research have shown that endothelial cells play a pivotal role in tissue morphogenesis and stem cell niche [13,14]. In the prostate, vasculature expansion has been shown to precede the expansion of the epithelium following castration and androgen treatment, suggesting the importance of endothelial derived signals or epithelial growth [15]. We have recently shown that microvessels are in close proximity with TDLUs [16]. A detailed description of the epithelial-endothelial interactions in the human breast gland however, has until recently been largely neglected. There are, however, few reports describing *in vitro* the interaction between endothelial- and epithelial cells in the human breast. Shekhar *et al.* [17,18] showed that interaction between endothelial cells and premalignant breast epithelial cells was necessary to allow sufficient proliferation of endothelial cells as well as to induce branching ductal-alveolar morphogenesis and hyperplasia of premalignant cells [17,18]. In these studies, they used human umbilical vein endothelial cells (HUVEC) instead of organ-specific endothelial cells. It is becoming increasingly recognized that endothelial cells from different organs vary in terms of morphology, marker expression and metabolic properties [19-23] highlighting the need to use organotypic endothelial cells in co-cultures with breast epithelial cells. We have recently improved the isolation protocol and the culture conditions for long term culture of breast endothelial cells (BRENCs) [16]. In this study, we describe a novel three dimensional co-culture system, where primary breast endothelial cells are seeded together with epithelial cells in three dimensional laminin rich gel. We provide evidence that BRENCs can induce proliferation of breast epithelial cells in three-dimensional culture. Furthermore, in co-culture with endothelial cells a subpopulation of luminal epithelial cells form bigger acini/ducts with larger lumens. Seeding normal and cancerous epithelial cells in rBM at clonal dilution with endothelial cells resulted in increased cloning efficacy and larger colony size. This data suggests that endothelial cells in addition to providing nutrient and oxygen to tissues, might be an important microenvironmental factor for normal morphogenesis and cancerous growth in the human breast gland.

## Methods

### Establishment of primary cell culture

Breast tissue specimens were obtained from reduction mammoplasties with informed consent from patients and approval from the National Bioethics Committee of Iceland, Reference number VSNa2001050056. Primary epithelial cells were processed as previously described and cultured on collagen I (Inamed, Gauting, Germany) coated culture flasks (BD Biosciences, Bedford MA) in serum free chemically defined medium (CDM3) [24,25]. Primary breast endothelial cells were isolated from the organoid supernatant as previously described [16]. Briefly, following centrifugation at 1000 rpm for 5 minutes, capillary organoids were isolated using CD31 coated magnetic beads (Invitrogen). Primary endothelial cells were cultured on collagen coated flasks in EGM-2 medium (Lonza, Basel, Switzerland) supplemented with 30% FBS (Invitrogen), heparin, FGF-2, EGF- VEGF, IGFR3, ascorbic acid and hydrocortisone. FBS concentration was reduced to 5% after 2 passages, this medium will be referred to as EGM5.

### Isolation of luminal- and myoepithelial cells

Luminal- and myoepithelial cells outgrown from organoids were isolated with MACS cell sorting system (Miltenyi Biotech, Bergisch Gladbach, Germany), with specific mAb for each cell type (see table 1). EpCAM and MUC-1 were used to isolate luminal epithelial cells and Thy-1 and β 4 integrin for myoepithelial cells. Purified luminal- and myoepithelial cells were cultured on CDM3 and -4 respectively as previously described [24].

### Cell lines

Human breast cell lines MCF10A, D382 [26], MCF7, T47-D and MDA-MB-231 were used in three dimensional culture (see table 2 for details). MCF10a and D382 were maintained on H14 medium [27]. MDA-MB-231, T47-D and MCF-7 were maintained on ATCC recommended culture medium.

### Three-dimensional cell culture

$1 \times 10^4$ primary epithelial cells were suspended in 300 μl rBM along with $2 \times 10^5$ endothelial cells and seeded in a 24-well plate. After incubation at 37°C for 30 minutes the cultures were supplemented with EGM5 medium. Co-cultures were maintained for 14 days and culture medium was changed three times per week.

The epithelial cell lines MCF10A, D382, MCF7, T47-D and MDA-MB-231 (table 2) were seeded at a clonal density (500 cells per gel) with $2 \times 10^5$ BRENCs and cultured as described above. Colony size and number was measured on days 5, 9 and 13.

To determine dose effect of endothelial cells in co-culture, BRENCs were seeded at increasing concentrations -

**Table 1: list of antibodies usied in the study**

| Antibody | Clone | Species | Isotype | Company |
|---|---|---|---|---|
| **β4-integrin** | 3E1 | Mouse | IgG1 | Millipore |
| **CD10** | SS 2/36 | Mouse | IgG1 | Dako |
| **CD31** | JC/70A | Mouse | IgG1 | Dako |
| **ck14** | LL002 | Mouse | IgG3 | Abcam |
| **ck19** | A53-B/A2 | Mouse | IgG2a | Abcam |
| **cl-caspase-3** | Polyclonal | Rabbit | IgG | Cell Signalling |
| **EpCAM** | VU1D9 | Mouse | IgG1 | Novocastra |
| **ki67** | Polyclonal | Rabbit | IgG | Abcam |
| **MUC-1** | 115D8 | Mouse | IgG2b | Biogenesis |
| **thy-1** | ASO02 | Mouse | IgG1 | Dianova |
| **ZO-1** | 1A12 | Mouse | IgG1 | Zymed |

ranging from 1,000 cells to 200,000 cells - with 250 MCF10A cells. Colony size and number was measured on day 10.

To prevent direct cell-cell contact, BRENCs were seeded on a 0.4 μm pore size Transwell (TW) filter (Corning Life Sciences, Lowell, MA) and cultured in a 12 well plate for 3 days. Epithelial cells (500 cells per well) were then seeded into 100 μl rBM in a separate plate and placed in an incubator at 37°C for 10 minutes. Confluent BRENCs on TW filters were then transferred on top of the gels. Cultures were maintained on EGM5 medium for 8 days.

Gradient co-cultures were conducted using $7 \times 10^4$ BRENCs embedded into 100 μl of rBM and seeded in a 4-well chamber slide. $3 \times 10^3$ epithelial cells were seeded in separate 100 μl rBM and placed 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.

**Table 2: List of cell lines used in the study**

| | Cell line | Origin | Phenotype |
|---|---|---|---|
| **Normal like cell lines** | MCF10a | F | Basal/Mixed |
| | D382 | RM | Luminal |
| **Cancer cell lines** | MCF7 | IDC (PE) | ER+ |
| | T47-D | IDC (PE) | ER+ |
| | MDA-MB-231 | AC (PE) | ER- MES |

**F**: Fibrocystic disease, **RM**: Reduction mammoplasty, **IDC**: Invasive ductal carcinoma, **PE**: Pleural effusion, **AC**: Adenocarcinoma, **ER**: Estrogen Receptor, **MES**: Mesenchymal

### Immunochemistry

Gels were frozen in n-hexane at the end of the culture period. For cryosectioning, gels were mounted in O.C.T. medium and sectioned in 9 μm slices in a cryostat. Primary tissue samples were sectioned in 9 μm slices for immunofluorescence and 5 μm slices for DAB staining. Cryostat sections were fixed in methanol at -20°C for 10 minutes and incubated with primary antibodies (table 1) mixed in PBS+10% FBS for 30 minutes. Slides were incubated with isotype specific fluorescent antibodies (Alexa fluor (AF, 488 (green), 546 (red) Invitrogen) mixed in PBS+10% FBS for 30 minutes in the dark. The specimens were then incubated with a fluorescent nuclear counterstain (TO-PRO-3, Invitrogen) and mounted with coverslips using Fluoromount-G (Southern Biotech). Co-culture gels were stained in a similar manner, with an initial blocking step using IF blocking solution [28] (10% goat serum (Invitrogen) and 1% Goat anti Mouse F(ab')2 Fragments (Invitrogen) in PBS) for 30 minutes. For F-actin staining sections were fixed in 3.7% formaldehyde for 10 minutes and permeabilized with 0.1% Triton-X-100 in PBS for 5 minutes. Slides were then incubated with AF488 conjugated Phalloidin (Invitrogen) for 30 minutes and counterstained with TOPRO-3.

#### In gel staining of endothelial cells

Endothelial cells were seeded on top or into rBM and cultured for two weeks. Visualization of CD31 was performed after 24 hours and Ac-LDL uptake after two weeks. For CD31 visualization, gels were fixed in methanol at -20°C for 10 minutes. Nonspecific binding was blocked using IF blocking solution for 30 minutes, followed by an overnight incubation with anti CD31 antibody. Secondary AF488 IgG1 antibody was incubated for 2 hours, followed by TOPRO-3 counterstaining for 15 minutes. LDL uptake of embedded endothelial cells was visualized by incubation of Alexa Fluor 488 AcLDL conjugate (Invitrogen) for 5 hours. Immunofluorescence was visualized using a Zeiss LSM 5 Pascal laser scanning microscope. See table 1 for list of antibodies used in this study.

#### Imaging and statistical analysis

All three-dimensional culture experiments were performed in triplicate for statistical accuracy. Imaging was performed using a Leica DMI3000 microscope and a Leica 310FX imaging system. Populations were compared using an unpaired two-tailed t test. Sample distribution was tested using an F-test. Welch correction was used for t-tests of samples with unequal variation. Graphs were created in Microsoft Excel. Error bars represent the standard error of the mean (SEM) unless stated otherwise.

## Results

#### Breast endothelial cells cultured in rBM are quiescent but metabolically active

When breast endothelial cells (BRENCs) are cultured on top of rBM they form a dense, capillary-like, network shortly after seeding (Figure 1a). However, after approximately 72 hours these structures detach from the gel (Figure 1b). The short lifespan of endothelial cells in this assay limits their use in long-term culture. In contrast, BRENCs that are embedded into the rBM appear as small round viable cells (Figure 1c, left). In these culture conditions the BRENCs stay proliferative quiescent but metabolically active for an extended time period (at least 14 days) (Figure 1d). Immunofluorescence staining demonstrate that BRENCs retain their marker expression in rBM as evidenced by CD31 staining (Figure 1c, right) and stay metabolically active as shown by uptake of acetylated low density lipoprotein (Ac-LDL) after two weeks in culture (Figure 1d insert). Prolonged proliferative quiescence of endothelial cells when seeded within rBM provides an opportunity to analyze endothelial contributions to epithelial growth and morphogenesis.

#### BRENCs facilitate growth of primary luminal and myoepithelial cells

BRENCs and isolated primary luminal epithelial (LEP) or myoepithelial cells (MEP) were embedded within rBM and co-cultured for 14 days (Figure 2). When breast epithelial cells were cultured alone in rBM at high density ($10^5$ cells within 300 μl rBM), LEPs formed acini-like colonies with a small central lumen (Figure 2A) as has previously been shown [29], whereas MEPs formed solid round colonies. At lower densities ($10^4$ cells per 300 μl rBM) growth was reduced and limited lumen formation was observed in LEP cultures. In contrast, co-culture of epithelial cells seeded at low density with endothelial cells, resulted in increased colony size, in both LEP and MEP co-cultures compared to low density control (Figure 2A). Interestingly, a dramatic increase in lumen size was observed in a subpopulation of LEP colonies in co-culture, (Figure 2A insert). Scatter plot reveals an increase in colony size in co-culture compared to both high density and low density LEP cultures (Figure 2B). Average colony size in high density and low density monoculture was 34 and 28 μm, respectively (Figure 2B). In contrast, average colony size in co-culture of BRENCs and LEP was 44 μm. In MEP cultures average colony size in high density and low density MEP culture was 71 and 58 μm, respectively (Fig 2C). In contrast, average colony size in co-culture of BRENCs and low density MEP was 72 μm. Interestingly, there was much more variation in colony size within each MEP culture than LEP culture. This data indicate that endothelial cells can signal to both luminal and myoepithelial cells to form larger colonies in co-culture than when cultured alone. Immunophenotypic characterization of high density (HD) culture and co-culture show clear apical to basal polarization in luminal epithelial cells. Luminal colonies are polarized with a central lumen and basally located nuclei, as evidenced by F-actin and nuclear stain (Figure 2D). No lumen formation is observed in MEP cultures. LEP colonies display basal polarization as seen with basal β4-integrin staining of both HD control and co-culture colonies. Apical polarization of LEP colonies is evidenced by staining against ZO-1. MEP colonies are negative for both CK19 and ZO1. Similar staining pattern is seen in HD and co-culture. Myoepithelial colonies also show a basal polarization as judged by β4 integrin expression (Figure 2D) but no apical polarization is observed.

#### Clonal colony formation is enhanced by BRENCs in normal and malignant breast epithelial cell lines

In order to analyze the effects of endothelial cells on proliferative and morphogenic phenotypes of established cell lines, several normal and cancerous breast epithelial cell lines were tested (table 2). When seeded within rBM at a clonal dilution (500 cells per 300 μl rBM), normal and malignant epithelial cells show limited or no proliferation (Figure 3A, left panel). In contrast, when co-cultured with BRENCs, a significant ($P < 0.0001$) increase in colony size is observed in all tested cell lines (Figure 3A, right panel). The phenotype of colonies in co-culture with BRENCs

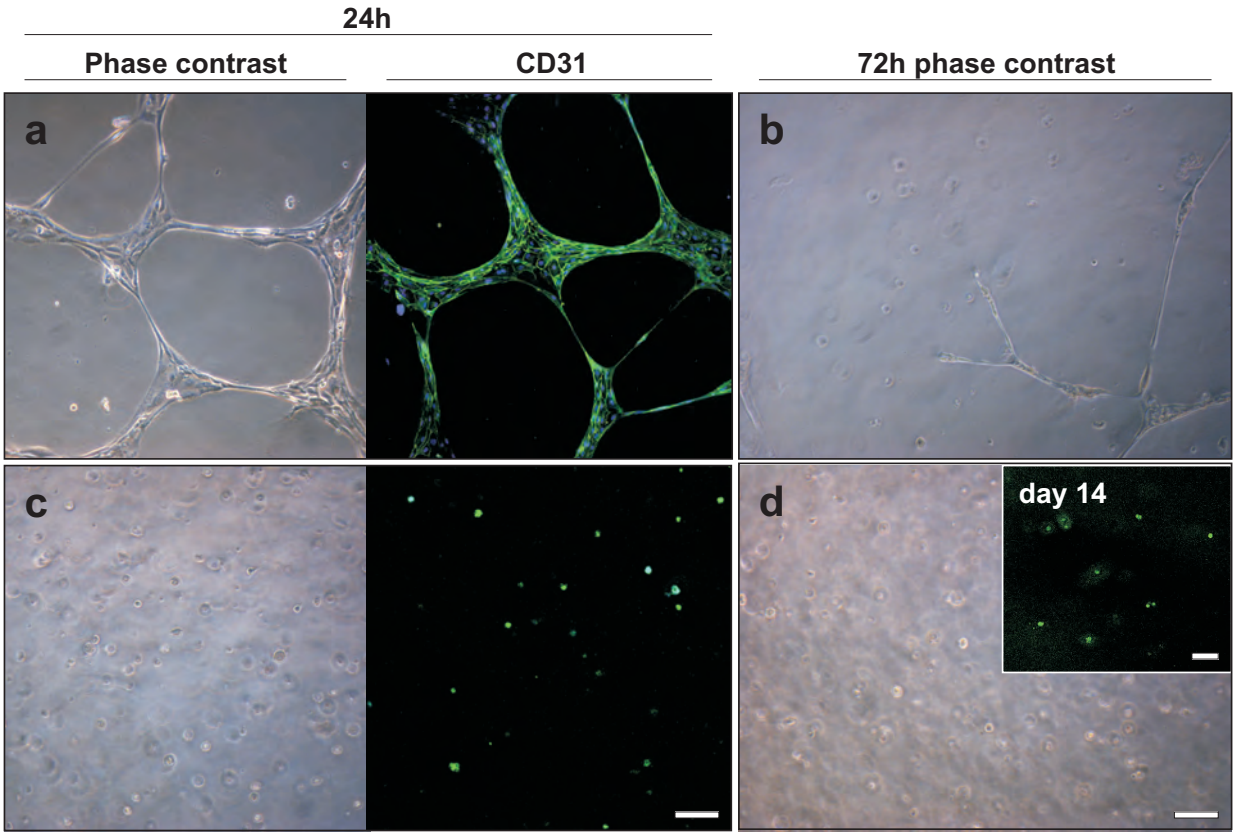


**Figure 1 Endothelial cells stay quiescent but metabolically active when cultured within rBM**. When BRENCs are seeded onto rBM they form network structures within a few hours that disassociate from the rBM within 72 hours (upper panel). In contrast BRENCS that are embedded into rBM (lower panel) stay quiescent but metabolically active for a prolonged time in culture. Fluorescent staining in a and c reveals CD31 expression. Note the insert in (**d**) that demonstrates the uptake of Ac-LDL at day 14. Bars = 50 μm.

differs between cell lines, ranging from multiacinar-like structures seen in MCF-10A, solid round (D382, MCF-7 and T47-D) and mesenchymal-like structures seen in MDA-MB-231 co-cultures (Figure 3A, right panel). When cryosectioned and immunostained against β4 integrin it was possible to see the organized and disorganized cell clusters in MCF-10A and MDA-MB-231, respectively (Figure 3B, a-b). In MCF10a cultures, β4 integrin expression is only seen on the periphery of individual acini, whereas in MDA-MB-231 cultures expression is ubiquitously seen, demonstrating a lack of polarity. Immunostaining for CD31 demonstrated the presence of BRENCs as single cells close to the epithelial colonies (Figure 3B, c-d). Immunostaining against ki67 demonstrates high levels of cell proliferation in both MCF-10A and MDA-MB231 colonies in co-culture (Figure 3B, e-f). The levels of apoptosis are low in both cell lines as evidenced by low staining for cleaved-caspase-3 (Figure 3B, g-h). Figures 3C and 3D show the colony size formed in co-culture between different epithelial cell lines and BRENCs compared to controls. A highly significant ($p < 0.0001$) increase in colony size was seen under co-culture conditions for all cell lines tested (Figure 3C). Colony size continued to increase throughout the culture period (Figure 3D). MDA-MB-231 colony size was dramatically increased from day 9 indicating possible endothelial independent effects after the colony has reached a certain size threshold.

In epithelial cultures without BRENCs, there was very limited colony formation, whereas in the co-culture conditions there was a marked increase in cloning efficiency. Figure 4 depicts the effects of BRENCs on cloning efficiency in the different cell lines, i.e. the number of colonies relative to the number of cells seeded. The cloning efficiency increased from less than 5% in controls to between 9% and 41% in co-cultures (Figure 4A). To analyze whether proliferative effects were dependent on the amount of BRENCs in co-cultures, MCF10a cells were embedded in rBM with increasing amount of BRENCs. In low density BRENCs co-cultures, there is limited cloning efficiency (less than 5%) and proliferation (colonies <100 μm). When BRENCs' density was increased, cloning effi-

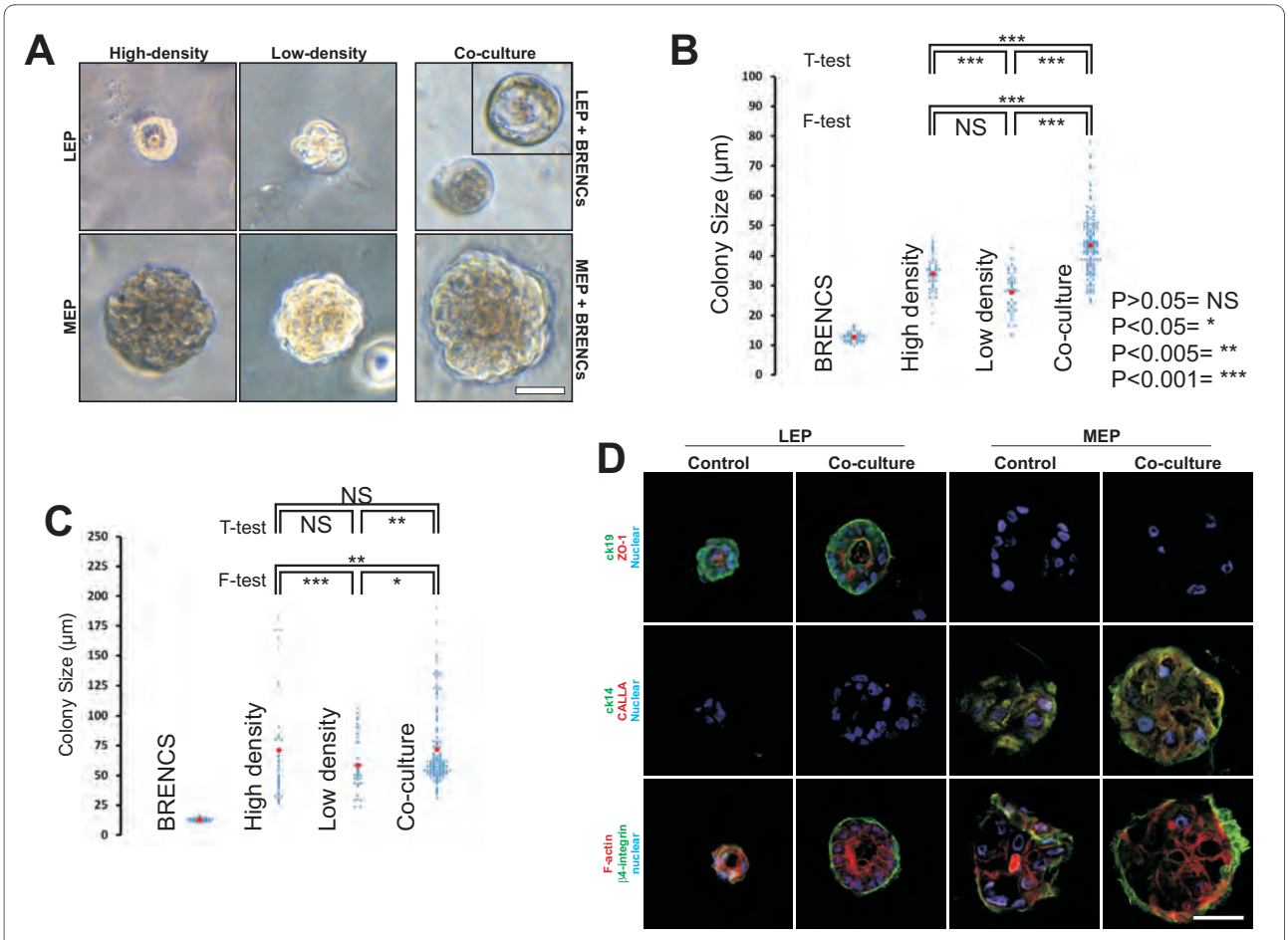


**Figure 2 BRENCs stimulate growth and morphogenesis of primary breast epithelial cells**. **A. BRENCs stimulate growth of both luminal epithelial and myoepithelial cells in rBM**. *Upper panel*: At high density, LEP cells form colonies with a small central lumen. At low density, no lumen formation is seen. In co-culture of low density LEP and BRENCS, large colonies with a large central lumen can be seen. *Lower Panel*: At high density, MEP cells form solid round colonies. At low density, colony size is reduced, but increases in co-culture with BRENCs. Bar = 50 µm. **B. Scatter plot demonstrate that co-culture of BRENCs and LEP result in larger colony size than LEP alone cultured in low and high density**. Colony size in high density control was significantly greater than in low density control. Average colony size in co-culture (n = 135) was significantly increased when comparing with high and low density controls (n = 45, both). Sample variance was also increased significantly when comparing controls and co-culture, but not between the controls. For samples of unequal variance, Welch's correction was used when performing the T-test. **C. Scatter plot show large variance in MEP culture size with and without BRENCs**. Colony size in high density control (n = 45) was significantly higher than in low density control (n = 45). Sample variance was also different. A significant difference was seen when comparing low density control and co-culture (n = 135), but not in high density control and co-culture. **D. Phenotypic characterization of luminal epithelial and myoepithelial colonies with and without BRENCs in rBM**. Immunofluorescence of cryosectioned colonies revealed that LEP colonies express CK19 and the tight junction protein ZO1 whereas MEP colonies were negative. MEP colonies, in contrast are positive for CK14 and CALLA. β4-integrin delineates the basal surface of both LEP and MEP colonies. Note the strong F-Actin staining subapical in co-culture of LEP and BRENCs. Cells were counterstained with TO-PRO-3 (blue). Bar = 50 µm.

ciency increased in a near-linear fashion, reaching its highest level with 200,000 BRENCs (24%) whereas colony size stopped increasing much earlier, reaching a plateau (~250 µm) at 50,000 BRENCs (Figure 4B).

