Modeling the role of the EGFR-receptor family in the normal and malignant breast gland

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Thesis for the degree of Philosophiae Doctor

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Hlutverk EGFR-viðtakafjölskyldunnar í eðlilegum og illkynja brjóstkirtli.

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Ritgerð til doktorsgráðu

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Fyrir Bogeyju Sigríði

Ágrip

Brjóstkirtillinn er gerður úr greinóttum kirtilgöngum með upphaf í geirvörtu en greinist svo inn á við og endar í kirtilberjum, sem eru seytunarhluti kirtilsins. Kirtilgangarnir eru umluktir æðaþelsríkum bandvef. Stofnfrumur í brjóstkirtlinum gera kirtlinum kleift að ganga í gegnum endurtekna hringrás frumufjölgunar, sérhæfingar og frumudauða tengt meðgöngu og mjólkurframleiðslu. Stofnfrumur í brjóstkirtli hafa á undanförnum árum verið tengdar myndun brjóstakrabbameina. Brjóstakrabbamein má flokka í marga undirhópa, byggt á tjáningu kennipróteina. Sýnt hefur verið fram á að EGF viðtakafjölskyldan gegnir mikilvægu hlutverki í bæði eðlilegum brjóstkirtli, en einnig í krabbameinsmyndun. Lokastig krabbameinsframvindu er myndun meinvarpa, þar sem frumur losna frá æxlinu og mynda meinvörp í öðrum líffærum. Hlutverk æðaþelsins í þessu ferli hefur yfirleitt verið álitið einskorðað við flutning næringarefna og súrefnis til æxlisins, en nokkrar rannsóknir hafa sýnt fram á beint hlutverk æðaþels í þroskun, meðal annars með því að ýta undir bandvefsummyndun þekjufruma (epithelial to mesenchymal transition, EMT). **Markmið** þessa doktorsverkefnis er að þróa þrívítt ræktunarlíkan til að kanna samspil æðaþels og þekjuvefjar í brjóstkirtli. Ennfremur er markmiðið að nota þetta ræktunarlíkan til að kanna hlutverk EGFR og ErbB2 í þroskun og sérhæfingu eðlilegra og illkynja brjóstaþekjufruma. **Í fyrstu greininni** sem lögð er fram með ritgerðinni sýnum við hvernig æðaþelsfrumur sem einangraðar voru úr heilbrigðum brjóstvef geta hvatað vöxt heilbrigðra óummyndaðra brjóstaþekjufruma. Samræktanir á æðaþeli og þekjufrumufrumulínum sýndi fram á að æðaþel virkar sem vaxtarhvetjandi þáttur í umhverfinu. **Í grein 2** könnum við áhrif þess að fjarlægja EGF úr ræktunaræti frumulínunnar A163 – þessi breyting leiðir til mikillar mögnunar á EGFR geninu í A163, sem breytir svipgerð hennar í þrívíðu umhverfi. Þessi breyting varð til þess að við skoðuðum hlutverk EGFR boðleiðarinnar í myndun greinóttrar formgerðar. Við notuðum stofnfrumulínuna D492 **í grein 3** og slógum niður Sprouty-2, sem er neikvæður afturkasthindri á Týrósín kínasa boðleiðum. Við sáum að með því að slá niður Sprouty fengum við fram mikla aukningu í myndun greinóttra frumuþyrpinga. Ennfremur sáum við að í samrækt með æðaþeli kom fram mikil aukning á myndun bandvefslíkra frumuþyrpinga, þar sem frumur höfðu undirgengist EMT. **Í fjórðu greininni** könnuðum við áhrif þess að yfirtjá EGFR og ErbB2 í D492. Við sáum að ErbB2 yfirtjáning leiddi til taps á bæði EGFR og þekjuvefspróteinum og aukningar á bandvefspróteinum, eða EMT. Þessari svipgerð gátum við snúið að hluta við með því að yfirtjá EGFR með ErbB2. Þegar þessar frumur voru græddar í mýs mynduðu ErbB2 yfirtjáandi frumur stór bandvefslík æxli, en æxli með bæði EGFR og ErbB2 voru mun minni, og sýndu þekjuvefssvipgerð. **Niðurstaða** verkefnisins er að æðaþel getur haft mikil áhrif á svipgerð þekjuvefsfruma í þrívíðri rækt, ennfremur að EGF

viðtakafjölskyldan, sérstaklega EGFR gegnir mjög mismunandi hlutverkum í frumum, og getur jafnvel þjónað hlutverki krabbameinsbælipróteins, en hingað til hefur EGFR verið álitinn hvetja krabbameinsmyndun. Líkanið sem við höfum þróað má nota til áframhaldandi rannsókna á samspili þessara viðtaka í klínísku samhengi