### Proliferative signals from BRENCs are delivered via soluble factors

To discriminate between direct contact and soluble factors in co-culture we used Transwell (TW) filters and a gradient co-culture system to physically separate the two cell populations. Endothelial cells were plated in monolayer on TW filters and allowed to grow to confluency. 500 epithelial cells were then seeded into 100 µl rBM. Endothelial coated TW filters were next placed on top of the gel (Figure 5A). Some colony formation was also evident in control cultures in this setup and could be explained by better diffusion of growth factors into the gel from the culture medium due to a higher area/volume. Colony size was significantly larger in Transwell separated co-cultures of the normal cell lines MCF10A

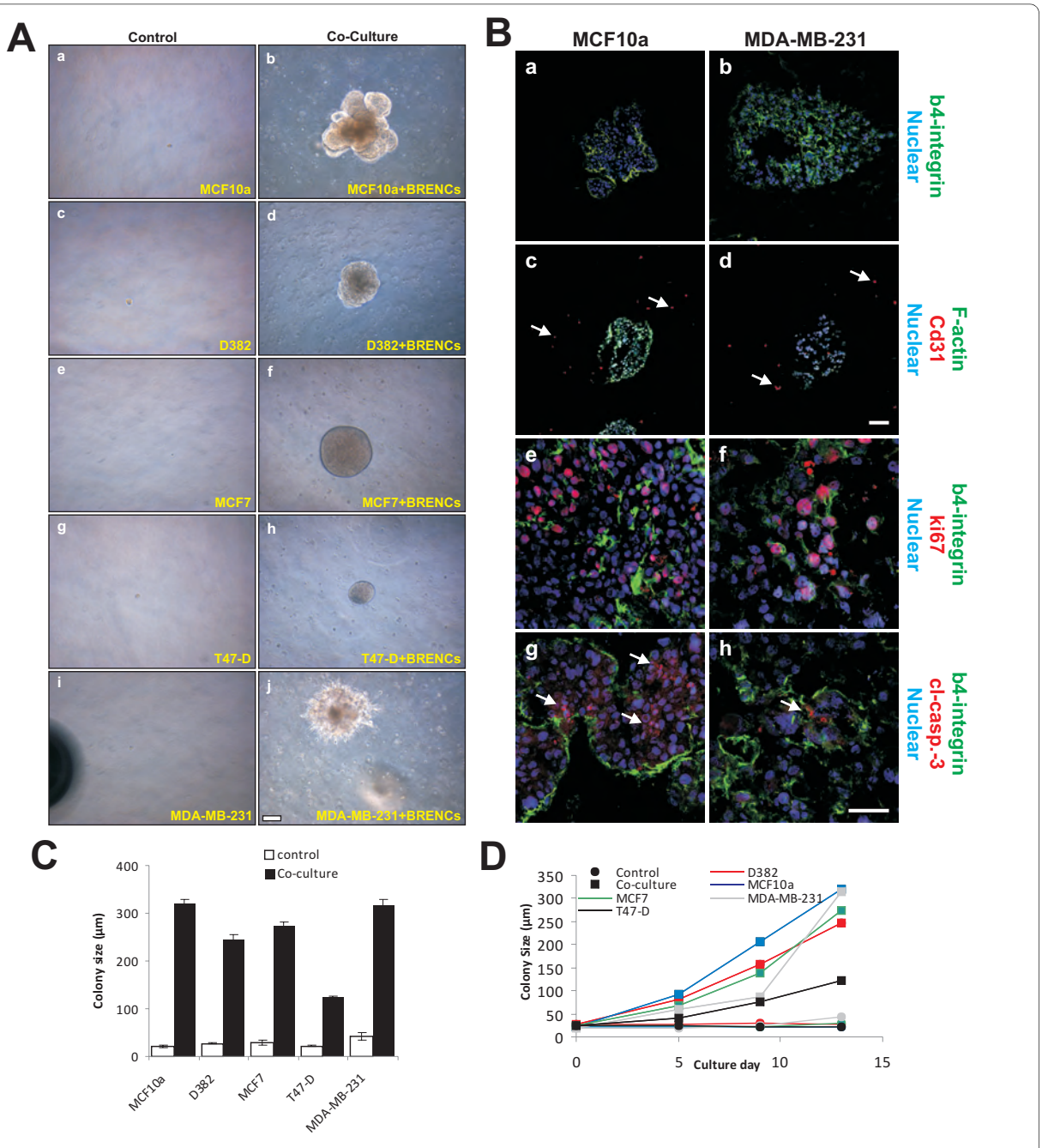


**Figure 3 BRENCs induce clonal growth of normal and malignant breast epithelial cell lines**. **A) BRENCs generate positive environment for clonal growth of breast epithelial cells**. MCF10A, D382, MCF7, T47-D and MDA-MB-231 cells were seeded at a clonal dilution, alone or in co-culture with BRENCs. When cultured alone there was very limited colony formation, most seeded cells stayed as non-proliferative single cells (**a**, **c**, **e, g, i**). In contrast, co-cultures of BRENCs with all cell lines resulted in dramatic increase in colony growth. MCF10A cells formed multiacinar-like structures (**b**), whereas D382 formed solid round colonies (**d**). both MCF7 and T47-D formed solid round colonies (**f**, **h**). MDA-MB-231 formed mesenchymal like colonies (**j**). Bar = 100 µm. **B) Immunophenotypic characterization of endothelial-epithelial co-cultures**. β4-integrin staining reveals the different phenotype of MCF10A and MDA-MB-231, where MCF10A forms dense multiacinar structures delineated by β4-integrin expression but MDA-MB-231 forms colonies of loosely connected cells with diffused staining pattern of β4-integrin. (**a-b**). Note the expression pattern of β4-integrin. CD31 staining shows the distribution of BRENCs around the colonies (**c-d**). ki67 expression levels are similar in both cell lines (**d-e**) and minimal expression of cleaved caspase 3 is seen (**g-h**) Bar = a-d 100 µm and e-h = 50 µm. **C) Colony size is increased in co-cultures with BRENCs**. Colony size (n > 100 for all cell lines) of epithelial cells co-cultured with BRENCs increased significantly compared with controls ($P < 0.0001$ for all co-cultures). 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. **D) Colony growth over time**. Most cell lines followed a linear growth pattern. Note however, the drastic change in growth of MDA-MB-231 cells after day 9 (light grey line).

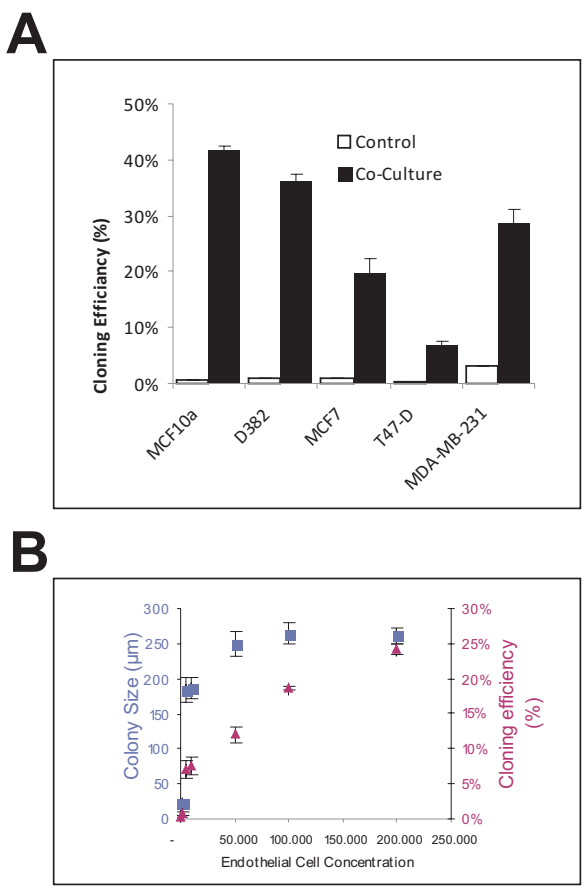


**Figure 4 BRENCs enhance cloning efficiency of normal and malignant breast epithelial cell lines in co-culture**. **A) Colony formation (cloning efficiency) is greatly increased in co-cultures**. MCF10A cells' colony forming ratio increased from 1% to 41.9%. MDA-MB-231 colony formation increased from 3.2% to 28.6%. Overall, increase in colony formation was greater for the normal cell lines than for the cancer cell lines. **B) Density of BRENCs determines the cloning efficiency of MCF10A cells in co-culture**. Increasing the density of BRENCs in co-culture resulted in an increase in colony formation (triangle). Colony size reached an apparent plateau at a BRENCs density of 50,000 (square box).

and D382, as well as MCF-7 ($P < 0.0001$) compared to co-cultures of the other malignant cell lines T47-D and MDA-MB-231, where no significant growth increase was detected.(Figure 5B and 5C). Cloning efficiency was increased in co-cultures with the normal epithelial cell lines MCF10a and D382, but not with the malignant epithelial cell lines. This possibly demonstrates a lower need for growth stimulation of the cancerous cell lines in comparison to the normal cell lines (Figure 5D).

To examine the spatial extent of BRENCs growth signals we setup a co-culture assay (Figure 6A) with a gradient in the densities of both BRENCs and epithelial cells. MCF10a co-culture showed that colony growth was most prominent in close contact with BRENCs and was comparable to regular co-culture but distal effects, however, were also visible (Figure 6B and 6C). This further demonstrated that BRENCs mediate the proliferative effects through soluble factors but the effects diffuse slowly through the gel. Growth of MDA-MB-231 showed a different pattern, where no significant proliferative effect was seen in either proximal or distal windows (Figure 6B and 6C). The appearance of spindle shaped colonies in the proximal window was also rarer than in co-culture, perhaps explaining this apparent size difference.

## Discussion

In this paper we have presented a novel three dimensional co-culture system that can be used to analyze cell-cell interaction in heterotypic co-culture. We have demonstrated that isolated primary breast endothelial cells exert a density dependant proliferative effect on epithelial cells when co-cultured. These growth signals are conveyed by soluble factors that disperse from the endothelial cells.

Paracrine interactions are important in the stromal-epithelial crosstalk within the breast gland. Various stromal cells such as fibroblasts produce growth factors and extracellular matrix that influence breast morphogenesis and cancer progression but very little is known about the inductive signals from vascular endothelium. Our data supports the notion that stroma is a vital regulator of tissue morphogenesis and could have a role in cancer progression in the human breast and thus adds a new key player, endothelial cells to this scenario. Studies on epithelial-endothelial interactions in the human breast are lacking. In contrast, studies in mice have shown that angiogenesis precedes the growth of epithelium during puberty and pregnancy when mammary epithelium undergoes a dramatic growth phase [30]. This suggests that endothelium may contribute to the breast morphogenesis. During pregnancy the mammary epithelium and its supporting intra-lobular vasculature rapidly expands to prepare for lactation, resulting in dramatic changes in the microenvironment [31]. The vasculature of the lactating gland is composed of well-developed capillary meshwork enveloping the secretory acini with basket-like structures [30]. During involution, apoptotic cell death returns the breast gland from active to resting state [30]. These morphological changes are also seen during each menstruation cycle where the breast gland undergoes a miniature version of this cycle observed during pregnancy, lactation and involution [32]. Vascular networks exist in most tissues where endothelial cell are in prime position to interact with parenchymal cells such as the epithelial cells. Indeed, recent data from various organs such as liver, pancreas, brain and bone marrow indicate that organ specific endothelial cells are important for fate control of stem cells, organogenesis and tissue mainte-

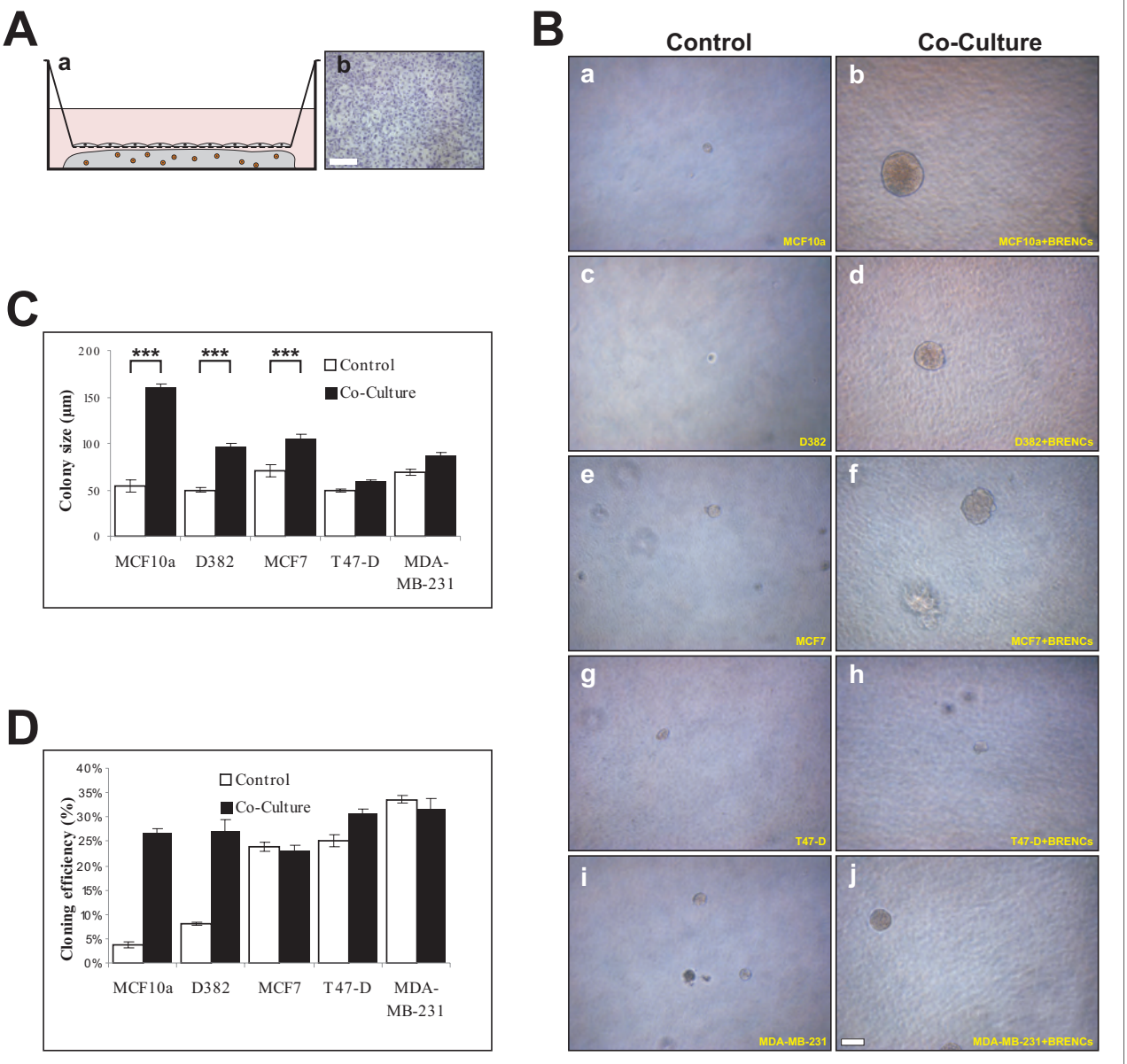


**Figure 5 Endothelial-derived effects are mediated trough soluble factors**. **A) Schematic figure of a Transwell co-culture assay**. BRENCs (grey) were seeded on Transwell (TW) filter inserts and allowed to reach confluency. Epithelial cells (orange) were embedded into Matrigel and seeded in the lower chamber (**a**). Hematoxylin stained BRENCs on a TW insert (**b**). Bar = 200 µm. **B) Phase contrast images of controls and co-cultures in rBM**. BRENCs were able to stimulate proliferation of MCF10a (**a**, **b**) and D382 (**c**, **d**) when separated by a Transwell filter. BRENCs were also able to stimulate proliferation of MCF7 (**e**, **f**), T47-D (**g**, **h**) and MDA-MB-231 (**i**, **j**) when separated by a Transwell filter. Bar = 50 µm. **C) Epithelial colony size is increased in co-culture with BRENCs**. A significant ($P < 0.0001$) increase in colony size of the cell lines MCF10a, D382 and MCF7 was seen in co-cultures (black bars) compared to controls (white bars). No significant proliferative effect was seen in T47-D and MDA-MB-231 co-culture with BRENCs. **D) Cloning efficiency of normal breast epithelial cells is enhanced in co-culture with BRENCs**. Colony formation was increased for the non-malignant cell lines only (MCF10a and D382). In contrast, similar colony count was seen in controls and co-cultures for the cancer lines.

nance (reviewed in [33]). Lammert *et al.* showed that endothelial cells are important for both pancreas and liver development before the endothelium takes up its usual roles [14]. In the nervous system Shen *et al.* [13] demonstrated that endothelial cells were enriched in the niche occupied by neural stem cells and that these endothelial cells regulate nerve stem cell proliferation and induce these stem cells to become neurons *in vitro*. Lai *et al.* [34] showed that endothelial cells induced proliferation and functional differentiation in embryonic stem cell-derived neural progenitor cells. In the bone marrow, hematopoietic stem cells are regulated by the vascular

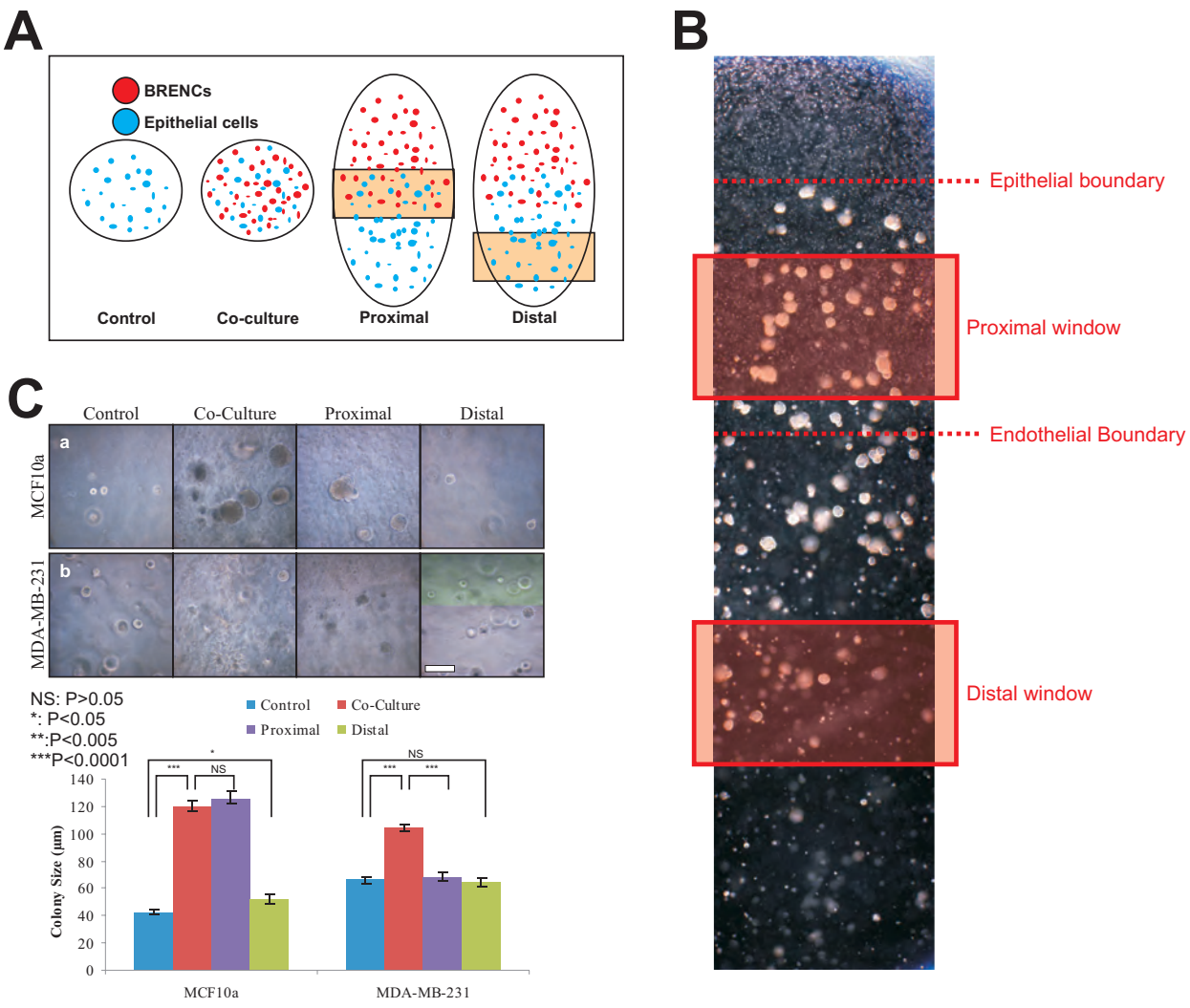


**Figure 6 Proliferation effects decrease in relation with distance from endothelial cells**. **A) Schematic representation of BRENCs and epithelial cells in gradient co-culture**. BRENCs and epithelial cells were embedded into separate gels on culture plates. The gels were allowed to fuse before culture medium was added. **B) Composite image of the gradient co-culture demonstrating the epithelial and endothelial boundaries**. Phase contrast image demonstrates the spatial distribution of epithelial colonies. The size of the colonies decreases proportionally with the distance from BRENCs. The distal colony growth, however, demonstrates that the endothelial effects are mediated at least partially by soluble factors. **C) Effects of endothelial cells are most prominent at close distance to epithelial cells**. Gradient co-culture show prominent proximal effects in MCF10A. The effects are however still present distally. No distal effects were seen in co-culture of BRENCs and MB-MDA-231. The apparent difference between co-culture and proximal windows can possibly be explained by the earlier appearance of mesenchymal colonies in co-culture.

niche [35]. *In vitro* experiments have shown that endothelial cells can provide the right environment for growth and differentiation of megakaryocytes [36].

In our 3D culture model BRENCS remain proliferatively quiescent but metabolically active and generate a stimulatory microenvironment for epithelial cells. This quiescence enables visualization of proliferating cells over a long time period, as the endothelial cells themselves do not form colonies that would limit visibility in the assay. Improvement of our *in vitro* three-dimensional cell culture model, for example incorporating fibroblasts is important. Nonetheless, these models remain superior systems to approach the situation found *in vivo*. Animal models, in particular mice, have provided extensive information regarding mammary development and cancer progression. Human and mouse mammary glands, however, have distinct differences [2]. In addition, an inherent limitation to *in vivo* models is the lack of information regarding cell-cell and cell-stroma interactions. Monolayer cultures (2D), due to their lack of physiological context are not suitable to study tissue morphogenesis. Breast epithelial cells cultured in 2D fail to form acinar-like structures and lose tissue specific differentiation such as apical-basal differentiation. In contrast, 3D models

have proven to be highly relevant when studying the tissue morphogenesis and cancer progression where they add critical elements not found in conventional two dimensional cell culture systems [37].

The observation that BRENCs stimulate a subpopulation of primary luminal epithelial cells to form colonies with a larger lumen is of interest and could indicate that these epithelial cells were derived from a ductal part of the epithelium rather than the small lobuli-derived acini. Using a Transwell assay we demonstrated that the proliferative effects of BRENCs are delivered by soluble factors. However, these factors do not diffuse effectively through the gel, and are most prominent at close proximity. These factors remain to be identified. Recent studies on endothelial-epithelial interaction by Neiva *et al.* have identified factors produced by endothelial cells that enhance migration and survival of epithelial cells [38]. The appearance of spindle shaped MDA-MB-231 colonies occurred most often in co-culture with complete mixing of the cell types (Figure 4), whereas in both the Transwell and gradient co-cultures the appearance rates were much lower (not shown). This suggests that even though proliferative effects are conferred, they are not as strong as in close cell-cell contact.

## Conclusions

Our co-culture model may help define some of the key components involved in heterotypic cell-cell interactions in normal breast morphogenesis and cancer progression. This model might be relevant for hard to culture cell types such as primary breast cancer cells where one might be able to grow these cells more readily *in vitro*. This study strengthens the notion that to understand tissue maintenance and tumor progression it is important to gain information on stromal components interacting with the epithelial cells. It is clear from other tissues that endothelial cells play an important role in organogenesis and tissue maintenance. Our data provides important hints that this might also be true in the breast gland. Furthermore, endothelial cells and their interaction with malignant breast cells might be an important factor to take into consideration in breast cancer biology.

## List of abbreviations

BRENC: Breast endothelial cell; LEP: Luminal epithelial cell; MEP: Myoepithelial cell; 3D: Three dimensional; rBM: Reconstituted basement membrane; TDLU: Terminal duct lobular unit; ECM: Extracellular matrix; CDM: Chemically defined medium; TW: Transwell; PBS: Phosphate buffered saline.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

SI planned and performed the experiments, performed data interpretation and statistical analysis and participated in writing the paper. VS, AJRF and MKM planned and discussed the experiments and participated in data interpretation and writing the paper. JGJ and JK provided access to primary breast tissue from reduction mammoplasties. TG planned and coordinated the study, participated in data interpretation and wrote the paper. All authors read and approved the final manuscript.

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**Author Details**

[1]Stem cell research unit, Department of anatomy, Faculty of medicine, University of Iceland and Department of laboratory hematology, Landspitali, university hospital, (Vatnsmýrarvegur 16), Reykjavík, (101), Iceland, [2]Department of cellular and molecular medicine, Faculty of health science, Copenhagen University, (Blegdamsvej 3), Copenhagen, (2200), Denmark, [3]Department of pathology, Landspitali, university hospital, (building 7/8), Reykjavik, (101), Iceland, [4]St. Josefs hospital, (Suðurgata 41), Hafnarfjörður, (220), Iceland and [5]Department of pharmacology and toxicology, Faculty of medicine, University of Iceland, (Hofsvallagata 53), Reykjavík, (101), Iceland

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# Paper #3

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# Endothelial Induced EMT in Breast Epithelial Cells with Stem Cell Properties

**Valgardur Sigurdsson[1,2], Bylgja Hilmarsdottir[1,2], Hekla Sigmundsdottir[1,2], Agla J. R. Fridriksdottir[3], Markus Ringnér[4], Rene Villadsen[3], Ake Borg[4], Bjarni A. Agnarsson[5], Ole William Petersen[3], Magnus K. Magnusson[1,2], Thorarinn Gudjonsson[1,2]***

**1** Stem Cell Research Unit, Biomedical Center, School of Health Sciences, University of Iceland, Reykjavik, Iceland, **2** Department of Laboratory Hematology, Landspitali University Hospital, Reykjavik, Iceland, **3** Department of Cellular and Molecular Medicine, Centre for Cell Biological Disease Analysis, and the Danish Stem Cell Centre, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark, **4** Department of Oncology, Clinical Sciences, Lund University, Lund, Sweden, **5** Department of Pathology, Landspitali University Hospital and School of Health Sciences, University of Iceland, Reykjavik, Iceland

## Abstract

Epithelial to mesenchymal transition (EMT) is a critical event in cancer progression and is closely linked to the breast epithelial cancer stem cell phenotype. Given the close interaction between the vascular endothelium and cancer cells, especially at the invasive front, we asked whether endothelial cells might play a role in EMT. Using a 3D culture model we demonstrate that endothelial cells are potent inducers of EMT in D492 an immortalized breast epithelial cell line with stem cell properties. Endothelial induced mesenchymal-like cells (D492M) derived from D492, show reduced expression of keratins, a switch from E-Cadherin (E-Cad) to N-Cadherin (N-Cad) and enhanced migration. Acquisition of cancer stem cell associated characteristics like increased $CD44^{high}/CD24^{low}$ ratio, resistance to apoptosis and anchorage independent growth was also seen in D492M cells. Endothelial induced EMT in D492 was partially blocked by inhibition of HGF signaling. Basal-like breast cancer, a vascular rich cancer with stem cell properties and adverse prognosis has been linked with EMT. We immunostained several basal-like breast cancer samples for endothelial and EMT markers. Cancer cells close to the vascular rich areas show no or decreased expression of E-Cad and increased N-Cad expression suggesting EMT. Collectively, we have shown in a 3D culture model that endothelial cells are potent inducers of EMT in breast epithelial cells with stem cell properties. Furthermore, we demonstrate that basal-like breast cancer contains cells with an EMT phenotype, most prominently close to vascular rich areas of these tumors. We conclude that endothelial cells are potent inducers of EMT and may play a role in progression of basal-like breast cancer.

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* E-mail: tgudjons@hi.is

## Introduction

Epithelial to mesenchymal transition (EMT) is associated with increased aggressiveness and adverse prognosis in carcinomas [1,2]. This conversion of cancer cells towards a more mesenchymal phenotype involves loss or lowered expression of epithelial markers (e.g. E-Cad and keratins), increased expression of mesenchymal markers (e.g. N-Cad, vimentin, fibronectin), increased mobility and an invasive phenotype [3,4,5]. EMT in breast cancer is tightly linked to the triple negative (ER-, PR- and ErbB2-) basal-like breast cancer subgroup and cancer stem cells [6,7,8,9,10,11,12]. Basal-like breast cancers express many markers associated with both myoepithelial and luminal epithelial cells suggesting the bipotential differentiation pattern and possible stem cell origin of these tumors [9,13,14]. Previous studies have demonstrated increased expression of EMT markers at tumor-stroma interfaces [15,16] and stromal cells are increasingly being recognized as major players in cancer progression [17,18]. Increasing number of factors are known that can induce EMT including transforming growth factor-β (TGF-β), ligands for receptor tyrosine kinases such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and hepatocyte growth factor (HGF) as well as components of the extracellular matrix [3,19]. These signaling events ultimately control transcriptional regulatory factors such as Snail, Slug, Twist, ZEB1, ZEB2 and FOXC2 leading to increased and decreased expression of mesenchymal and epithelial markers, respectively. Defining the cellular and microenvironmental cues that trigger EMT during the progression of breast cancers is critical and could provide new therapeutic targets.

Vascular endothelial cells have attracted increased attention as important regulators of organogenesis and stem cell maintenance in various tissues, such as bone marrow, brain, liver and pancreas [20,21,22,23]. Furthermore, intratumoral angiogenesis is also one of the hallmarks of cancer progression and increased microvessel density in tumors is an indicator of poor prognosis [12]. In the

breast gland, Shekhar et al. have previously shown that human umbilical vein endothelial cells (HUVEC) induce ductal-alveolar morphogenesis of preneoplastic MCF10A cells [24]. We have recently improved methods to propagate breast endothelial cells (BRENCS) in culture and shown that BRENCS can mediate proliferative and morphogenic signals to breast epithelial cells in coculture [25,26]. In the lung, we have shown that endothelial cells induce branching morphogenesis in lung epithelial cells when cocultured in a 3D model. Interestingly, these structures mimic phenotypic traits of lung histology *in vivo* including bronchio-alveolar like structures [27]. Thus, data from diverse organs shows that endothelial cells are important players in tissue remodeling making this cell type particularly interesting as a regulator of morphogenesis.

We have previously established a breast epithelial cell line, referred to as D492, which has a basal-like phenotype as evidenced by expression of both luminal (K8, K19) and myoepithelial (K5/6, K14) cytokeratins. Furthermore, D492 has stem cell properties as demonstrated by its ability to differentiate into luminal- and myoepithelial cells and to form branching TDLU-like structures in a 3D reconstituted basement membrane (rBM) [28,29]. Here, we demonstrate in 3D coculture that endothelial cells are potent inducers of EMT in D492 and this process is partially inhibited by blocking HGF. Furthermore, we show in basal-like breast cancer that N-Cad a marker of EMT is upregulated in proximity to vascular rich areas. These data suggest that the vascular rich stroma in breast cancer lesions might serve as an ideal niche for the stimulation of epithelial cancer cells to undergo EMT, and might especially apply to the highly aggressive basal-like breast cancers, a subtype rich in stem cells.

## Materials and Methods

### Cell culture

D492 and D382 were cultured in H14 medium as described previously [28]. W2320 cell line was cultured in DMEM/F12+5% FBS [33]. The MCF-7, MCF10A and MDA-MB-231 cell lines where purchased from ATCC (American Type Culture Collection) and are routinely authenticated with genotype profiling according to ATCC guidelines. Primary human BRENCs were isolated from breast reduction mammoplasties as previously described by Sigurdsson et al. [25] and cultured on endothelial growth medium (EGM) (Lonza) containing 50 IU/ml penicillin, 50 μg/ml streptomycin, hydrocortisone, FGF, EGF, VEGF, R3-IGF-1, Ascorbic acid, Heparin, GA-1000 and supplemented with 5% FBS (EGM5). Growth factor reduced reconstituted basement membrane (rBM, purchased as Matrigel, BD Biosciences) was used in direct 3D coculture. Transwell coculture was conducted in a 24 well setup with a 0,4 μm polyester membrane seperating the chambers (Costar). $5\times10^4$ endothelial cells were seeded in the upper chamber as a monolayer and 250 D492 cells in 100 μl matrigel on the bottom of the lower chamber maintained on EGM5. For additional information on cell culture and 3D coculture see Methods S1.

### Blocking experiments

Direct coculture of 500 D492 cells with $2\times10^5$ BRENCs in 300 μl of rBM were treated with 8 μg/ml anti-HGF neutralizing antibody (#MAB294, R&D Systems) in the rBM and in the medium. In transwell coculture HGF was blocked with 8 μg/ml anti-HGF in the rBM and in the medium in the lower transwell chamber and the controls were treated with mouse IgG1 in the same manner.

### Immunochemistry and tumor samples

Formalin-fixed, paraffin embedded tissue blocks were cut into 5 μm serial sections and mounted on slides. Sections were deparaffinized and rehydrated in xylene and ethanol. Antigen retrieval was done by boiling in citrate buffer for 15 min. The following primary antibodies were used; fibronectin (LabMab, gift from D.E. Mosher [30], CD-31 (M0823, DakoCytomation), Keratin 19 (ab7754, Abcam), Keratin 14 (NCL-LL002, NovoCastra), E-Cad (#13-1700, Zymed), N-Cad (#610920, BD), EpCAM (NCL-ESA, Novocastra). For double and triple labelling experiments we used fluorescence iso-type specific secondary antibodies (Invitrogen). Fluorescent nuclear counterstain, TO-PRO-3 (Invitrogen) was used in immunofluorescence. Specimens were visualized on a Zeiss LSM 5 Pascal laser-scanning microscope (Carl Zeiss). Breast cancer specimens were from the clinical Department of Pathology, Landspitali, University Hospital and included 9 basal-like and four estrogen receptor positive (ER-positive) breast cancers. This work has been approved by the National Bioethics Committee of Iceland, Reference number VSNa2001050056.

### Western blotting

Equal amounts (5 μg) of proteins were separated on 10% NuPage Bis-Tris gels (Invitrogen) and transferred to a PVDF membrane (Invitrogen). Antibodies: E-Cad (1:500; Zymed), N-Cad (1:1000; BD), β-actin (1:5000; Abcam), GAPDH (1:5000; Abcam), K5/6 (1:1000; Zymed), K8 (1:1000; Abcam), K14 (1:1000; Abcam), α-SM-Actin (1:500; Dako) K17 (1:500; Dako), K19 (1:1000; abcam), Vimentin (1:1000;Dako) and FOXC2 (1:2000; Abcam) were used. Membranes were visualized with ECL+ after incubation with anti-mouse or rabbit secondary antibody (1:5000) (GE healthcare).

### Migration, anchorage independence and mammosphere assays

For migration experiments a total of $1\times10^4$ and $2{,}5\times10^4$ starved cells were seeded in DMEM/F12 basic medium on collagen coated transwell filter in a transwell Boyden chamber (Corning) with an 8 μm pore size. The transwell filter were incubated in collagen (0.06 μg/μl) in PBS for 24 h at 4°C, then excess collagen solution was rinsed off with PBS before cells were seeded. EGM5 medium was used as a chemoattractant in the lower chamber. After 12 h incubation cells in the upper chamber were removed with a cotton swab and migrated cells on the bottom surface stained with 0.1% crystal violet. Cells were counted in three representative fields in each transwell. Soft agar assay was performed by mixing $1\times10^4$ D492 and D492M cells to 1.5 ml of 0.5% low melting agar (Invitrogen) that was overlaid on 1% agar solution in 6 well plates and cultured on H14 medium. After 20 days the colonies were stained with crystal violet and counted. Mammosphere assay was done in 24 well Ultra-Low attachment plates (Corning) where 500, single cell filtered, D492 and D492M cells were seeded and cultured on EGM5 medium. Number and size of spheres was evaluated after 8 days.

### Apoptosis resistance

D492 and D492M were seeded into 6 well culture plates (BD) and grown to 70% confluency. Cells were treated with 10 μM of Camptothecin (Sigma) in EGM5 medium and counted on culture days 0–3.

### Flow cytometry analysis

Adherent cells were trypsinized and filtered through a 30 nm nylon filter (Millipore). Cells were incubated for 20 minutes with

fluorochrome-conjugated antibodies against CD44 (clone IM7, BD), CD24 (clone ML5, BD) or isotype-matched controls, subsequently washed and resuspended in PBS with 4% formaldehyde (cell-fix). Cells were collected ($2\times10^4$ events) on a FACS-Calibur (BD) and analysed using CellQuest (BD).

### Statistical analysis

Data is presented as mean +SEM from number of independent experiments as indicated. Statistical analysis was performed by two-tailed Students T-test using GraphPad. P values of $<0.05$ were considered to be statistically significant.

## Results

### Immortalized breast epithelial cell line with stem cell properties generate mesenchymal-like cells in coculture with endothelial cells

The D492 cell line forms branching structures in reconstituted basement membrane (rBM) [28,29]. Growth of D492 alone in rBM requires, however, moderate cell density ($1\times10^4$ cells per 300 µl rBM) [28]. In order to test the effects of breast endothelial cells (BRENCs) on growth, and morphogenesis of D492 cells we set up a coculture with BRENCS and D492 cells inside a rBM. In this assay BRENCs remain viable and metabolically active but non-proliferative (Fig S1). No growth was seen when D492 cells were cultured alone at clonal dilution (500 cells per 300 µl rBM) (Fig. 1A). In contrast, in coculture with BRENCs the total number of D492 colonies increased with increasing amount of endothelial cells reaching a cloning efficacy of 23.5% (117.3±3.5 colonies; $p<0.01$) (Fig. 1A). In addition to solid round and branching structures that have previously been shown to form when D492 are cultured alone, spindle shape, mesenchymal-like colonies emerged in coculture with BRENCs (Figs. 1B and S2). No effect was seen on endothelial cell morphology under coculture conditions. These data suggest that BRENCs stimulate growth and morphogenesis of D492 and furthermore induce the formation of spindle-shaped colonies reminiscent of EMT in a 3D environment.

To see if the endothelial induced EMT-like phenotype was breast-endothelial specific we also cocultured D492 with human umbilical vein endothelial cells (HUVECs). HUVECs were also able to induce a similar phenotype to what was seen in coculture with BRENCs (data not shown) suggesting a general endothelial-derived effect rather than an endothelial organ-specific effect.

As D492 has an immunophenotype similar to the cells of basal-like breast cancer, we also tested W2320 which is a basal-like metaplastic breast cancer cell line [31]. W2320 generated solid epithelial colonies when cultured alone in 3D rBM. In contrast, when cocultured with BRENCs there was a marked increase in total colony formation and induction of spindle-like colonies (Fig. 1C). We also tested several other cell lines in our 3D coculture model. D382 is E6E7 immortalized cell line generated from differentiated, normal, luminal breast epithelial cells [28] and MCF10A is a non-tumorigenic epithelial cell line. MCF-7, is an estrogen receptor positive breast cancer cell line, while MDA-MB-231 is a highly malignant basal-like breast cancer cell line. When these cell lines were cocultured with BRENCs in a rBM assay, MDA-MB-231 generated mesenchymal colonies while D382 and MCF10A, generated only round epithelial colonies (Fig. S3). Furthermore, the estrogen receptor positive breast cancer cell line

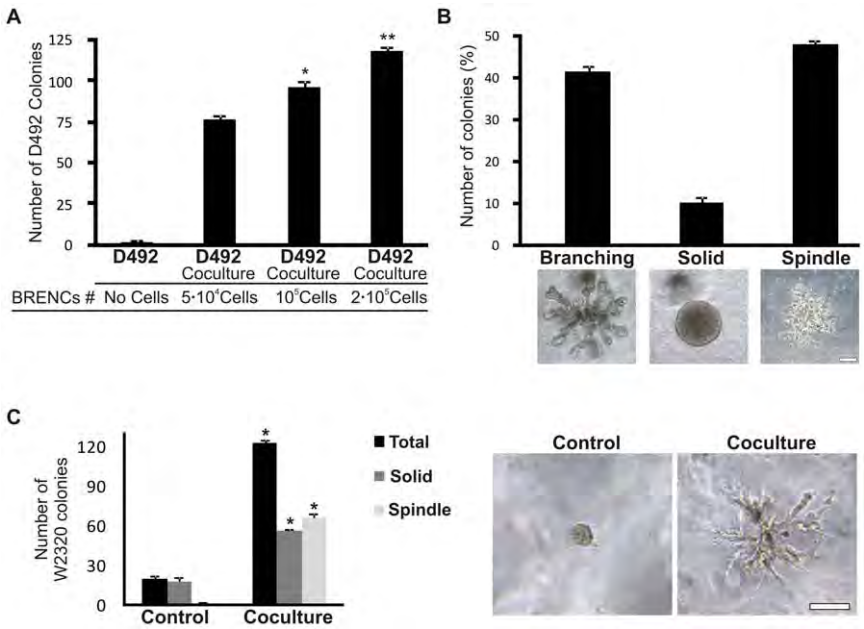


**Figure 1. Breast epithelial cells with stem cell properties generate spindle-like cells in coculture with endothelial cells. A**, Colony growth of D492-derived epithelial structures increases proportional with increased number of BRENCs in coculture. When 500 D492 cells are cultured in 300 µl rBM they fail to grow (control). With BRENCs, colony growth increases from 76 ($5\times10^4$ BRENCs), 96 ($1\times10^5$ BRENCs) to 117 colonies (23,5% cloning efficacy) when $2\times10^5$ BRENCs are inoculated with 500 D492 cells. Average (AVG) number of colonies +SEM in three experiments. *, $p<0.05$; **, $p<0.01$; compared to $5\times10^4$ BRENCs. **B**, D492 generate spindle-like cells in coculture with BRENCs ($2\times10^5$ cells). D492 cells (500 cells incubated) form three distinct structures, branching, solid, and spindle-like colonies. Appearance of the spindle colonies from D492 is novel and occurs only in coculture with endothelial cells. Average % of colony type +SEM in three experiments. Bar 100 µm. **C**, Using a primary metaplastic breast cancer cell line, W2320, we were able to show that these cells could also produce spindle-like colonies in coculture with BRENCs (right). Data shown as AVG number of colonies +SEM in three experiments (left). *$p<0.05$. Bar 100 µm.
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MCF-7, generated only large solid round colonies in coculture with BRENCs (Fig. S3). This indicates that breast cancer cell lines with basal-like characteristics have the plasticity for mesenchymal conversion, in coculture with endothelial cells, while other more differentiated cell lines are unable to undergo this transition.

## Isolation and characterization of a D492-derived EMT cell line

To analyze the origin and morphogenic capacity of branching and spindle-like colonies from cocultures, we isolated single colonies and plated them into monolayer culture. Cells derived from branching colonies showed cuboidal epithelial phenotype whereas cells from spindle-like colonies showed a spindle shaped phenotype (Fig. 2). Spindle-like colonies were isolated and expanded as sublines, one of them is referred to as D492M (mesenchymal) (Fig. 2). When replated into secondary rBM cocultures, cells from spindle-like colonies were fixed in making similar colonies whereas cells from branching colonies retain the ability to make both branching and spindle-like colonies (Fig. 2).

The parental cell line D492 was initially established by transfection with a retroviral vector containing the E6 and E7 oncogenes and the neomycin resistant gene [28]. To eliminate possible endothelial-derived contamination, the D492M subline was selected in medium containing neomycin. Furthermore, we cloned and sequenced an insertion site of the retrovirus (Methods S1). We showed the presence of this insertion in D492M and four different single cell-derived mesenchymal colonies as well as being present in 5 different single cell derived D492 sub-clones (Fig. S4A). To further confirm the epithelial origin of the mesenchymal colonies we generated a D492 subline containing a GFP expressing vector. When these GFP positive D492 cells were cocultured with BRENCs all mesenchymal-like colonies were green (Fig. S4B). This confirms the epithelial origin of the mesenchymal colonies and furthermore confirms the clonal origin of D492M from the D492 cell line.

Immunophenotypic characterization of D492M confirmed that the spindle cell morphology was a direct consequence of EMT. Thus, as opposed to the parent cell line, D492M has lost expression of E-Cad and shows reduced expression of keratins 5/6, 8, 14, 17, and 19, while showing increased expression of Vimentin, N-Cad, and alpha-smooth muscle actin (Figs. 3A and B). Using an *Illumina BeadChip* expression microarray (HumanWG-6 v3.0) we screened the expression pattern in the two cell lines. There was significantly different expression level of 9399 genes of the 13105 genes that had detectable expression levels (for an FDR of $<1\%$). Clustering pattern for the top 50 genes demonstrates the clear differences between the two cell lines (Fig. S5). E-Cad, keratins 5, 6, 14, and 19 were all downregulated in D492M compared to D492. Likewise, mesenchymal markers such as N-Cad, Thy-1, thrombin receptor (PAR1), and CD70 were all highly up-regulated in D492M. Global gene expression shows EMT-associated transcription factors that are upregulated in D492M, including FOXC2 (3.96 fold), and FOXC1 (1.29 fold) (Fig. 3C). FOXC2 upregulation in D492M was confirmed with western blot and compared to D492, MDA-MB-231 and D382 (Fig. 3D). To confirm that the EMT is causally driven by the endothelial-induced EMT, rather than reflecting the properties of a single clonal cell sub-line we isolated four other sublines from D492

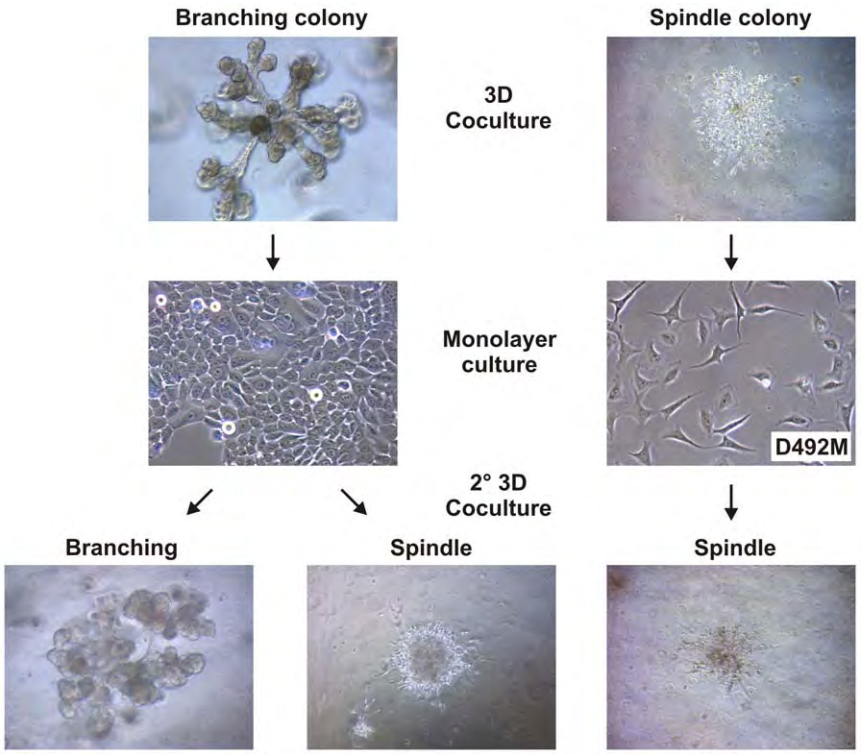


**Figure 2. Isolation of D492-derived mesenchymal-like cells (D492M).** Six branching and six spindle-like colonies were isolated and plated in monolayer culture. Cells from branching structures retain cuboidal epithelial phenotype in monolayer (left panel). When cocultured with BRENCs these cells generate branching TDLU-like (40%) and spindle-like colonies (50%) in secondary 3D culture (°2 3D). Cells from spindle-like colonies (right panel) showed mesenchymal/spindle like morphology in monolayer and cells isolated from one of these colonies gave rise to D492M. When cocultured with BRENCs these cells only gave rise to spindle like colonies in secondary 3D coculture.
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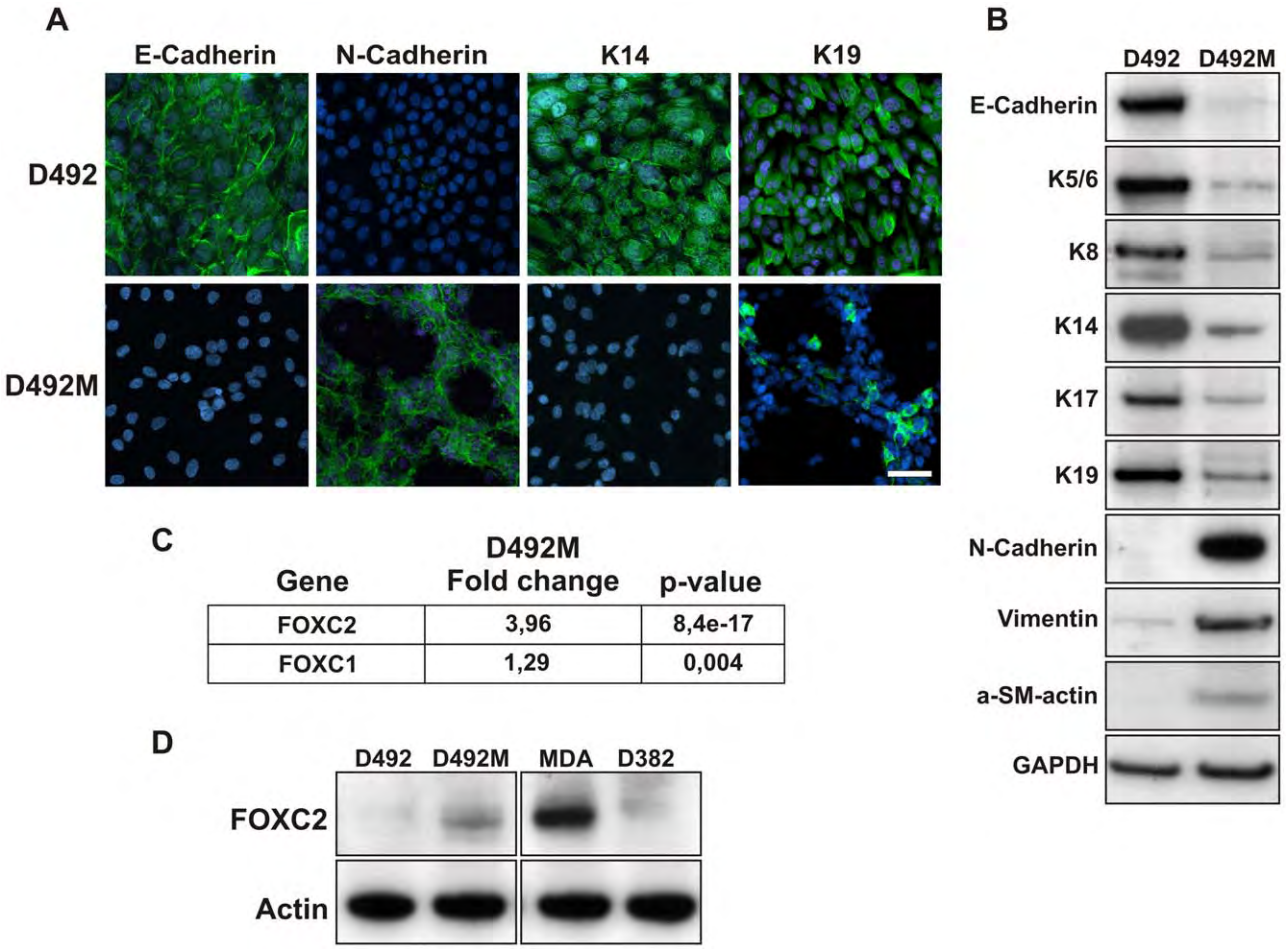


**Figure 3. D492M has acquired an EMT phenotype. A**, Immunofluoresence staining on D492M show switch from E- to N-Cad and reduced expression of K14 and K19. Counterstain TO-PRO-3, Bar 100 μm. **B**, Western blotting confirms downregulation of epithelial markers such as E-Cad, K-5/6, 8, 14, 17 and 19 in D492M. In contrast, the mesenchymal markers N-Cad, Vimentin and alpha-smooth muscle actin were expressed more intensively in D492M than D492. GAPDH loading control. **C**, EMT associated transcription factors are upregulated in D492M. Gene expression data showed upregulation of FOXC2 (3.96 fold,p: 8.4e-17) and FOXC1 (1.29 fold,p: 0.004) transcription factors in D492M. **D**, FOXC2 is strongly expressed in breast epithelial cell lines with EMT phenotype. Western blotting shows strong expression of FOXC2 in D492M and MDA-MB-231, an EMT-like breast cancer cell line, compared to no or low expression in D492 and D382. Actin, loading control.
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derived spindle-like colonies (D492M1-4). All these sublines were shown to have acquired an EMT phenotype (Fig. S6).

## D492M has acquired a functional EMT and cancer stem cell phenotype

A major characteristic of the mesenchymal phenotype is increased motility. In a transwell migration assay when compared to D492, the D492M cells showed increased migration, 3.8 fold ($p<0.05$) and 7.4 fold ($p<0.01$) when plated at $1\times10^4$ or $2.5\times10^4$ cells, respectively (Fig. 4A). Functionally, the D492M cells also showed signs of transformation by growth in soft agar assay. While D492 fail to grow, D492M grew well in this assay showing 6% cloning efficacy ($p<0.01$) (Fig. 4B). In addition, when cultured in monolayer, D492M formed multilayered ridges further indicating a loss of contact inhibition (Fig. 4B, right). The $CD44^{high}$, $CD24^{low}$ phenotype has been associated with cancer stem cell phenotype in the breast [32] and recently EMT-like traits have been added to this profile [6,7]. Flow cytometry analysis showed that the D492 cells contain a mixture of $CD44^{high}$,$CD24^{high}$ cells (81%) and $CD44^{high}$,$CD24^{low}$ cells (19%). In contrast, D492M showed marked increase in the proportion of $CD44^{high}$, $CD24^{low}$ cells (70%) (Fig. 4C).

Papers have demonstrated a strong correlation between the EMT phenotype and the ability to form mammospheres, an assay that functionally tests for breast stem cell properties [6,33]. When cultured in low attachment plates both D492 and D492M generated mammospheres demonstrating the self-renewal and cancer stem cell properties of these cell lines, respectively (Fig. 4D). However, D492M generated significantly larger and higher number of colonies (size>100 μm; $p<0.01$ and size>150 μm; $p<0.05$) in this assay (Fig. 4D). One of the hallmarks of cancer stem cells and EMT is the acquisition of apoptosis resistance [6,34]. D492M showed increased resistance ($p<0.05$) to chemically induced apoptosis (Fig. 4E). Thus, D492M has acquired phenotypic and functional characteristics of EMT cells and cancer stem cells.

## Endothelial induced EMT in D492 is generated through soluble factors partially mediated by HGF

To analyze if endothelial induced EMT in D492 was mediated through soluble factors we used transwell coculture with BRENCs cultured on top of a filter and D492 cells embedded in rBM, in the lower well (Fig. 5A). In this setup, BRENCs were even more

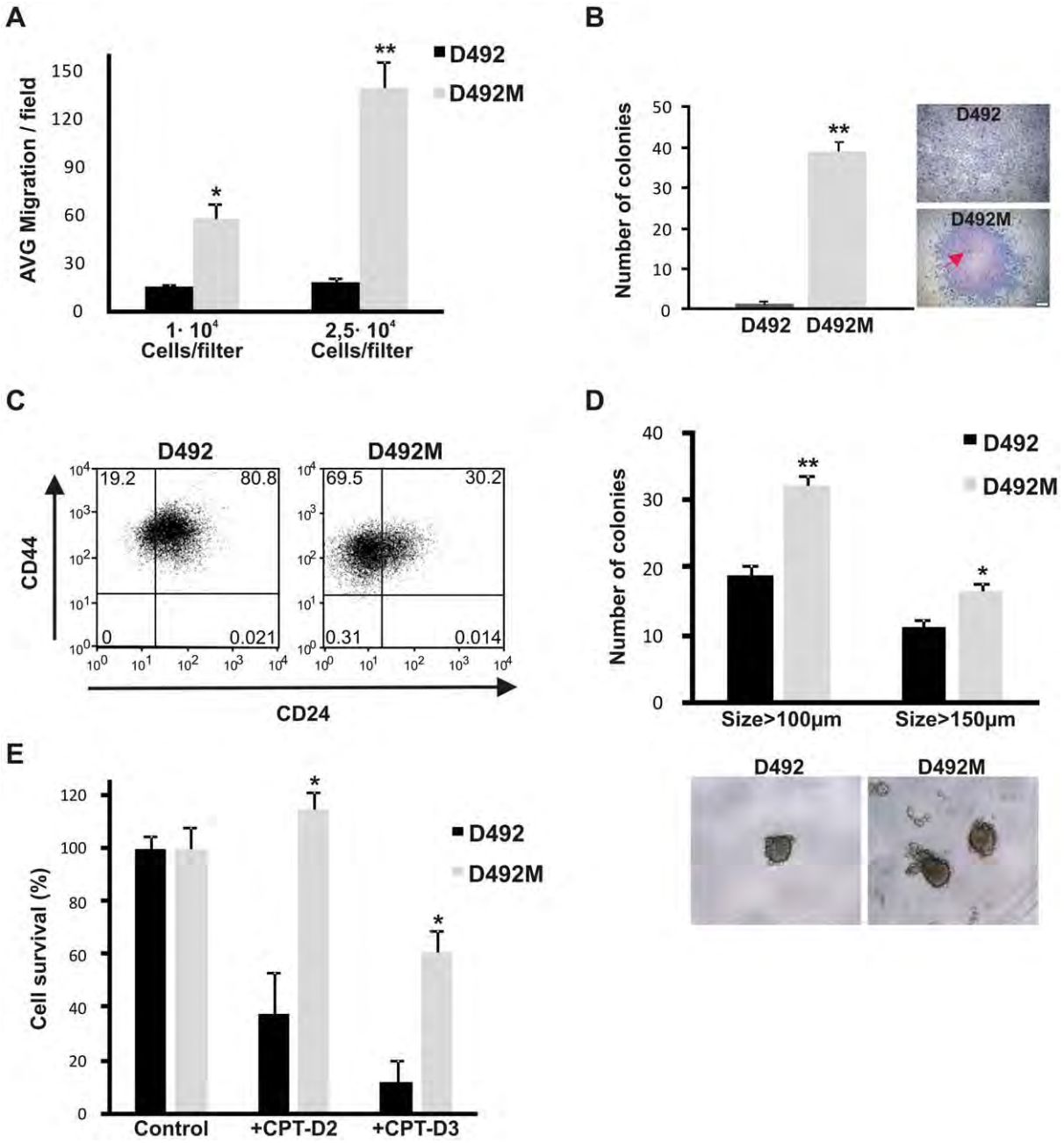


**Figure 4. D492M has acquired cancer stem cell-like phenotype. A**, D492M show increased migration compared to D492. Increased migration was seen at the two cell concentrations ($1\times10^4$ and $2.5\times10^4$). **B**, D492M grow anchorage independently. In 0.5% soft agar D492 cells fail to form colonies. In contrast, the D492M cells are able to grow, indicating acquisition of anchorage independent growth. In monolayer culture (right) D492 cells are contact inhibited while D492M piles up in the culture flask indicating lack of contact inhibition (arrows). Counterstain hematoxylin. Bar 100 µm. **C**, D492M cells are $CD44^{high}$,$CD24^{low}$ consisting with the breast cancer stem cell phenotype. D492 contain a subpopulation (19%) of cells that are $CD44^{high}$, $CD24^{low}$. This population increases to 70% in the D492M cell line. **D**, D492 and D492M differ in their ability to form mammospheres. Both D492 and D492M can generate colonies in mammosphere assay, however, D492M generates more and larger (>100 µm: 1.7 fold; >150 µm:1.5 fold) mammospheres than D492 cells. **E**, D492M cells show delayed chemically induced apoptosis. D492 and D492M show distinct responses to Camptothecin, an apoptosis inducing agent. D492 cells underwent immediate apoptosis and showed cell survival under 40% on day 2 while having no effect on D492M. On day 3 D492M cells showed cell survival of 60% where only few D492 cells were left. Data shown as AVG number of cells per field (A,E) or AVG number of colonies (B,D) +SEM in three experiments. *$p<0.05$; **$p<0.01$.
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effective in inducing the emergence of spindle-like colonies (Fig. 5B) suggesting endothelial-derived soluble factor/s. These spindle-like colonies show an EMT phenotype with an E- to N-Cad switch, reduced K14 and K19 expression and increased expression of vimentin and fibronectin (Fig. 5C). It should, however, be noted that in this setup a few small colonies grew in D492 monoculture and were either of solid round or spindle-like morphology. The reason for this is unknown but may be due to the difference in the experimental setup of the transwell compared to the direct coculture 3D experiments.

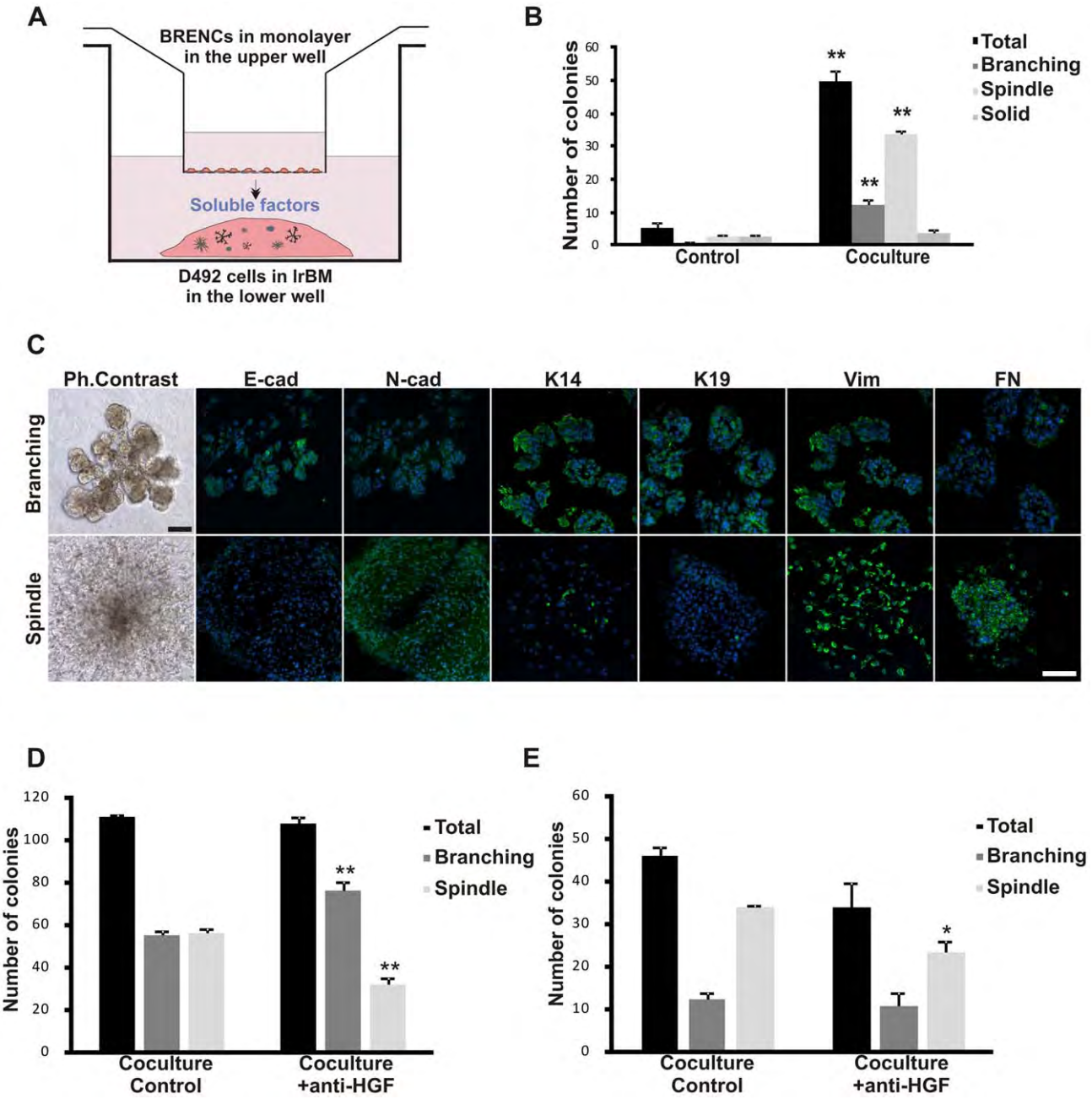


**Figure 5. Endothelial induced EMT is mediated through soluble factors and is partially blocked by inhibition of HGF. A**, In the transwell coculture setup endothelial cells were cultured as a monolayer in the upper well and D492 cells in 3D rBM on the bottom of the lower well. **B**, BRENCs induce spindle-like colony formation in transwell coculture. D492 cells without BRENCs showed limited growth (less than 1% of seeded cells, control). coculture the BRENCs induced a significant increase in number of spindle-like colonies. **C**, D492-derived branching colonies generated in transwell culture show characteristical epithelial phenotype including expression of E-Cad, K14 and K19. In contrast, D492-derived spindle like colonies show EMT phenotype including expression of N-Cad, Vimentin and fibronectin (FN). Bar 100 μm. **D–E**, Formation of spindle-like colonies is partially blocked by inhibition of HGF. Spindle-like colony formation is reduced with anti-HGF by 44% in direct coculture (D) and by 30% in transwell coculture (E). Data shown as AVG number of colonies +SEM in three experiments. *$p<0.05$; **$p<0.01$.
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There are a number of factors that can elicit EMT such as TGF-β1, FGF, EGF and HGF. As D492 did not form any EMT in the EGM5 coculture media that contains EGF, FGF and VEGF we set focused on TGF-β1 and HGF, known morphogenic and EMT inducing factors [3]. We treated 3D cocultures with a small molecule inhibitor targeting the TGF-β receptor-1 (ALK5) and with a TGF-β1 neutralizing antibody. We observed no changes in the number of spindle colonies using the ALK5 kinase inhibitor or the anti-TGF-β1 (not shown) indicating that other factors were responsible for the endothelial induced EMT.

HGF is expressed in endothelial cells and other stromal cells and can induce both scattering (including EMT) and morphogenic effects on epithelial cells [35]. In our 3D rBM assay BRENCs secreted HGF into the surrounding culture media as

measured by ELISA. They secreted over four times higher concentrations than D492 in this setup (Fig. S7). When coculture of D492 and BRENCS was treated with a neutralizing antibody against HGF a significant decrease ($p<0.01$) in spindle colonies was observed in contrast to a significant increase ($p<0.01$) in the formation of branching colonies (Fig. 5D). We also tested this in transwell coculture and as before BRENCs induced the emergence of spindle colonies. Neutralizing antibody against HGF significantly decreased ($p<0.05$) their number but had no effects on branching colonies (Fig. 5E). Collectively, this suggests that the balance in formation of branching or spindle colonies from D492 cells can be modulated by HGF signaling and that soluble HGF, at least partially, mediates endothelial induced EMT in our 3D coculture model.

### EMT phenotype in basal-like breast cancers is associated with vascular-rich areas

Circumstantial evidence suggests that basal-like breast cancers originate in epithelial stem or progenitor cells [14]. Furthermore, studies show that these tumors are highly vascularized [36,37] and rich in EMT associated markers such as N-Cad with low or no E-Cad expression [9,11]. Because both EMT and angiogenesis are associated with increased metastatic potential, we explored the possible connection between vascularization and the EMT phenotype within basal-like breast cancer. We stained 9 basal-like and four estrogen receptor positive (ER-positive) breast cancers with antibodies against E-Cad, N-Cad, K14, K19 and CD-31. While all ER-positive cancers were N-Cad and K14 negative, basal-like cancers were positive for N-Cad and K14, with some tumors showing medium-to-low expression of N-Cad (Fig. 6A). To study the possible association between vascularization and the EMT-phenotype, we quantified the microvascular density (MVD) in N-Cad medium-to-low areas and in N-Cad high areas. Microvessel density (MVD) was significantly higher in areas containing cells with high expression of N-Cad (MVD: $86.77\pm3.52$) compared to areas with low N-Cad expression (MVD: $36.66\pm4.01$) (Fig. 6B, 6C and Fig. S8). Low or no expression of E-Cad was seen in all basal-like biopsies tested (Fig. 6D). Thus the cellular context in basal like breast cancers reveals an interesting pattern of cancer cells showing an EMT phenotype closely associated with vascular rich components. Based on these findings we hypothesize that the endothelial compartment might contribute to the EMT phenotype of tumor cells within basal like breast cancer.

## Discussion

We report here, that in a 3D coculture model EMT-like cells arise from immortalized breast epithelial cells with stem cell properties upon interaction with breast endothelial cells. These effects are at least partially mediated through HGF with other endothelial-derived factors possibly involved. The endothelial induced transition resulted in a characteristic EMT phenotype as evidenced by marked difference in protein and gene expression with loss of many adhesion and epithelial specific markers and gain of mesenchymal markers. Functionally, the EMT cells showed increased migratory abilities and an increase in cancer stem cell phenotype. Furthermore, we show that basal-like breast cancers are rich in cells showing a potential EMT phenotype with highest intensity of N-Cad expression close to vascular rich areas.

EMT has recently been linked to basal-like breast cancer as demonstrated by upregulation of EMT markers (Vimentin, alpha-smooth muscle actin, and N-Cad) together with reduction of characteristic epithelial markers (E-Cad and keratins) [9,11]. This is supported by our observation that basal like breast cancers have features of EMT as evidenced by no or reduced expression of E-Cad and high expression of N-Cad. Interestingly, the strongest expression of N-Cad was seen in vascular-rich areas suggesting that endothelial cells may provide a favorable environment for the EMT phenotype. Intratumoral angiogenesis, assessed by microvessel density, has been proposed to identify patients at high risk of recurrence, especially in node-negative breast cancer. Meta-analyses have confirmed this association, although being a relatively weak risk factor [38]. More recent studies have shown that microvessel density might be a major risk factor in triple negative breast cancer [39] and vascular endothelial growth factor (VEGF), a marker of angiogenesis, has also been shown to be significantly higher in this subclass of breast cancer [40]. High MVD has also been associated with medullary breast tumors, which are a subtype of the basal-like group and with breast tumors with a predominant $CD44^{high}/CD24^{low}$ cancer stem cell phenotype [37,41]. Niu et al. have also showed in hepatocellular carcinoma, that tumors expressing Twist, a marker of EMT, have higher MVD [42].

EMT is a complex process and there have been numerous factors shown to elicit EMT in culture. Of these, TGF-β1 and ligands for various receptor tyrosine kinases have received much attention [34]. We report here that inhibition of TGF-β1 with a neutralizing antibody or an ALK5 inhibitor did not affect the formation of spindle-like colonies in coculture suggesting that TGF-β1 is not involved in endothelial induced EMT in the 3D-context. Interestingly, Mostov et al. reported that HGF induces partial EMT in MDCK cells cultured in 3D collagen gel [35]. The HGF receptor, c-Met has also been shown to have a higher expression in basal-like breast cancer than in other subtypes. Basal-like breast cancer are also enriched for gene sets indicating transcriptional activation induced by c-Met signaling [43]. Hypoxia, a major effector of endothelial cells has been shown to increase HGF mRNA stability through overexpression of HIF-1alpha [44]. Hypoxia has also been shown to increase the expression of c-Met, leading to increased sensitivity to HGF and an invasive phenotype in the tumor cells [45]. In our study, endothelial cells were shown to secrete HGF in 3D culture and when HGF was blocked with a neutralizing antibody in direct- and indirect (transwell) coculture a significant reduction in the number of EMT colonies was observed demonstrating that endothelial-derived HGF is, at least partially, responsible for EMT in our culture model. These findings suggest a novel role for endothelial cells and angiogenesis in cancer progression in addition to the more classical role of oxygen and nutritional delivery.

Defining the cellular and microenvironmental cues that trigger EMT during cancer progression is important. Studies have shown increased expression of EMT markers at the tumor-stroma interface [15,16] and stromal cells are now recognized as major players in cancer progression (reviewed in [17,18]). The stromal compartment includes various cell types, e.g. fibroblasts (and myofibroblasts), immune cells and endothelial cells. Fibroblasts and myofibroblasts have received attention as important players in tissue morphogenesis and neoplasia [17,46]. We have previously shown that breast cancer cells can generate non-malignant fibroblast-like cells that can facilitate growth and invasion of cancer cells [31]. Myofibroblast have been shown to induce EMT and tumor progression in a hepatocellular carcinoma mouse model through PDGF and TGF-beta signaling [47]. Recently, CD8 positive T cells have been shown to induce EMT in mouse mammary cancer cells. Following T cell-induced EMT, these

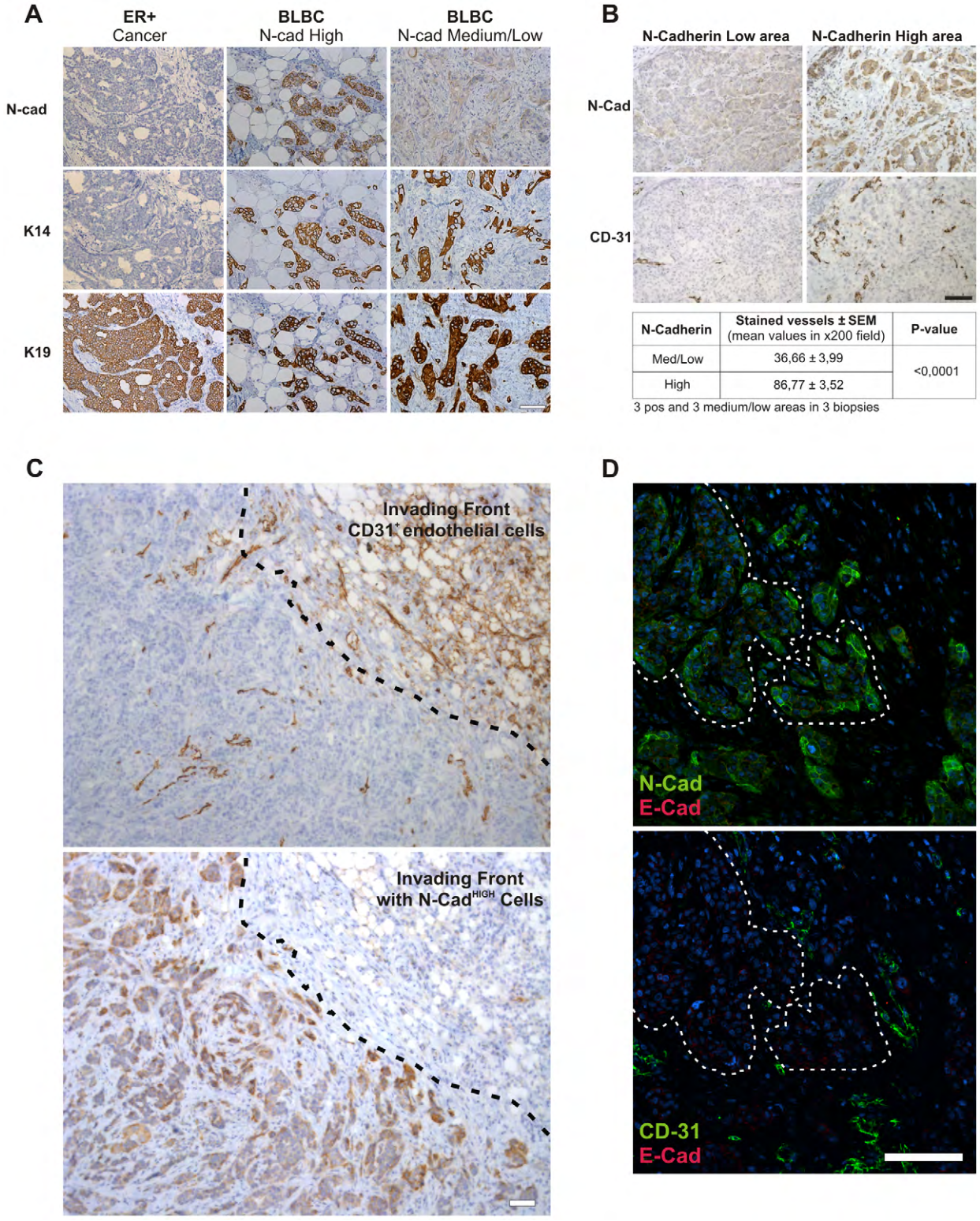


**Figure 6. The EMT phenotype is most prominent close to vascular rich areas in basal-like breast cancer. A**, N-Cad expression is most prominently found within basal-like breast cancer. ER tumors are K19 positive but negative for N-Cad and K14. In contrast basal-like breast cancers (BLBC) are positive for all three markers. Bar 100 μm. **B**, Increased microvessel density in basal-like breast cancer is associated with areas containing cells with high expression of N-Cad. Immunostaining show increased CD31 positive microvessels in areas with high N-Cad expression. Statistcal analyzis (bottom) from three basal-like breast cancer biopsies show significant increase in microvessels within areas with high N-Cad expression. **C**, Expression of CD-31 reveals highly vascularized area at the tumor stroma interface. N-Cad expression was seen in most cancer cells. Note the strong expression of N-Cad close to the vascular rich area (dashed line). **D**, Double-labeling against E- (red) and N-Cad (green) in the same area shows strong expression of N-Cad only. Low or no expression of E-Cad (red) was seen close to the CD31 positive (green) endothelial cells. Cells were counterstained with hematoxylin (A B and C) and TO-PRO-3 (D). Bar, 100 μm.
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cancer cells acquired cancer stem cell phenotype including increased $CD44^{high}/CD24^{low}$ ratio, drug resistance and increased tumorigenicity [48].

Although EMT can easily be recognized in monolayer culture of cells, recognizing these cells *in situ* is more troublesome, due to its transient nature. In contrast to monolayer cultures, 3D culture models capture more closely the *in vivo* situation [49]. Papers from our laboratory and others have shown the importance of 3D cultures to elucidate the functional role of the stroma as an instructive factor in normal breast morphogenesis and cancer progression [17,18,49,50,51]. Numerous cell lines, such as MCF10A and MCF-7, have been reported to be susceptible to EMT in traditional monolayer culture [52]. Our results, however, show that in 3D culture EMT induction by BRENCs is only achieved in selected cell lines, i.e. those harboring stem/progenitor characteristics (D492) and/or cell lines that have cancer initiating abilities (MDA-MB 231). We also show that primary metaplastic breast cancer cells, W2330 [31], can be facilitated to undergo EMT in 3D coculture with BRENCs. In contrast the luminal epithelial cell line D382, MCF10A and MCF-7 show no signs of EMT in coculture with BRENCs. Even though MCF10A has been shown to have a basal-like phenotype, they lack fundamental stem cell properties that D492 has, such as branching morphogenesis that may explain why they are non-responsive to endothelial induced EMT in 3D cultures.

Recent studies have shown that induction of EMT in immortalized human breast epithelial cells was associated with acquisition of cancer stem cell associated properties, measured by increased expression of $CD44^{high}/CD24^{low}$ cells accompanied by the ability to form mammosphere colonies in culture [6,7]. In these studies, immortalized breast epithelial cells (HMECs) were induced to undergo EMT in 2D culture conditions with TGF-β1 or transfected with potent inducers of EMT such as snail, Twist or the ras oncogene. These studies are in line with our data where D492M show cancer stem cell and tumorigenic phenotype as evidenced by an increased ratio of $CD44^{high}/CD24^{low}$ cells, ability to form mammospheres, increased motility, anchorage independent growth and resistance against chemically induced apoptosis. It is noteworthy that in our study, D492, a cell line with epithelial stem cell properties, appear to lose the normal epithelial stem cell properties (i.e. generating differentiated luminal and myoepithelial cells and forming branching TDLU-like structures) after undergoing EMT and acquire a phenotype associated with cancer stem cells. This suggests an important difference between the properties of breast epithelial stem cells and epithelial cancer stem cells. Studies linking cancer stem cells and EMT also raise interesting questions about the cell renewal, developmental plasticity and signaling pathways involved in cancer progression.

In this paper we show that in basal like breast cancer, cells undergoing EMT are enriched in the vascular-rich areas and furthermore, we show that endothelial cells can directly induce EMT. This endothelial-induced EMT is at least partially facilitated by HGF making this a potential novel therapeutic target for patients with the basal-like subtype of breast cancer. Furthermore, our findings suggest a role for endothelial cells in basal-like breast cancer suggesting that therapy targeting the neovascular compartment might be relevant.

## Supporting Information

**Figure S1 Endothelial cells cultured in rBM appear as single, non proliferative but metabolically active cells.** Endothelial cells cultured for 10 days within rBM remain as single non proliferative but metabolically active as seen by the uptake of fluorescent labeled Ac-LDL (green). Insert shows single endothelial cells that have taken up Ac-LDL in higher magnification.
(TIF)

**Figure S2 Spindle-like colony formation increases proportionally with the amount of endothelial cells.** Increased number of BRENCs in coculture with D492 results in decreased and increased number of solid and spindle-like colonies. No effect was seen on branching colonies. AVG % of colonies +SEM in triplicate. *, $p<0.05$; **, $p<0.01$; compared to $5\times10^4$ BRENCs.
(TIF)

**Figure S3 BRENCs facilitate mesenchymal phenotype in MDA-MB-231 a poorly differentiated breast cancer cell line.** To explore if BRENC could induce EMT in other cell types we set up cocultures of BRENCs ($2\times10^5$ cells) with MCF10A, MCF-7, D382 and MDA-MB-231 (500 cells). Coculture of BRENCs with MCF-10A, D382 and MCF-7 resulted in non-branching, non-EMT-like epithelial colonies. In contrast coculture of BRENCs with the highly malignant cancer cell line MDA-MB-231 resulted in large EMT-like colonies. Bar 100 μm.
(TIF)

**Figure S4 D492 and D492M share a common origin. A**, Origin of D492M confirmed by viral insertional analysis. D492 cell line contains a retroviral insertion of E6 and E7 genes. The insert site was identified (schematic) on chromosome 20q13.1 close to the gene PTP1N that codes for the protein tyrosine phosphatese 1B (PTP1B). PCR analyzes identified the same insert in D492M confirming its origin from D492. **B**, GFP positive D492 cells give rise to mesenchymal colonies in coculture with BRENCs. The origin of mesenchymal colonies from D492 was confirmed by using GFP positive D492. All colonies in the 3D culture were GFP positive. Bar = 100 μm.
(TIF)

**Figure S5 Gene expression analysis demonstrates global changes in D492-D492M transition.** Heat map showing the top 50 genes discriminating D492 and D492M. Red and green shows up- and down regulation of genes, respectively.
(TIF)

**Figure S6 Characterization of four mesenchymal-derived cell lines from D492.** D492-derived mesenchymal cell lines designed D492M1-M4 were characterized in terms of expression profile and for functional mesenchhymal properties. **A.** D492M1 show reduced expression of E-cadherin and EpCAM, weak expression of N-Cad and strong expression of fibronectin (FN) and vimentin. **B.** D492M-1 show increased migration compared to D492. **C.** Mesenchymal cell lines derived from D492 show advanced growth in soft agar. **D.** Summary of phenotypic and functional characteristics of D492M1-M4.
(TIF)

**Figure S7 BRENCs secreted HGF into the surrounding culture media.** BRENCs secreted HGF into the surrounding culture media as measured by ELISA. BRENCs secreted over four times higher concentration of HGF than D492 when cultured rBM.
(TIF)

**Figure S8 N-cadherin expression is prominent around vascular rich area of basal-like breast cancers.** Two basal like breast cancer were stained with antibodies against N-Cad and CD31. Figures show N-Cad high and N-Cad medium/low areas

within the same cancer stained with N-Cad and CD31. Cells counterstained with heamotoxylin. Bar = 100 μm.
(TIF)

**Methods S1** Supplementary material and methods.
(DOC)

## Author Contributions

Conceived and designed the experiments: VS MKM TG. Performed the experiments: VS HS AJRF MR RV BAA BH. Analyzed the data: VS HS MR AB BAA. Wrote the paper: VS OWP MKM TG BH.

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## Supplementary material and methods

### Cell culture

The breast epithelial stem cell line D492 and daughter cell line D492M were maintained in H14 medium (Briand, Petersen et al. 1987), consisting of DMEM/F12, 50 IU/ml penicillin, 50 µg/ml streptomycin (Invitrogen), 250 ng/ml insulin, 10 µg/ml transferrin, 2,6 ng/ml sodium selenite, 0,1 nM estradiol, 0,5 µg/ml hydrocortisone, 5 µg/m l prolactin (SIGMA) and 10 ng/ml EGF (Peprotech). Luminal epithelial cell line referred to as D382 and MCF10A were also maintained on H14 medium. MDA-MB 231 was cultured on RPMI-1640 supplemented with 5% FBS. W2320 and MCF-7 on DMEM/F12 with 5% FBS. The MCF-7, MCF10A and MDA-MB-231 cell lines where purchased from ATCC and are routinely authenticated with genotype profiling according to ATCC guidelines. To further ensure cell line integrity D492, D492M and D382 cell lines were analyzed with the same method.

### Generation of GFP positive D492 cells

Lentiviral pGIPZ vector (RHS4346) expressing green fluorescent protein (GFP) (Open Biosystems, Huntsville, AL) was transfected into HEK-293T cells using Arrest-In transfection reagent (ATR1740; Open Biosystems) according to instructions. Virus-containing supernatants were collected at 48 hours after transfection and target cells infected in the presence of 8ug/ul polybrene. 24 hours later, drug selection was done with 3ug/ul puromycin to establish stable cell line expressing GFP.

### Preparation of 3D cocultures

Coculture experiments were carried out in 24 well culture plates (BD Falcon) with 500 D492 cells alone (monoculture) and with , $5x10^4$, $1x10^4$ and $2x10^5$ BRENCs (cocultures). The two cell types were mixed and suspended in 300µl rBM and cultured in EGM5 for 15 days. Cocultures of BRENCs with normal breast epithelial lines MCF10A and D382, estrogen receptor positive breast cancer cell line MCF-7, basal-like / EMT breast cancer cell line MDA-MB-231

and primary metaplastic breast cancer cell line W2320 were done with 500 epithelial cells and $2x10^5$ BRENCs in EGM5 medium.

**Isolation of 3D coculture colonies, replating and secondary 3D coculture**

Branching, solid and spindle-like structures were isolated from 3D cocultures with gentle shaking on ice in PBS - EDTA (5mM) solution. Single structures were placed in a 24 well plate and cultured on H14 medium. Monolayer cultured cells from branching, solid and spindle like colonies were then put back into 3D coculture of 500 cells with $5x10^4$ BRENCs in rBM.

**ELISA and additional blocking experiments**

BRENCS and D492 were seeded in 3D monocultures in rBM and the HGF concentration in the culture media was determined using comercially available HGF ELISA kit (DHG00; R and D, MN, USA) according to instructions. ALK5 kinase inhibitor (SB431542, Tocris Bioscience) was used to block signals through the ALK5 receptor and was diluted in the rBM (10μM) and in the medium (10μM) in coculture of 500 D492 cells and $5x10^4$ BRENCs. We also blocked TGFβ1(8μg/ml) in 3D coculture with a neutralizing antibody (ab10517, Abcam) in the rBM and in the medium.

**Microvessel density scoring**

Microvessel counting was conducted as previously described [1,2]. Briefly, microvessel density was evaluated by immunohistochemistry of tumor vessels for CD31 in whole tissue sections. An immunopositive cell or cluster of cells clearly separated from adjacent clusters, was considered an individual vessel. Microvessels were counted in three different areas of low and high N-cadherin expression, respectively, in three different biopsies in a 200x field.

**Endothelial uptake of AcLDL in 3D rBM cultures**

Endothelial cells have the ability to take up AcLDL and this trait has become routine to identify them in culture. BRENCs were treated with 15 μg/ml AcLDL conjugated to A488 fluorecent dye (Invitrogen) for 4 hrs. The uptake of AcLDL-A488 was monitored on day 10 in 3D cultures of BRENCs.

**Retroviral insertion analysis**

The D492 cell line was initially established by transfection with a retroviral vector containing the E6 and E7 oncogenes and the neomycin resistant gene for selection [3]. To identify the genomic insertion site of the E6/E7 containing retrovirus we performed an inverse PCR (I-PCR) (Suzuki et al., 2002) by using 5 μg of cell line DNA digested with 60 U of BamHI overnight in 40 μl. After heat inactivation DNA was diluted to 200 μl, circularized by ligation with T4 DNA ligase at 16°C overnight, ethanol precipitated, and resuspended in 30 μl of Tris-EDTA. PCR was performed in 25 μl with 1 μl of the DNA template, 0,2 mM deoxynucleoside triphosphates, 10 pmol of each primer, 1,3 U of Expand Long Template Polymerase, and Expand Buffer 1 (Roche). The primers used were I-1F (CTAGCTTGCCACCTACGGGT) and I-1R (TGAGGAAATTGAGGCACAGC). The cycling conditions were 94°C for 2 min, followed by 10 cycles of 94°C for 10 s, 65°C for 30 s, and 68°C for 6 min and 20 cycles of 94°C for 10 s, 65°C for 30 s, and 68°C for 6 min with a 20-s autoextension and a final extension at 68°C for 10 min. Amplified products were cloned into the TA cloning TOPO vector (Invitrogen) and clones selected and sequenced. We identified with this method a single insertion on chromosome 20, 95 kb upstream of the gene PTPN1 (encoding for the phosphatase PTP1B).

**Gene expression analysis**

RNA was isolated from D492 and D492M at 50% and 90% confluency in monolayer culture using RNeasy mini kit (QIAGEN). Experiments were conducted in triplicate, on three different time points (36 samples). RNA was analysed on NanoDrop ND-1000 spectrophotometer and run on Agilent 2100 Bioanalyzer chip. Microarray analysis was carried out using the Illumina BeadChip expression microarray (HumanWG-6 v3.0) platform. The data was background subtracted and normalized using cubic spline with all samples as a group using BeadStudio. Probes were quality filtered such that if p detect >0,01 then the intensity was replaced with a missing value. Probes with missing values for all 36 hybridizations were omitted from future analysis. This left 16547 probes which had p value <=0,01 in at least one hybridization. To

identify differentially expressed genes we used the MeV software (www.tm4.org) and the significance of microarrays (SAM) method [4]. Genes that had detectable expression levels in 50% of the samples were used in the comparison. All raw data are available at http://stofnanir.hi.is/rle/sites/stofnanir.hi.is.rle/files/EMT-expression%20profile%20D492-vs-D492M.xlsx

**Supplementary figure legends**

**Supplementary figure 1. Endothelial cells cultured in rBM appear as single, non proliferative but metabolically active cells.** Endothelial cells cultured for 10 days within rBM remain as single non proliferative but metabolically active as seen by the uptake of fluorescent labeled Ac-LDL (green). Insert shows single endothelial cells that have taken up Ac-LDL in higher magnification.

**Supplementary figure 2**. **Spindle-like colony formation increases proportionally with the amount of endothelial cells.** Increased number of BRENCs in coculture with D492 results in decreased and increased number of solid and spindle-like colonies. No effect was seen on branching colonies. AVG % of colonies +SEM in triplicate. *, p<0,05; **, p<0,01; compared to $5x10^4$ BRENCs.

**Supplementary figure 3**. **BRENCs facilitate mesenchymal phenotype in MDA-MB-231 a poorly differentiated breast cancer cell line.** To explore if BRENC could induce EMT in other cell types we set up cocultures of BRENCs ($2x10^5$ cells) with MCF10A, MCF-7, D382 and MDA-MB-231 (500 cells). Coculture of BRENCs with MCF-10A, D382 and MCF-7 resulted in non-branching, non-EMT-like epithelial colonies. In contrast coculture of BRENCs with the highly malignant cancer cell line MDA-MB-231 resulted in large EMT-like colonies. Bar 100 µm.

**Supplementary figure 4. D492 and D492M share common origin. A,** Origin of D492M confirmed by viral insertional analysis. D492 cell line contains a retroviral insertion of E6 and E7 genes. The insert site was identified

(schematic) on chromosome 20q13.1 close to the gene PTP1N that codes for the protein tyrosine phosphatese 1B (PTP1B). PCR analyzes identified the same insert in D492M confirming its origin from D492. **B,** GFP positive D492 cells give rise to mesenchymal colonies in coculture with BRENCs. The origin of mesenchymal colonies from D492 was confirmed by using GFP positive D492. All colonies in the 3D culture were GFP positive. Bar =100 µm.

**Supplementary figure 5. Gene expression analysis demonstrates global changes in D492-D492M transition.** Heat map showing the top 50 genes discriminating D492 and D492M. Red and green shows up- and down regulation of genes, respectively .

**Supplementary figur 6. Characterization of four mesenchymal-derived cell lines from D492**

D492-derived mesenchymal cell lines designed D492M1-M4 were characterized in terms of expression profile and for functional mesenchhymal properties. **A**. D492M1 show reduced expression of E-cadherin and EpCAM, weak expression of N-Cad and strong expression of fibronectin (FN) and vimentin. **B.** D492M-1 show increased migration compared to D492. **C.** Mesenchymal cell lines derived from D492 show advanced growth in soft agar. **D.** Summary of phenotypic and functional characteristics of D492M1-M4.

**Supplementary figure 7. BRENCs secreted HGF into the surrounding culture media.** BRENCs secreted HGF into the surrounding culture media as measured by ELISA. BRENCs secreted over four times higher concentration of HGF than D492 when cultured rBM.

**Supplementary figure 8. N-cadherin expression is prominent around vascular rich area of basal-like breast cancers.** Two basal like breast cancer were stained with antibodies against N-Cad and CD31. Figures show N-Cad high and N-Cad medium/low areas within the same cancer stained with N-Cad and CD31. Cells counterstained with heamotoxylin. Bar =100 µm.

## Supplementary references


1. Lopes N, Sousa B, Vieira D, Milanezi F, Schmitt F (2009) Vessel density assessed by endoglin expression in breast carcinomas with different expression profiles. Histopathology 55: 594-599.
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3. Gudjonsson T, Villadsen R, Nielsen HL, Ronnov-Jessen L, Bissell MJ, et al. (2002) Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties. Genes Dev 16: 693-706.
4. Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 98: 5116-5121.

**Figure S1**

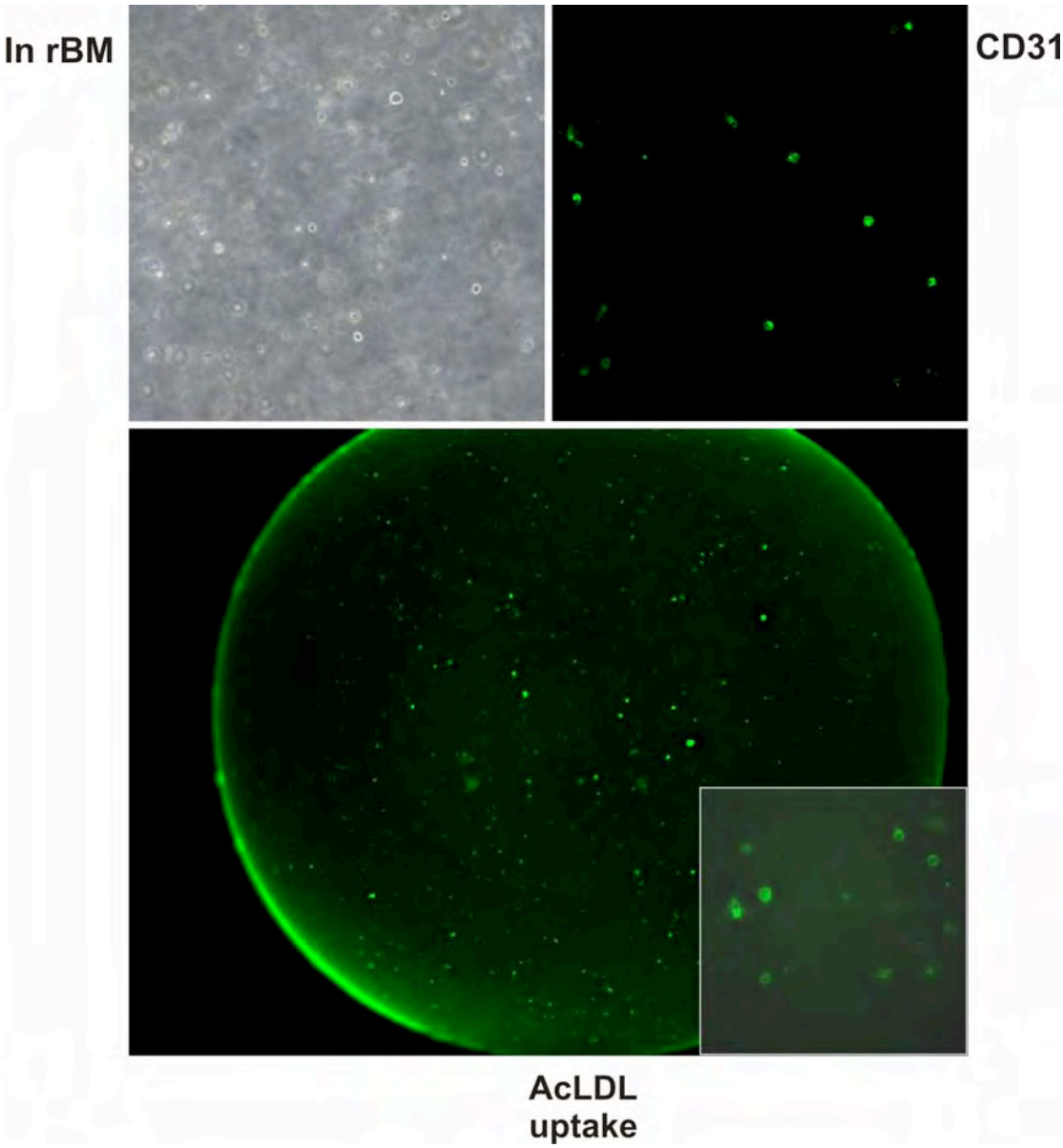
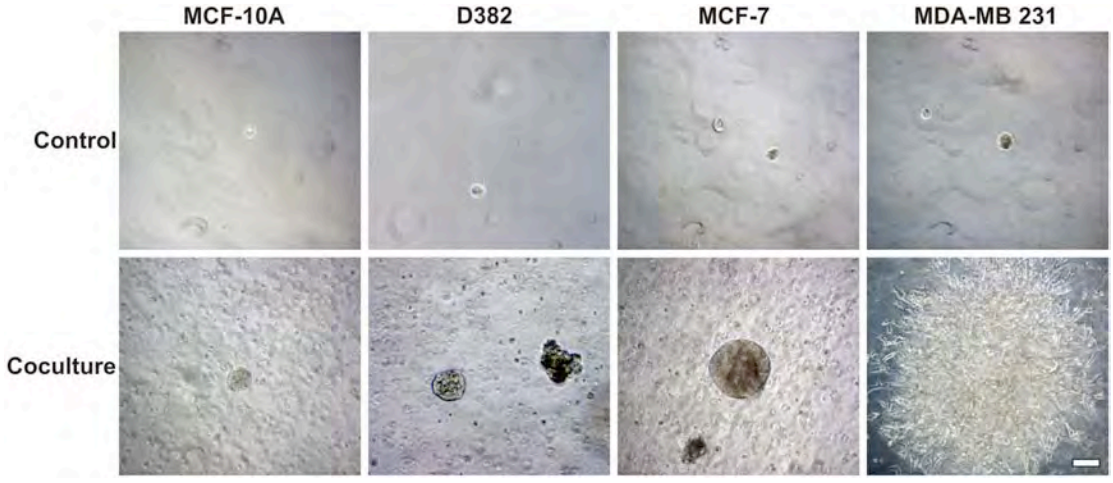


**Figure S2**


**Figure S3**



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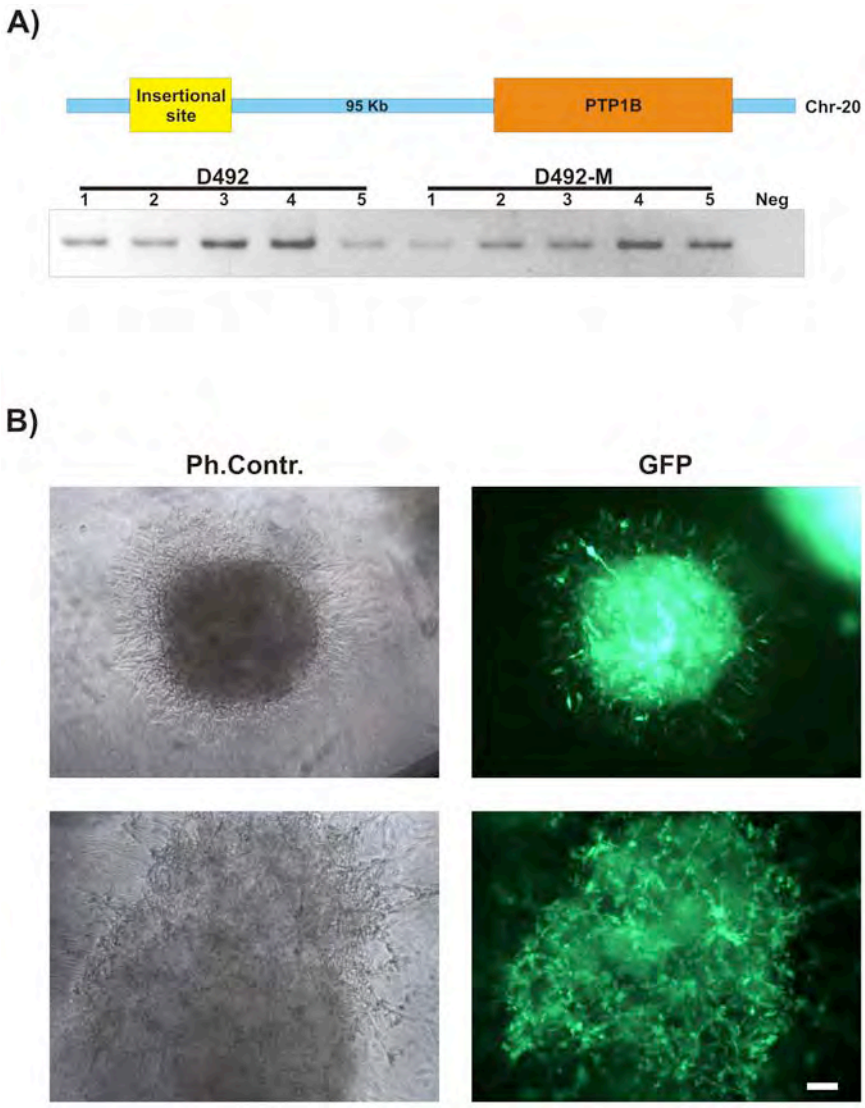
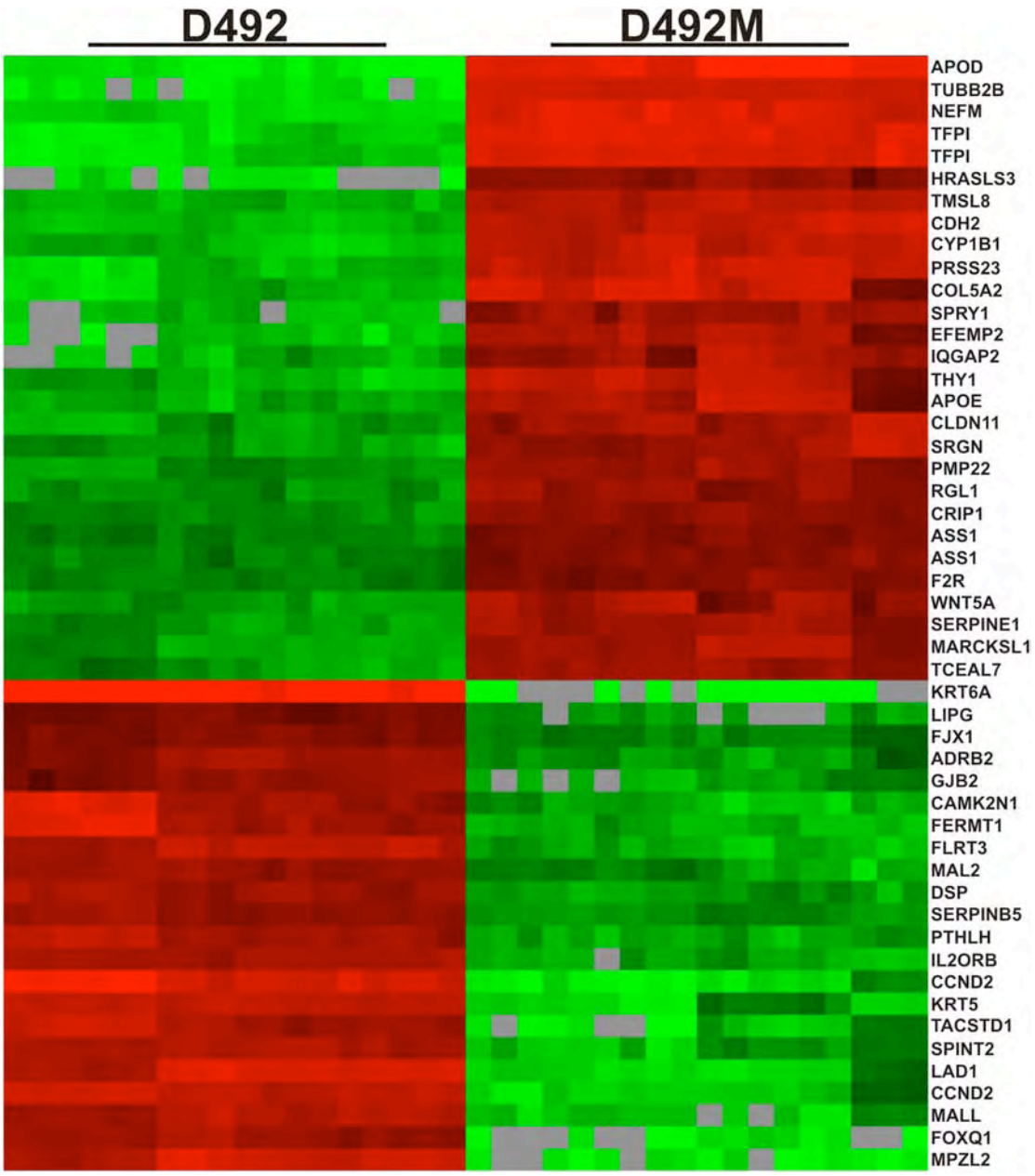

A)
Insertional site
95 Kb
PTP1B
Chr-20
D492
D492-M
1 2 3 4 5
1 2 3 4 5
Neg
B)
Ph.Contr.
GFP

**Figure S5**


D492
D492M
APOD
TUBB2B
NEFM
TFPI
TFPI
HRASLS3
TMSL8
CDH2
CYP1B1
PRSS23
COL5A2
SPRY1
EFEMP2
IQGAP2
THY1
APOE
CLDN11
SRGN
PMP22
RGL1
CRIP1
ASS1
ASS1
F2R
WNT5A
SERPINE1
MARCKSL1
TCEAL7
KRT6A
LIPG
FJX1
ADRB2
GJB2
CAMK2N1
FERMT1
FLRT3
MAL2
DSP
SERPINB5
PTHLH
IL2ORB
CCND2
KRT5
TACSTD1
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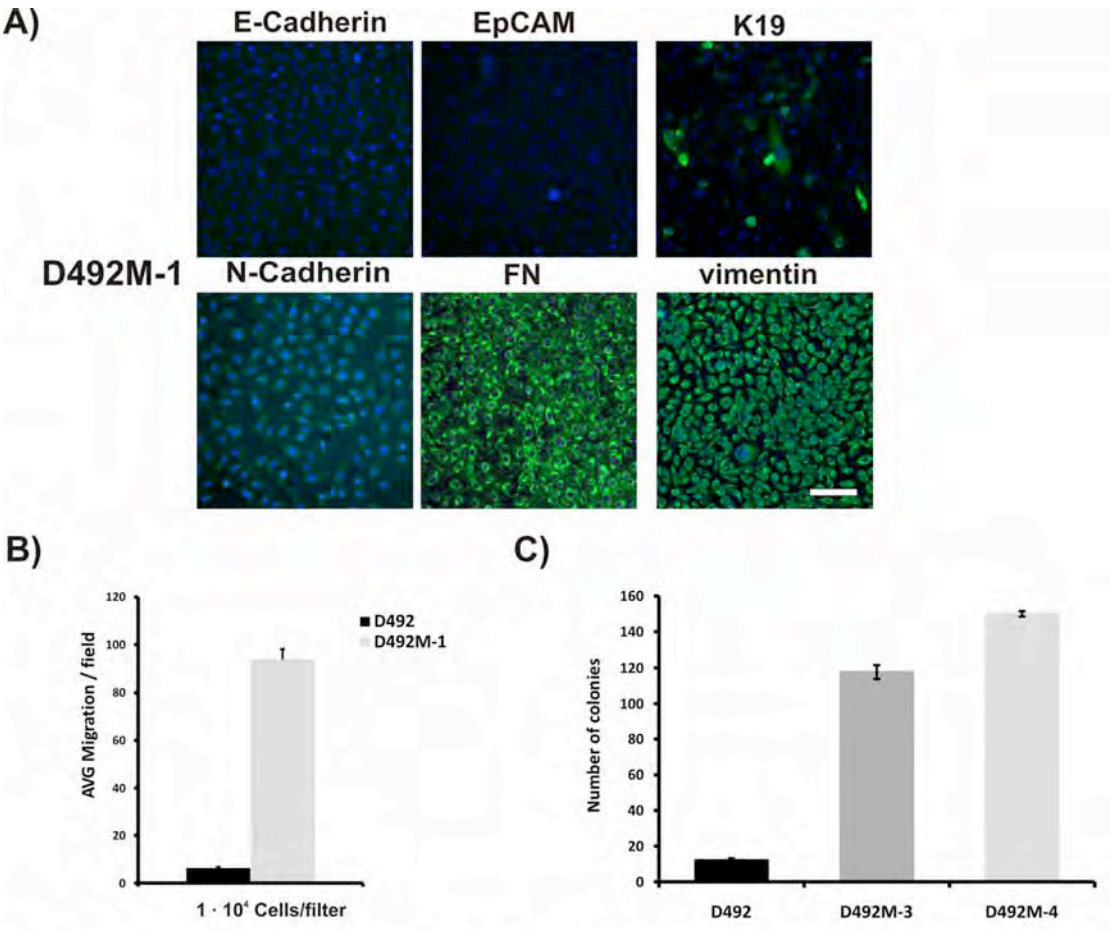
LAD1
CCND2
MALL
FOXQ1
MPZL2

**Figure S6**




D)

| | N-cadh | E-cadh | K14 | K19 | Migration | Soft agar | Colonies in 3D |
|---|---|---|---|---|---|---|---|
| D492M-1 | + | - | +/- | -/+ | +++ | + | Mesenchymal |
| D492M-2 | +/- | - | +/- | -/+ | ++ | ND | Mesenchymal |
| D492M-3 | + | - | -/+ | +/- | +++ | + | Mesenchymal |
| D492M-4 | + | - | -/+ | +/- | ++ | + | Mesenchymal |

**Figure S7**

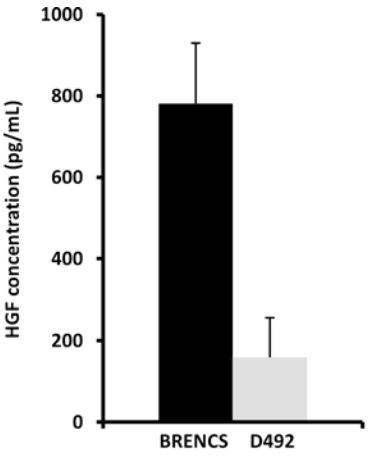
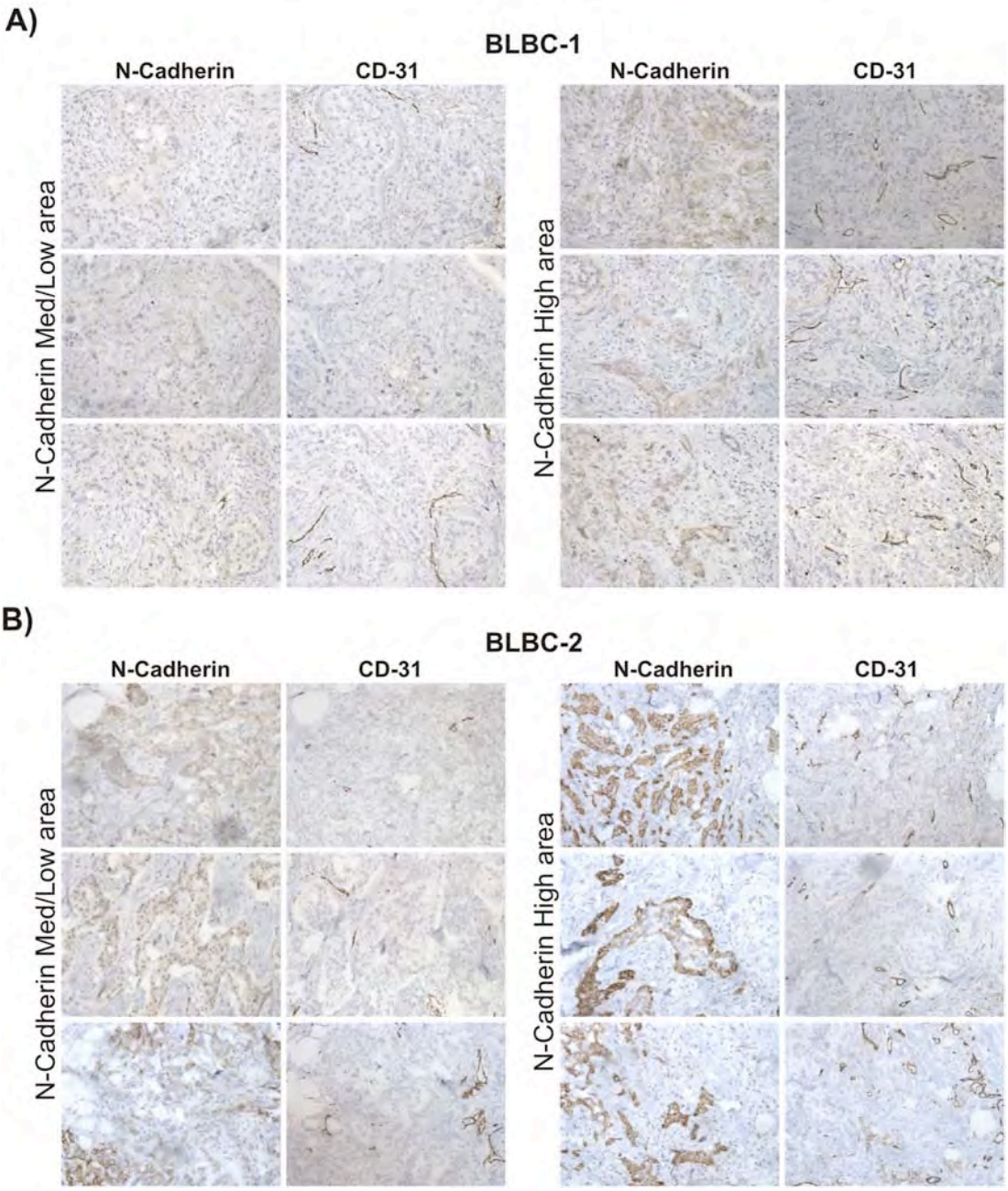


**Figure S8**


A)
BLBC-1
N-Cadherin
CD-31
N-Cadherin
CD-31
N-Cadherin Med/Low area
N-Cadherin High area
B)
BLBC-2
N-Cadherin
CD-31
N-Cadherin
CD-31
N-Cadherin Med/Low area
N-Cadherin High area

# Paper #4

# Branching morphogenesis in the mammary gland is regulated by sprouty-2

Valgardur Sigurdsson[1,2,], Bylgja Hilmarsdottir, Saevar Ingthorsson, Sigridur Rut Franzdottir, Eirikur Steingrimsson, Magnus K. Magnusson[1,2]& Thorarinn Gudjonsson[1,2]

[1]Stem Cell Research Unit, Biomedical Center, School of Health Sciences, University of Iceland.
[2]Department of Laboratory Hematology Landspitali University Hospital, Iceland.

Keywords: Breast morphogenesis/ RTK / sprouty/ Breast cancer /EMT

Corresponding authors:

Thorarinn Gudjonsson, Email: tgudjons@hi.is

Magnus Karl Magnusson, Email: magnuskm@hi.is

***Abstract***

Branching morphogenesis is a conserved mechanism used by many species for organogenesis and tissue maintenance. Receptor tyrosine kinases (RTKs) including the epidermal growth factor receptor (EGFR) and their intracellular regulators sprouty protein family are believed to be critical regulators of branching morphogenesis. In this study, we show that, Sprouty-2 (Spry-2) is predominantly expressed in the luminal epithelial cells both in ducts and lobuli in the human breast gland. We have also analyzed the expression of Spry-2 and EGFR pathway in virgin, lactating and pregnant mouse mammary gland. Spry-2 is expressed at branching epithelial buds during pregnancy with increased expression during lactation. The expression of pEGFR show similar expression pattern as Spry-2. Using D492 a breast epithelial cell line with stem cell properties, which generate branching structures in 3D culture we show that Spry-2 expression increases during the formation of branching. Immunostaining locates expression of Spry-2 and pEGFR at the tip of lobular-like, branching ends. Interestingly, Spry-2 knock down (KD) results in increased migration and in larger and more complex branching structures indicating loss of negative feedback to control spatial location of cells and branching morphogenesis. In co-culture with endothelial cells D492 sprouty KD generate spindle like colonies reminiscent of epithelial to mesenchymal tansition. In conclusion, these data indicate that Spry-2 is an important regulator of branching morphogenesis and epithelial to mesenchymal transition in the mammary gland.

***Introduction***

Branching morphogenesis is a highly conserved developmental process, where epithelial-based organs are able to increase their surface area and form the correct functional histoarchitecture. (Davies, 2002; Horowitz and Simons, 2009). This process gives rise to the airways of the lungs (Metzger et al., 2008), the urine collecting ducts (Costantini and Kopan), the prostate (Cunha et al., 2004), salivary glands (Hsu and Yamada, 2010) and the mammary glands (Ewald et al., 2008; Fata et al., 2004).

The molecular events that induce and regulate branching morphogenesis are highly conserved between different organs and between different species (Lu et al., 2006). Receptor tyrosine kinases (RTKs), such as fibroblast growth factor receptors (FGFRs) and epidermal growth factor receptors (EGFRs) are key mediators of signals that regulates proliferation, differentiation and branching morphogenesis in the mammary gland (Davies, 2002; Dillon et al., 2004). (Extracellular cues such as FGFs and EGFs act via their respective receptors to activate, among other pathways, the mitogen-activated protein kinase (MAP-kinase) pathways and phosphatidylinositol 3-kinase (PI-3 kinase) pathways which are critical for proper development of branched organs (Davies, 2002). Indeed, aberrant expression of RTKs such as the EGFR family is common in number of cancers including breast cancer (reviewed in (Gutierrez and Schiff, 2011)). Signaling must be precisely regulated both spatially and temporally to ensure normal homeostasis. Recent studies have underscored the importance of negative feedback control of RTKs signaling in ensuring correct cell fate and morphogenesis (Amit et al., 2007). Sprouty, initially shown to be critical for tracheal development in drosophila (Hacohen et al., 1998), is now known to act as a conserved antagonist of RTK signaling in higher eukaryotes (Gross et al., 2001; Hanafusa et al., 2002; Impagnatiello et al., 2001; Lee et al., 2001; Sasaki et al., 2001; Tefft et al., 2002; Yusoff et al., 2002). There are four mammalian sprouty proteins (Sprouty1-4) and they have been proposed to participate in a classical negative feedback loop on RTK signaling through ras/Erk/Mek pathway. However, detailed molecular mechanism of the action of the sprouty proteins has not been fully elucidated. The studies of sprouty in the mammalian system have thus far mostly focused on the regulation of FGFR

and EGFR (Mason et al., 2006).. Sprouty proteins have been identified as antagonists of FGFR, c-Met and EGFR in lung, kidney and vasculogenesis but their role in the human breast gland morphogenesis has not been systematically analyzed (Kim and Bar-Sagi, 2004).

In mouse mammary glands the branching ducts ends in terminal end buds (TEB) whereas in humans, breast ducts are more elaborate and terminates in the lobuli commonly referred to as the terminal duct lobular units (TDLU) (Rønnov-Jessen et al., 1996). The TDLUs are composed of differentiated luminal- (LEP) and surrounding myoepithelial (MEP) cells, separated from the stroma by basement membrane. Branching morphogenesis in the mammary gland is believed to occur through collective migration of both LEP and MEP cells where epithelial cells at the branching end loose adhesion and acquire transient mesenchymal-like phenotypes through epithelial to mesenchymal transition (EMT) that enables cells with active motility and invasion (Ewald et al., 2008; Micalizzi et al., 2010). Indeed, temporal EMT phenotypes have also been linked to cancer progression and metastasis (Hanahan and Weinberg, 2011; Mani et al., 2008; Micalizzi et al., 2010). This temporal activation of EMT in both cancer progression and branching morphogenesis highlights the importance of understanding the molecular regulators of breast morphogenesis. Indeed, disruption in the regulation of RTKs, critical regulators of branching morphogenesis, is also major factor seen in many cancer forms, including breast cancers (Davies, 2002). Lo et al. have recently shown that sprouty-2 gene expression is suppressed in breast cancers suggesting that sprouty-2 could function as a tumor suppressor (Lo et al., 2004), linking candidate morphogenic pathways to cancer progression.

Three-dimensional cultures have proven to be important tools to recapitulate in-vivo like context in the mammary gland (Gray et al., 2010; Kenny et al., 2007). We have previously shown that D492 a suprabasal epithelial cell line with stem cell properties generates TDLU-like structures in 3D culture (Gudjonsson et al., 2002; Villadsen et al., 2007). D492 is thus a good model to dissect molecular mechanisms regulating branching morphogenesis. We have recently shown that breast endothelial cells (BRENCs) stimulate growth and morphogenesis of breast and lung epithelial

cells (Franzdottir et al., 2010; Ingthorsson et al., 2010). Interestingly when-cultured with BRENCs in rBM D492 generate spindle-like colonies with EMT phenotype (Sigurdsson et al., 2011). In this paper, we show that sprouty-2 is abundant in luminal epithelial cells of duct and lobuli in human breast tissue. We also show that sprouty-2 is abundant in lactating mouse mammary gland and its expression is associated with expression of phosphorylated EGFR and downstream MAPK signaling pathway. Using D492 cell line that generates TDLU-like structures in rBM we show that sprouty-2 is expressed at the branching tips. Suppression of sprouty-2 through shRNA gene knockdown increases branching morphogenesis and promotes epithelial to mesenchymal transition when cultured with endothelial cells.

## *Material and methods*

### Cell culture

The breast epithelial stem cell line D492 was maintained in H14 medium (Briand, Petersen et al. 1987), consisting of DMEM/F12, 50 IU/ml penicillin, 50 µg/ml streptomycin (Invitrogen), 250 ng/ml insulin, 10 µg/ml transferrin, 2.6 ng/ml sodium selenite, 0.1 nM estradiol, 0.5 µg/ml hydrocortisone, 5 µg/ml prolactin (SIGMA) and 10 ng/ml EGF (Peprotech). Primary LEPs and MEPs on CDM3 and CDM4 as previously described (Inthorsson et al. 2010). Primary human BRENCs were isolated from breast reduction mammoplasties as previously described (Sigurdsson et al. 2006) and cultured on endothelial growth medium (EGM-2) (Lonza) + 5% FBS (Invitrogen).

### Preparation of 3D mono- and co-cultures

3D monocultures were carried out in 96 well culture plates (BD Falcon), 7 x $10^3$, 1 x $10^4$ and 1,3 x $10^4$ D492 cells were suspended in 300µl of rBM (Matrigel, BD). Co-culture experiments were carried out with 1 x $10^3$ D492 mixed with 5 x$10^4$ BRENCs. 100µl of mixed cells / rBM were seeded in each well in a 96 well plate and cultured on H14 (MC) or EGM5 (CC) for 16 days.

### Isolation and processing of mammary glands and 3D cell cultures

Tissue from human breast reductions were used for IHC and isolation of primary breast epithelial cells. Primary LEPs and MEPs were isolated by magnetic cell sorting (MACS) as previously described (REF). Mammary glands were isolated from C57BL/6 mice from 6 week old virgins, on day 15 of pregnancy and on day 2 of lactation. Mammary glands were snap frozen in liquid nitrogen and preserved at -80°C. The tissue was cryosectioned into 15µm sections and mounted on slides. Isolation of whole colonies from 3D cell culture was done as previously described by gentle dissociation in PBS-EDTA buffer (Lee et al., 2007).

**Immunochemistry**

Formalin-fixed, paraffin embedded tissue blocks were cut into 5µm serial sections and mounted on slides. Sections were deparafinized and rehydrated in xylene and ethanol. Antigen retrieval was done by boiling in EDTA buffer for 15 min. Frozen mouse mammary glands were cryosectioned at 15µm setting following formalin fixation. The following primary antibodies were used; Sprouty-2 (#07-524, Upstate/Millipore), CD-31 (M0823, DakoCytomation), Keratin 19 (ab7754, Abcam), Keratin 14 (NCL-LL002, NovoCastra), PCNA (ab29, Abcam), EGFR (#4267, Cell Signaling), p-EGFR (Tyr1068) (#3777, Cell Signaling). Fluorescent nuclear counterstain, TO-PRO-3 (Invitrogen) was used in IF. Specimens were visualized on a Zeiss LSM 5 Pascal laser-scanning microscope (Carl Zeiss).

**Western blotting**

Equal amounts (5 µg) of proteins were separated on 10% NuPage Bis-Tris gels (Invitrogen) and transferred to a PVDF membrane (Invitrogen). Antibodies: Sprouty-2 (1:2000, #07-524, Upstate/Millipore), Erk (1:2000, #4695, Cell Signaling), p-Erk (1:2000, #9101, Cell Signaling), β-actin (1:5000; ab3280, Abcam) were used. Membranes were visualized with ECL+ after incubation with anti-mouse or rabbit secondary antibody(1:5000) (GE healthcare).

**Q-RT-PCR**

Total RNA was extracted with Trizol (Invitrogen), DNAase treated and reverse transcribed with Hexanucleotides using ReverAid (#K1622, Fermentas). Resulting cDNA was used for Q-RT-PCR, in master mix (Applied Biosystems) with primer pairs and probes for Spry2 (Hs00183386_m1, AB) and GAPDH (AB). Experiments were done in triplicate on 7500 Real Time PCR System (AB). Quantitations of Spry2 mRNA levels were normalized to GAPD and relative mRNA difference was calculated with the $2^{-\Delta Ct}$ Method.

**Spry2 KD by shRNA**

pGIPZ lentiviral shRNA constructs targeting Sprouty2 transcripts were purchased from Open Biosystems (RHS4430-101098640, RHS4430-101103852, RMM1766-96881511). A non-silencing construct (RHS4346) was used as a control. Viral particles were produced in HEK-293T cells using Arrest-In transfection reagent (ATR1740; Open Biosystems) according to instructions. Virus-containing supernatants were collected at 48 hours after transfection and target cells were infected in the presence of 8ug/ul polybrene. Stable, D492, Spry-2 KD cells were established by puromycin selection (3µg/µl) as well as selection for green fluorescent protein (GFP) expression.

**Migration and proliferation assay**

For migration experiments a total of $2,5 \cdot 10^4$ starved cells were seeded in DMEM/F12 basic medium on collagen coated upper compartment of a transwell Boyden chamber (Corning) with an 8µm pore size. EGM5 medium was used as a chemoattractant in the lower chamber. After 18h incubation, cells in the upper chamber were removed with a cotton swab and migrated cells on the bottom surface stained with 0,1% crystal violet. Cells were counted in three representative fields in each transwell. In the proliferation assay, $10^4$ cells were seeded per well in a 24 well plate (Falcon, BD). Cells were fixed with formalin and stained with 0,1% crystal violet (days 1-5), washed and left to dry. The crystal violet staining in each well was dissolved in acetic acid and measured at 570nm in a plate reader.

**Statistical analysis**

Data is presented as mean +SEM from number of independent experiments as indicated. Statistical analysis was performed by two-tailed Students T-test using GraphPad . P values of <0,05 were considered to be statistically significant.

## *Results*

### *Sprouty-2 is predominantly expressed in luminal epithelial cells in the human breast gland*

To explore the expression of Sprouty-2 (Spry-2) in the human breast gland we stained tissues from reduction mammoplasties and analyzed the expression of isolated breast epithelial cells in culture. Characterization of Spry-2 expression in normal human breast tissue revealed a distinct expression pattern between the epithelium and stroma. Spry-2 expression was seen in all epithelial cells, both in large ducts and in the terminal duct lobular units (TDLU) (Fig. 1A). The same expressional pattern was seen when we used in situ hybridization (Fig. S1). Spry-2 was also detected in discreet areas in the stroma, most likely endothelial cells (Fig. 1A). Dual labeling with antibodies targeting linage restricted markers, i.e. keratin 18 (luminal epithelial cells), keratin 14 (myoepithelial cell) and Spry-2, demonstrated that Spry-2 was predominantly expressed within the luminal epithelial compartment (Fig. 1A). This was supported by analyzing the expression of Spry-2 in purified myoepithelial and luminal epithelial cells using quantitative real-time PCR. Luminal epithelial cells showed 15-58 fold higher expression of Spry2 compared to myoepithelial cells (Fig. 1B). A disadvantage of studying sprouty expression in tissue from reduction mammoplasty specimens is that we are unable analyze expression during different developmental stages of branching morphogenesis. We therefore moved to mouse mammary gland for such analysis.

### *Sprouty-2 expression in the pregnant and lactating mouse mammary gland is associated with activated EGFR signaling*

Accumulating evidence exist that the sprouty protein family is an important regulator of branching morphogenesis in various organs (Kim and Bar-Sagi, 2004). To see how Spry-2 is expressed during mammary gland branching morphogenesis we isolated mammary glands from virgin, pregnant and lactating mice and analyzed EGFR and Spry-2 expression during these critical developmental periods. Expression of phosphorylated EGFR (p-EGFR) was low in the virgin mammary gland but increased focally at branching end buds

in the pregnant gland. Furthermore, a dramatic increase in p-EGFR was seen in end buds during lactation (Fig. 2A). Increased Spry-2 expression was seen during pregnancy that reached its highest expression level during lactation associated with p-EGFR expression (Fig. 2A). p-EGFR and Spry-2 expression was inversely correlated with cell proliferation as evidenced by dramatic reduction in cell proliferation (PCNA staining) during lactation (Fig. 2A). Spry-2 expression was also accompanied with increased signals through EGFR downstream signaling pathway measured by total- and pospho-ERK, especially during the lactational period (Fig. 2B). Thus increased Spry-2 expression was associated with activation of the EGFR/Erk/MEK signaling pathway. These data suggest that Spry-2 and pEGFR/ERK/MEK signaling work together to maintain maximal differentiation state during mouse mammary lactation.

***Spatial and temporal expression of sprouty-2 and EGFR interactive pathway during branching morphogenesis of breast epithelial cells in 3D culture***

To explore the functional role of Spry-2 in branching morphogenesis in the human breast we used the D492 cell line cultured in 3D rBM. D492 has stem cell properties, can differentiate into luminal- and myoepithelial cells and through branching morphogenesis forms TDLU-like colonies when cultured within a 3D rBM (Gudjonsson et al., 2002; Villadsen et al., 2007). We first analyzed temporal expression of Spry-2 during TDLU formation in 3D rBM. D492 cells undergo most of their branching during days 8-16 in 3D rBM culture (Fig. 3A). Initially, D492 forms solid round colonies that start to branch on days 10-12 (primary branching). After the first branching event ductal structures elongate and secondary branching occurs with bifurcation or trifurcation on the lobular-like ends (Fig. 3A). To analyze Spry-2 expression we isolated mRNA from culture days, 8, 10, 12, 14 and 16. Pre-branching, round colonies show high expression of Spry-2. Interestingly, during the formation of primary branching (day 10-12) the expression of Spry-2 decreases. At day 16 elaborated TDLU-like structures have formed and the expression of Spry-2 is increased to more than 4fold compared to day 10 (Fig. 3B). Expression was also confirmed with an immunoblotting on D13, D16 and

D19 showing the increase in Spry-2 expression from D13 to D16. Interestingly, p-EGFR is expressed at similar level as evidenced by increased expression from D13 to D16 but is lowered on D19 when further branching has stopped (Fig. 3C). This expression pattern suggests that Spry-2 might have a regulatory role during the temporal formation of branching structures and the formation of lobular units at the ductal ends. In support of this, immunofluorescence staining of branching colonies at day 16 shows that Spry-2 expression is mainly concentrated at the branching, lobular-like tips but is lowered at sites of cleft formation (Fig. 3D). The location of Spry-2 at day16 is similar to that of p-EGFR at branching tips while staining for total EGFR has a more general distribution in the branching colonies (Fig. 3D). Staining for **β**4-integrin and F-actin expression show the general outlines of the branching structures and its connection to the surrounding basement membrane (Fig. 3D).

**Sprouty-2 knock down in D492 promotes increased branching morphogenesis**

To further explore the functional role of Spry-2 in the regulation of branching morphogenesis we knocked down Spry-2 in D492 and explored their proliferation migration and morphogenic potential. We used a lentiviral approach where D492 was transfected with a GFP-containing non-silencing (NS) control and 3 different knock down (KD) shRNA constructs (Spry2 KD1, Spry2-KD2 and Spry2-KD3) targeting Spry2. The Spry-2 KD3 construct was most effective, decreasing Spry-2 levels 4 fold (Fig. 4A-B) and continuing work focused on this knock down construct and a single cell subclone referred to as Spry2 KD3a. There was no morphological difference seen between NS cells and KD cells when visualized in monolayer (Fig. 4C) but $D492^{Spry2\text{-}KD3}$ and $D492^{Spry2\text{-}KD3a}$ showed increased migration compared to $D492^{NS}$ cells (Fig. 4D). There was, however, no significant difference in the proliferation of $D492^{Spry2\text{-}KD3}$ cells and $D492^{NS}$ (Fig. 4E). To analyze the effects of sprouty-2 knockdown on branching morphogenesis we compared the cell lines in 3D rBM culture. $D492^{Spry2\text{-}NS}$ generated *in vivo*-like 3D branching colonies similar to wild type D492 while $D492^{Spry2\text{-}KD3}$ and $D492^{Spry2\text{-}KD3a}$ showed increased branching (Fig. 5A). The effect of sprouty-2 knockdown was quantified by

counting colonies of simple/early branching, complex/late branching and other (mostly solid round colonies) morphology (Fig. 5B). In a setup with $1x10^4$ cells both $D492^{Spry2\text{-}KD3}$ and $D492^{Spry2\text{-}KD3a}$ cell lines formed more branching colonies in total and substantially more colonies that showed the complex branching phenotype in comparison to $D492^{Spry2\text{-}NS}$ cells (Fig. 5C). Large complex colonies (>250μm) were twofold more common in both $D492^{Spry2\text{-}KD3}$ and $D492^{Spry2\text{-}KD3a}$ cell lines compared to $D492^{Spry2\text{-}NS}$ cells (Fig. 5D). All cell lines were cultured in three different cell concentrations ($1.3\ x10^4$, $1x10^4$ and $7x10^3$) due to the fact that different degree of branching is observed with different number of cells seeded in the rBM. In general less branching was seen in culture with higher cell density but the $D492^{Spry2\text{-}KD3}$ cells formed more branching colonies in all cell concentrations (Fig. 5E). When we looked at the expression of Spry-2 at D16 in $D492^{Spry2\text{-}NS}$ and $D492^{Spry2\text{-}KD3}$ cells we could see that the $D492^{Spry2\text{-}NS}$ cells showed normal expression of Spry-2 at the lobular tips while the $D492^{Spry2\text{-}KD3}$ cells showed markedly suppressed expression (Fig. 5F).

**Endothelial cells in co-culture stimulate branching morphogenesis and induce EMT in $D492^{Spry2\text{-}KD}$ cells**

As previously published, endothelial cells stay as single viable, nonproliferative, and functionally active cells in 3D culture in rBM (Ingthorsson et al., 2010) and when cocultured with epithelial cells they support morphogenesis and improve clonal efficiency (Franzdottir et al., 2010; Ingthorsson et al., 2010). Furthemore, we have recently shown that in coculture, breast endothelial cells (BRENCs) induce breast epithelial cells (e.g. D492 cells) to undergo EMT (Sigurdsson et al., 2011). When D492 cells were cocultured with BRENCs we saw marked stimulation in branching morphogenesis of D492 cells. Branching TDLU-like structures in coculture were generated from as few as 100 D492 cells in 300 μl rBM compared to the usual amount of 10,000 cells used in monoculture in this assay (Fig. 6A). Immunophenotypic characterization of the TDLU-like structures generated in co-cultures revealed distinct luminal- and myoepithelial differentiation as shown by expression of cytokeratin 19 and 14, respectively (Fig. 6A). Dual immunostaining against cytokeratin 14 and CD31 demonstrates the location

of endothelial cells surrounding the TDLU structures (Fig. 6A). Thus, TDLU-like colonies generated in co-culture with BRENCs mimic TDLU´s *in situ* with a bi-layered epithelium consisting of inner layer of luminal epithelial cells, outer layer of myoepithelial cells and extralobular location of endothelial cells.

When D492 cells were co-cultured with BRENCS they formed larger branching colonies in coculture but as reported previously they also underwent an epithelial to mesenchymal transition (Sigurdsson et al., 2011). D492 NS cells formed50% EMT-like colonies and 40% branching colonies while D492$^{Spry2\text{-}KD3}$ cells formed over 65% EMT-like colonies. The D492$^{Spry2\text{-}KD3a}$ clone which produced larger and a higher number of branching colonies in the monoculture was also used in coculture and interestingly they exclusively produced EMT-like colonies. Partial EMT is known to occur during branching and this suggests that Spry2 might regulate branching through temporal suppression of EMT during the branching process.

**Discussion**

The sprouty protein family is increasingly recognized as a key regulator of receptor tyrosine kinases signaling in different species and organs where Spry-2 has captured most attention (Basson et al., 2005; Chi et al., 2004; Taniguchi et al., 2007). In this study, we have analyzed the expression of Spry-2 in mouse and human mammary gland. In the human breast gland sprouty-2 were equally expressed in ducts and TDLU, however, its expression was most prominent within the luminal epithelial cells. We demonstrated that expression of Spry-2 is low in virgin mouse mammary gland but is focally increased at branching tips during pregnancy and reaches maximum expression during lactation. The expression of p-EGFR/ERK correlated with expression of sprouty-2 but correlated inversely with cell proliferation. Previously, Lo et al demonstrated by *in situ* hybridization that Spry-2 was highly expressed in pregnant mouse mammary gland but disappeared during lactation (Lo et al., 2004). The difference between their data and ours could be explained by the fact that they were analyzing mRNA expression but in our study we were focusing on protein expression.

The functional role of sprouty in branching morphogenesis during trachea development in drosophila was first evidenced in spry-/- mutants were excessive morphogenesis was seen (Guy et al., 2003). Tefft et al. (Tefft et al., 1999) demonstrated that inhibition of mSpry-2 expression in mouse embryo at E11,5 produced a significant increase in lung branching. Development of the uretic bud is an example of controlled branching morphogenesis that is regulated by sprouty proteins (Basson et al., 2005). In the nephric duct cells with higher Ret tyrosine kinase receptor expression preferentially moves to the dorsal nephric duct adjacent to the metanephric mesenchyme where they form the first uretic bud. Interesting Spry1-/- mutant show elevated expression of RET and increased branching (Costantini and Kopan, 2010). These data demonstrate the regulatory role of sprouty proteins during branching morphogenesis.

Data from mouse studies show that the mammary organoid branches and migrates by bifurcation and collective migration (Fata et al., 2007). Furthermore end buds and TDLU formation requires growth factor induced cell proliferation and studies show that this cell proliferation is mediated

through Erk1/2 (Ewald et al., 2008; Fata et al., 2007). These results correlate with our data where D492 captures in 3D culture by collective migration the morphogenic process in the mammary gland including the formation of TDLU like structures. Sprouty-2 expression was most prominent at the pheripheral branching buds and lost or significantly reduced in clefts/furrows similar to the expression pattern seen in the growing mammary gland in pregnant mice. The same expression pattern was also seen for p-EGFR. This indicates that D492 cell line can in addition to generation of TDLU-like structures capture important signaling pathways involved in morphogenesis. During branching morphogenesis epithelial cells need to transiently activate critical mesenchymal properties to be able to invade the surrounding matrix. This mesenchymal transition proceeds gradually under tight control of morphogenic signals and under regulation of the microenvironment (Andrew and Ewald, 2010). Branching morphogenesis can therefore be regarded as partial EMT that is under tight control from the surrounding microenvironment or from within the invading cell. When we suppress sprouty-2 expression in D492 we see hyperplasia-like effect and increased branching morphogenesis.

It is becoming clear that the stromal microenvironment plays a critical role in tissue morphogenesis including breast morphogenesis (Bissell et al., 2002). It is also widely acknowledged that cancer progression is dependent on signals from the surrounding microenvironment (Ronnov-Jessen and Bissell, 2009). Fibroblasts and extracellular matrix molecules, such as laminin, fibronectin and extracellular matrix-entrapped growth factors have received much attention (Hanahan and Weinberg, 2011). Our recent results demonstrate that endothelial cells stimulate growth and morphogenesis of breast epithelial cells (Ingthorsson et al., 2010) and induce EMT (Sigurdsson et al., 2011). We have also shown that endothelial cells can induce bronchial epithelial cells with stem cell properties to generate bronchioalveolar branching structure in 3D culture (Franzdottir et al., 2010). Interestingly, in coculture of endothelial cells and D492 we see dramatic increase in TDLU formation. This demonstrates the proliferative and morphogenic induction from endothelial cells. When we coculture endothelial cells with D492$^{\text{Spry2-KD3}}$ we see increase in the induction of EMT-like colonies and the single cell derived clone D492$^{\text{Spry2-KD3a}}$ show almost complete transition to mesenchymal

phenotype when cocultured with BRENCs. These data provide possible insights into the cellular mechanisms behind sprouty induced morphogenesis. Sprouty might act as a temporal modulator of RTK signaling through suppression of EMT.

In summary, we show here that sprouty-2 which is expressed at branching buds in pregnant and lactating mouse mammary glands is also expressed in human breast gland predominantly in the luminal epithelial compartment. Furthermore, in 3D culture D492 capture the histarchitecture of the breast gland including the spatial expression pattern of sprouty-2 and pEGFR seen during mouse mammary gland remodeling. Interestingly knock down of sprouty-2 result in increased migration and advanced branching phenotype. In addition, coculture with endothelial cells result in facilitated epithelial to mesenchymal transition. In conclusion, these data show that generation of branching TDLU-like structures are regulated by sprouty-2. Furthermore, our data indicate that sprouty-2 is an important regulator of epithelial integrity as sprouty-2 KD in D492 cells make the prone to endothelial induced EMT.

**Figure legends**

***Figure 1. Expression of sprouty-2 in lobules and ducts in the normal human breast gland***

*A) Expression of spry-2 is most prominent in the luminal epithelial cells. Sprouty expression was predominantly found within the epithelial compartment of duct and lobuli (top panel).* Sprouty-2 expression was predominantly expressed in luminal epithelial cells both in ducts and lobuli. Sprouty was costained for the K14 (myoepithelial, middle panel) and K18 (luminal, bottom panel)). Sections were counterstained with TOPRO-3. Bar =100μm.

*B) Expressional differences of sprouty-2 in luminal- and myoepithelial cells.* Real time PCR was used to quantify expressional difference of sprouty-2 between luminal- and myoepithelial cells. Sprouty-2 expression was generally low in myoepithelial cells compared to luminal epithelial cells that expressed up to 58 fold more sprouty-2.

**Figure 2. Expression of sprouty-2 in virgin, pregnant and lactating mouse mammary gland.** *A) Expression of sprouty-2 and pEGFR is inversely correlated with cell proliferation.* pEGFR expression is not seen in the virgin mouse mammary gland but focal expression is seen at terminal buds in pregnant gland. Dramatic increase in pEGFR expression is seen in the lactating gland. Similar expression is found for sprouty-2. Low expression is found in the virgin gland with few positive stromal cells. In the pregnant gland there is small increase in the epithelium. Note the strong expression in the stroma which disappears during lactation. Proliferation is increased from virgin to pregnant gland but disappear during lactation with only few PCNA positive cells left. Cells counterstained with TOPRO-3, Bar=100μm.

*B) Sprouty-2 expression is highest during lactation accompanied by activation of Erk/MAPK pathway.* Western blot demonstrated the expression differences of sprouty-2 in virgin, pregnant and lactating gland. There is over 38 fold increase in sprouty-2 expression during lactation compared to virgin state.

Total Erk and pERK is also significantly increased during lactation. Actin used as a loading control.

***Figure 3. Spry2 expression is correlated with critical points in branching morphogenesis of D492 breast stem cell line***

*A) D492 generate branching structures when cultured in rBM.* When seeded in rBM D492 generate TDLU-like structures. By following in vitro TDLU formation it is possible to follow individual steps in the branching morphogenesis process. Until day 8 or 9 cells grow as single colonies. First sign of initial budding occurs at day 10 and 11(yellow arrows) followed by duct elongation and bifurcation blue and red arrows, respectively.

*B-C) Sprouty-2 expression shows a dramatic shift during TDLU formation in 3D culture*

Colonies were isolated from 3D culture at different time points as indicated. Initially at day 8 there is relative high expression of Spry2 but its expression is reduced during initial budding but increases again during duct elongation and further bifurcation of complex branching. Western blot confirms that Spry2 levels increase up to day 16 and remain high while p-EGFR is slightly decreasing for day 16 to day 19. Actin used as a loading control.

*D) pEGFR and Sprouty-2 are expressed at the growing tips of TDLU-like structures.* TDLU-like structures were stained with antibodies against sprouty-2, EGFR, pEGFR, β4-integrin and F-actin. pEGFR was predominantly expressed at the branching tips while total EGFR had a more general distribution. Sprouty-2 was also expressed on branching tips but not in clefts. F-actin staining gives a general outlook of a branching colony while β4-integrin outlines their connection to the surrounding rBM matrix. Counterstained with TOPRO3 nuclearstain. Bar=100μm.

***Figure 4. Sprouty-2 Knock down in D492 results in increased migration***

*A and B) D492 show significant knock down of Sprouty-2.* D492 were transfected with non-silencing(NS) shRNA and different version of knock

down(KD) shRNA against sprouty-2. KD-3 showed most efficient knock down (70%) measured by western blot.

*C)D492$^{Spry\text{-}2KD\text{-}3}$ retain epithelial phenotype in monolayer culture.* No phenotypic difference was observed in monolayer between D492$^{NS}$ and *D492$^{Spry\text{-}2KD\text{-}3}$* upper panel. Transfection efficacy was evaluated by GFP.

D) *D492$^{Spry\text{-}2KD}$has acquired increased migration.* When plated on porous transwell filter *D492$^{Spry\text{-}2KD\text{-}3}$* showed increased migration compared to D492$^{NS}$. Single cell derived clone KD-3a from KD-3 had the highest migrational abilities.

*E) Spry-2 knock down has no effect on cell proliferation*. Monolayer proliferation of D492$^{NS}$ , D492$^{Spry\text{-}2KD\text{-}3}$ and D492$^{Spry\text{-}2KD\text{-}3a}$ was evaluated on different time points as indicated. There was no remarkable difference in the proliferation rate of the NS and KD cells although at day 4 D492$^{Spry\text{-}2KD\text{-}3a}$ seemed to proliferate slightly less.

***Figure 5. D492$^{Spry\text{-}2KD}$ result in increased branching***

*A) Spry-2KD result in increased branching colonies in 3D culture.* Phasecontrast images of representive 3D rBM gels for D492$^{NS}$ , D492$^{Spry\text{-}2KD\text{-}3}$ and D492$^{Spry\text{-}2KD\text{-}3a}$.

*B) Quantification of 3D rBM morphogenesis*. Epithelial colonies were divided into three morphotypes, simple branching, complex branching and other. Representive images of simple- and complex branching are shown.

*C) 3D morphogenesis of D492$^{NS}$ , D492$^{Spry\text{-}2KD\text{-}3}$ and D492$^{Spry\text{-}2KD\text{-}3a}$ cells.* In a setup with $10^4$ cells both Spry-2 KD cells showed an increase in simple- and complex branching.

*D) Large complex colonies in 3D rBM culture.* Complex branching colonies over 250μm were counted and that showed a 2 fold increase in the Spry-2 KD cells.

*E) 3D morphogenesis of D492$^{NS}$ , D492$^{Spry\text{-}2KD\text{-}3}$ and D492$^{Spry\text{-}2KD\text{-}3a}$ cells with variable amount of cells.* In a setup using 1.3x10$^4$, 10$^4$ and 7x10$^3$ cells the Spry-2 KD cells showed superior branching abilities compared to NS cells in all setups.

*F) Spry-2 expression in branching colonies from D492$^{NS}$ and D492$^{Spry\text{-}2KD\text{-}3}$*. As seen before Spry-2 was located at branching tips in NS cells. Spry-2 KD cells showed reduced expression of Spry2 with some areas of diffused staining.

***Figure 6. D492$^{Spry\text{-}2KD}$ are prone to EMT in coculture with endothelial cells.***

*A) Endothelial cells stimulate growth of D492 cells.* When plated in 3D rBM culture with endothelial cells D492 cells can form complex branching colonies from as little as 100-1000 cells compared to 7x103 -104 in monoculture in 3D. These colonies are bilayered and polarized as they express K14 on the outer side and K19 on the inner side (upper right). In coculture endothelial cells stay as single cells as seen with CD31 staining (lower right).

*B) Phenotypes in coculture.* In coculture with endothelial cells D492 cells form branching- and spindle-like colonies.

*C) Spry-2 KD cells show an increase in the spindle-like morphology.* While D492$^{NS}$ cells form about 40% spindle-like colonies there is a significant increase in the D492$^{Spry\text{-}2KD\text{-}3}$ cells up to 65%. The D492$^{Spry\text{-}2KD\text{-}3a}$ form almost exclusively spindle-like colonies in coculture with endothelial cells.

***Figure S1. In situ hybridization shows high expression of Sprouty-2 in breast epithelial cells.***

Sprouty-2 is expressed at high levels in breast epithelial cells in contrast to the surrounding stromal cells. High expression is seen in lobules and ducts.

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**Figure 1**

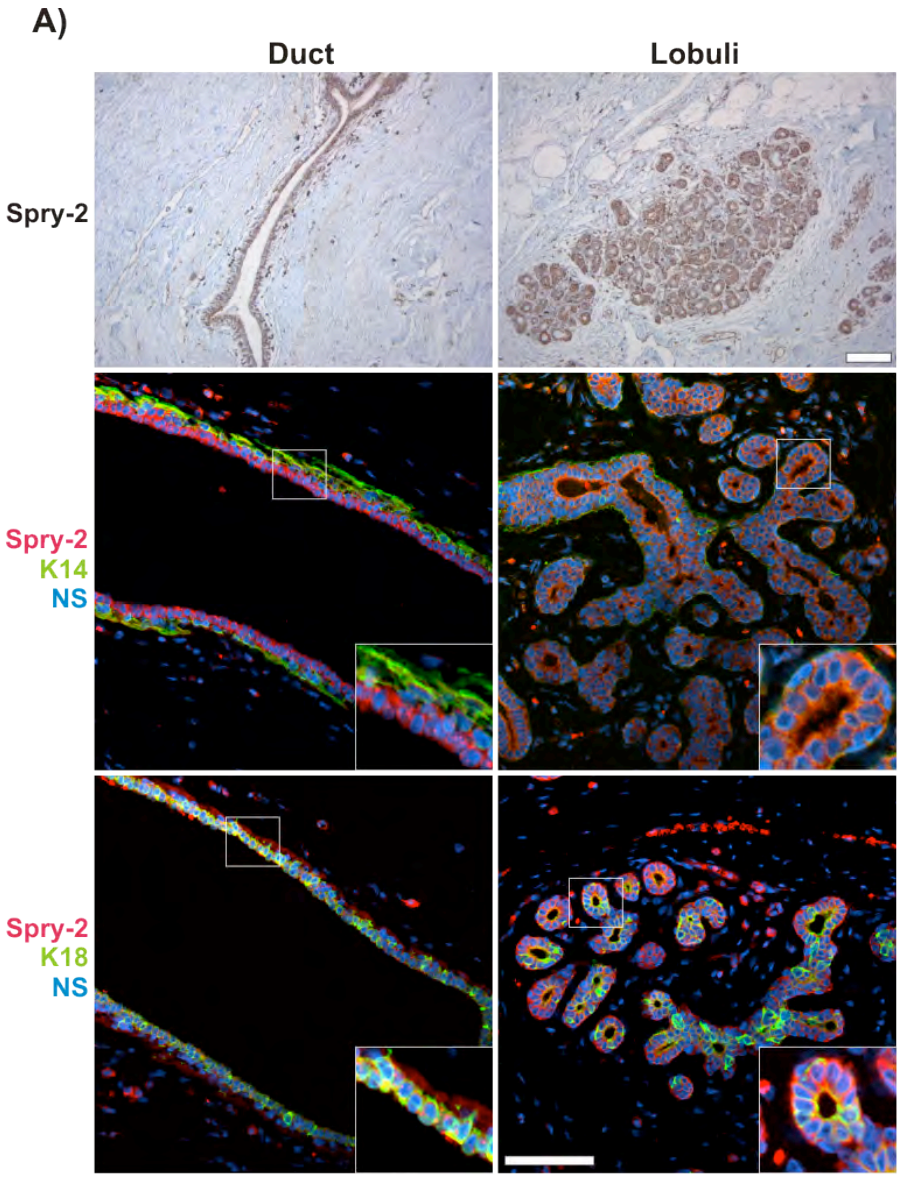
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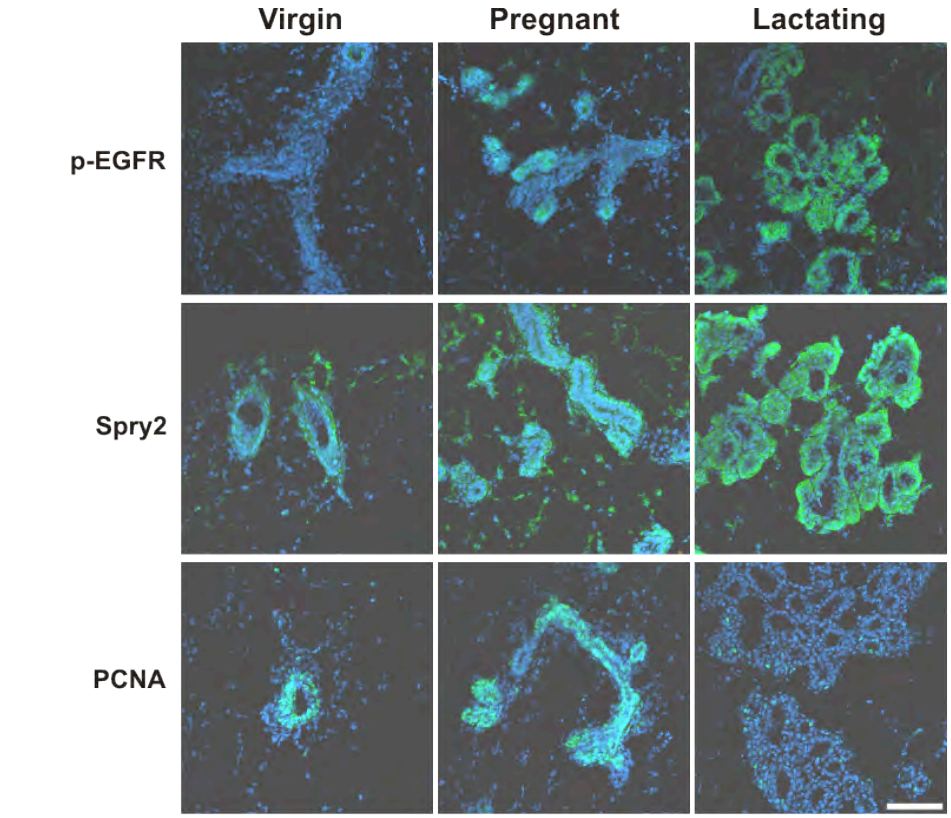
**Figure 2**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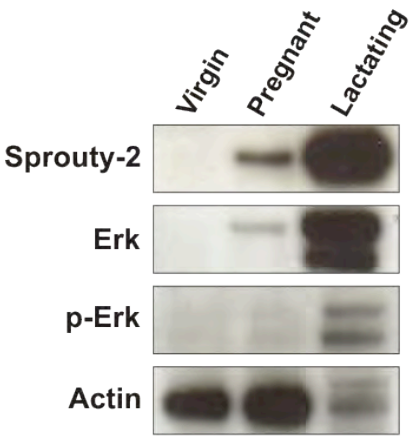
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p-Erk
Actin

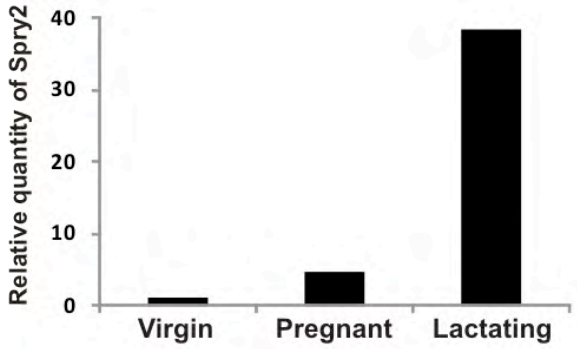

Relative quantity of Spry2
40
30
20
10
0
Virgin
Pregnant
Lactating

**Figure 3**

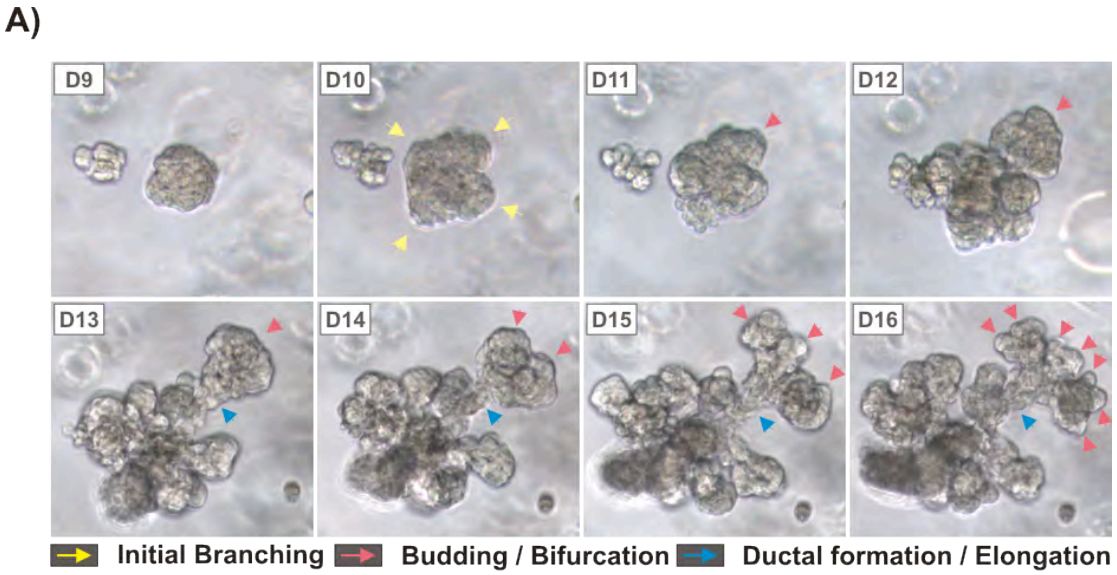
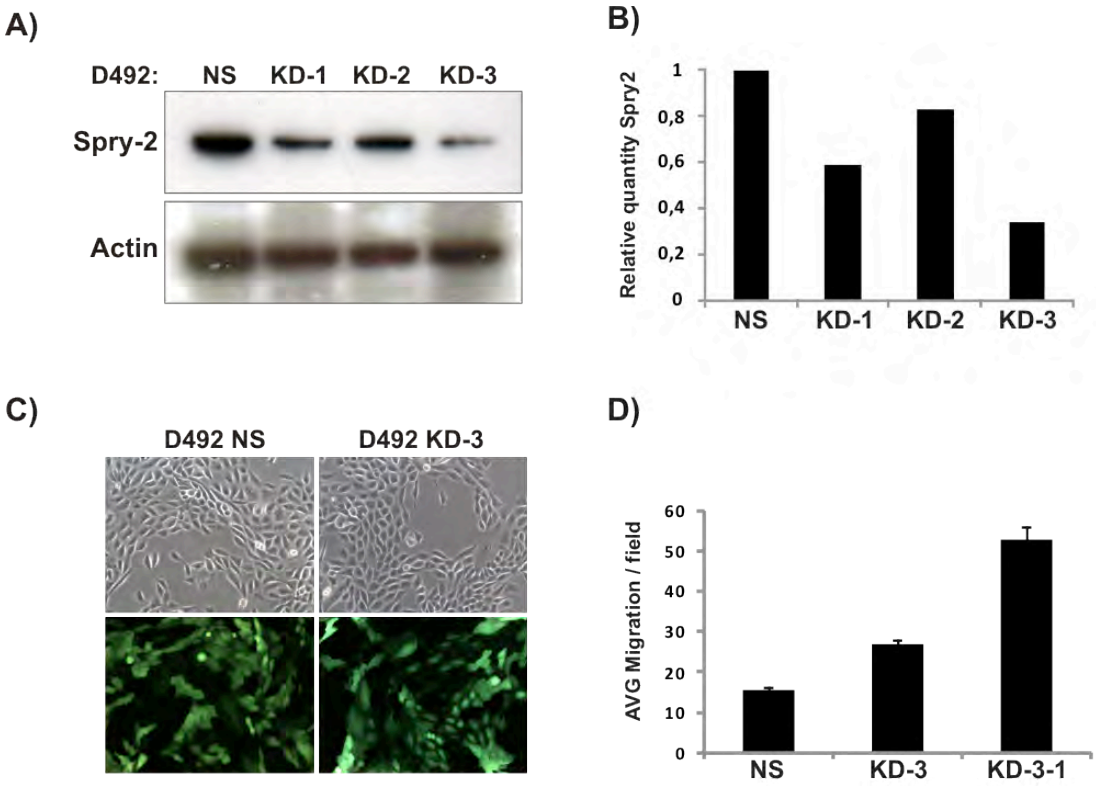

A)
D9
D10
D11
D12
D13
D14
D15
D16
Initial Branching
Budding / Bifurcation
Ductal formation / Elongation
B)
Fold change Spry2 mRNA
0
1
2
3
4
5
6
D8
D10
D12
D14
D16
C)
D13
D16
D19
p-EGFR
Spry2
Actin
D)
F-actin
NS
EGFR
NS
p-EGFR
NS
β4-Intgr
NS
Spry2
NS

**Figure 4**


A)
D492: NS KD-1 KD-2 KD-3
Spry-2
Actin
B)
Relative quantity Spry2
1
0,8
0,6
0,4
0,2
0
NS KD-1 KD-2 KD-3
C)
D492 NS
D492 KD-3
D)
AVG Migration / field
60
50
40
30
20
10
0
NS KD-3 KD-3-1

D)

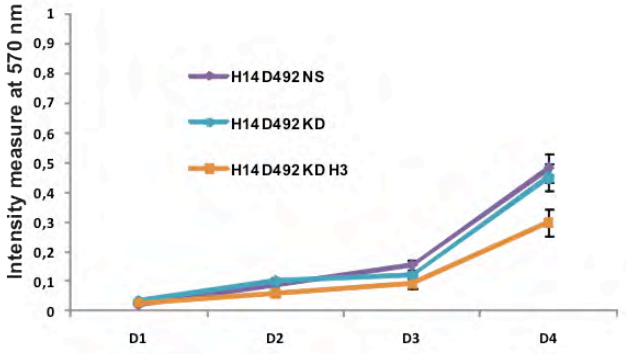

Intensity measure at 570 nm
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
H14 D492 NS
H14 D492 KD
H14 D492 KD H3
D1
D2
D3
D4

**Figure 5**

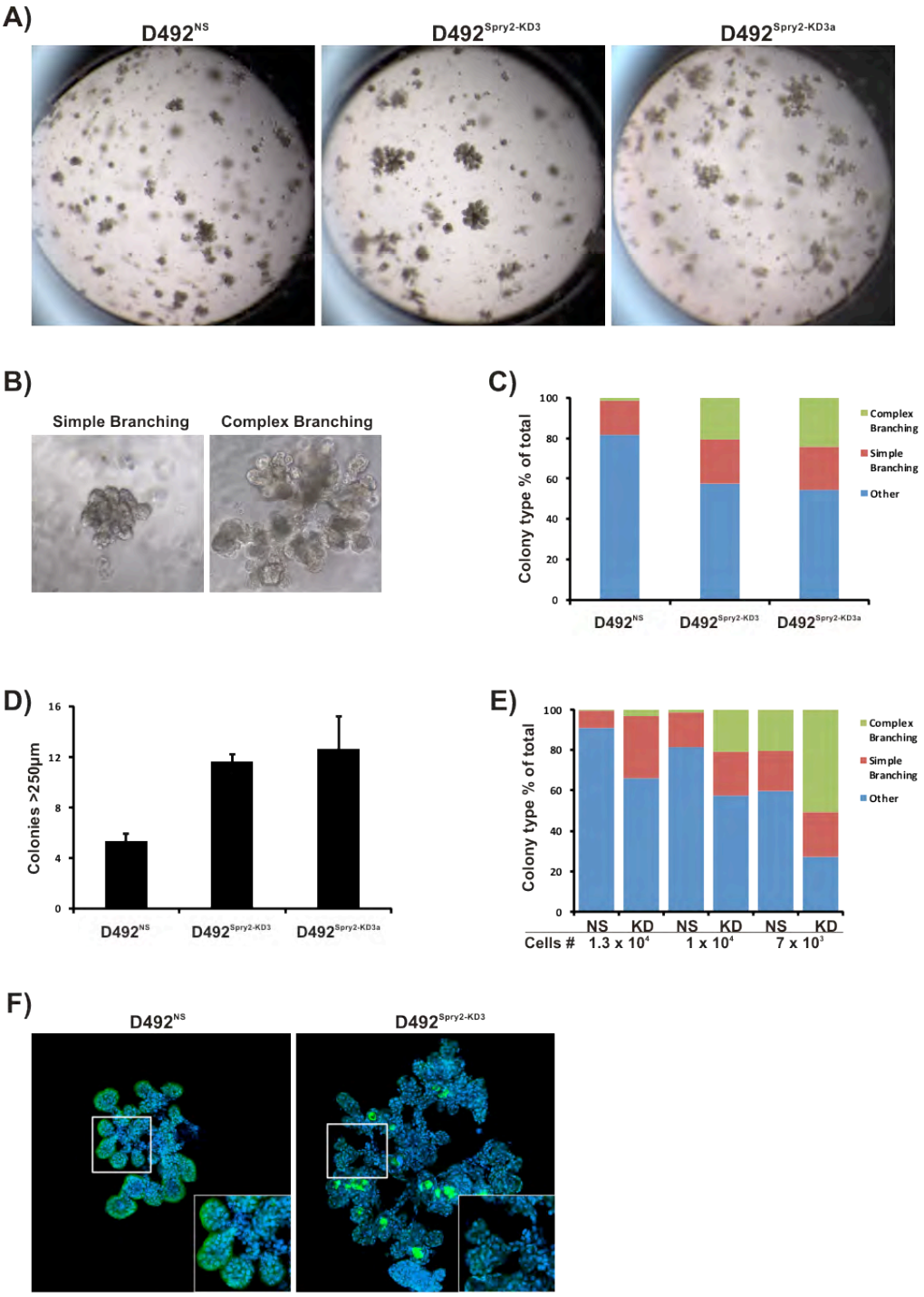

A)
D492NS
D492Spry2-KD3
D492Spry2-KD3a
B)
Simple Branching
Complex Branching
C)
Colony type % of total
100
80
60
40
20
0
Complex Branching
Simple Branching
Other
D492NS
D492Spry2-KD3
D492Spry2-KD3a
D)
Colonies >250µm
16
12
8
4
0
D492NS
D492Spry2-KD3
D492Spry2-KD3a
E)
Colony type % of total
100
80
60
40
20
0
Complex Branching
Simple Branching
Other
NS KD NS KD NS KD
Cells # 1.3 x 10⁴ 1 x 10⁴ 7 x 10³
F)
D492NS
D492Spry2-KD3

**Figure 6**

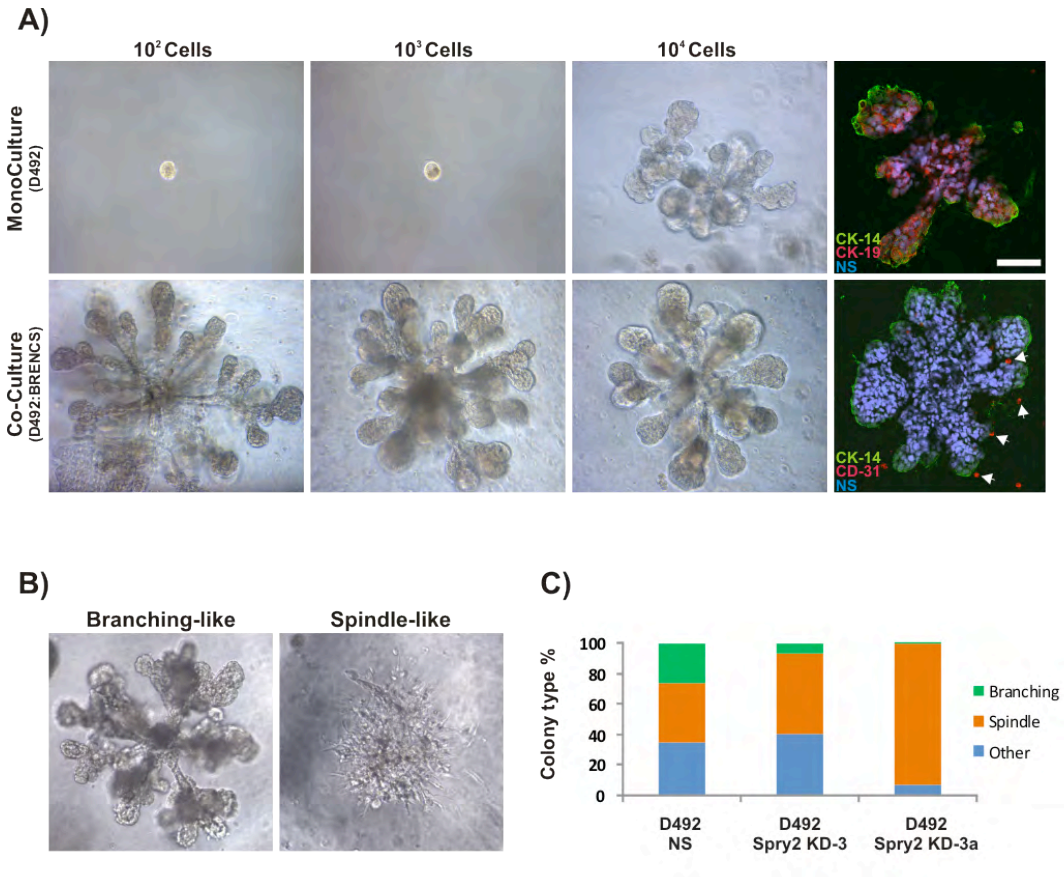

A)
10² Cells
10³ Cells
10⁴ Cells
MonoCulture (D492)
Co-Culture (D492:BRENCS)
CK-14
CK-19
NS
CK-14
CD-31
NS
B)
Branching-like
Spindle-like
C)
Colony type %
100
80
60
40
20
0
Branching
Spindle
Other
D492 NS
D492 Spry2 KD-3
D492 Spry2 KD-3a

**Figure S1**