Lykilorð:

Stofnfrumur, brjóstakrabbamein, greinótt formgerð, bandvefsumbreyting, þrívíð ræktun

Abstract

The mammary gland consists of epithelial ducts originating from the nipple and branches inwards, terminating in acini, the functional, milk producing unit of the breast. Stem cells within the mammary gland enable repeated cycles of proliferation, differentiation and involution during pregnancy and lactation, and also to a lesser degree during each menstrual cycle. Breast cancer can be stratified into several subgroups, based on marker expression. Formation of distant metastases represents the final progression of breast cancer. The role of endothelium during development and cancer progression has been considered that of oxygen and nutrient transport, in addition to facilitating cancer cell metastasis, while some research has shown the importance of endothelial derived signals during normal development. **The aim** of this thesis was to design a culture system that could be utilized for modeling endothelial-epithelial interaction. Additionally, the aim was to use this culture system to analyze the role of EGFR and ErbB2 signaling pathways in the epithelial stem cell line D492, which can form branching colonies when cultured in a three-dimensional environment. **The first paper** submitted with this thesis demonstrates how normal primary endothelial cells facilitate increased proliferation of primary epithelial cells. This was further supported by co-culturing endothelial cells with established cell lines. **Paper two** shows how removal of EGF from the culture media results in strong upregulation of EGFR in the breast cell lines A163 through amplification of the EGFR gene. This change results in a stronger basal-like phenotype, loss of polarity and abnormal three-dimensional behavior. These data emphasized the importance of EGFR signaling during morphogenesis. We used the D492 cell line in **paper three** to analyze the role of sprouty-2, a modulator of receptor tyrosine kinase signaling. We observed that knockdown of Sprouty-2 resulted in greatly enhanced branching morphogenesis. Additionally, we observed an increased tendency for D492 cells to undergo epithelial to mesenchymal transition (EMT) in co-culture with endothelial cells. **In the fourth paper** we overexpressed ErbB2 and EGFR in D492 cells. We saw that ErbB2 overexpression resulted in downregulation of EGFR, in addition to loss of epithelial cadherin and keratin expression, and gain of mesenchymal markers and N-cadherin. This phenotype could be partially reversed by overexpressing EGFR along with ErbB2. ErbB2 overexpression also enhanced tumor formation in mice, where large tumors with a mesenchymal phenotype were observed. Dual expression of EGFR and ErbB2 reduced tumor growth, and tumors exhibited a stronger epithelial phenotype. **The result of this thesis** is that endothelial cells can greatly influence

proliferation and morphology of epithelial cells in three-dimensional culture. Additionally, the EGF receptor plays a highly varied role in epithelial cells, and could have different prognostic values in breast cancer. Traditionally EGFR has been described as an oncogene, facilitating tumor formation, but in the context of ErbB2 expression, EGFR could serve as a tumor suppressor, by maintaining epithelial marker expression, thereby reducing invasiveness. These data indicate that EGFR and ErbB2 must be valued together when tumors are evaluated for treatment options.

Keywords:

Stem cells, breast cancer, branching morphogenesis, Epithelial to mesenchymal transition, three-dimensional cell culture.

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Contents

List of Abbreviations

2D	Two dimensional
3D	Three dimensional
ALDH	Aldehyde Dehydrogenase
BRENC	Breast Endothelial Cell
BLBC	Basal-like breast cancer
CK	Cytokeratin
CSC	Cancer Stem Cell
DCIS	Ductal Carcinoma *in situ*
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
ErbB2	EGFR2
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
GFP	Green Fluorescent Protein
GH	Growth Hormone
HGF	Hepatocyte Growth Factor
IGF	Insulin-like Growth Factor
LEP	Luminal Epithelial Cell
MAPK	Mitogen-Activated Protein Kinase
MEP	Myoepithelial Cell
miR	MicroRNA
MMP	Matrix-Metalloprotease
PCR	Polymerase Chain Reaction
PLA	Proximity Ligation Assay
RTK	Receptor Tyrosine Kinase
SPRY	Sprouty
TAM	Tumor Associated Macrophage
TDLU	Terminal Duct Lobular Unit
TEB	Terminal End Bud
TGFβ	Transforming Growth Factor beta
TIC	Tumor Initiating Cell
TNBC	Triple Negative Breast Cancer
VEGF	Vascular Endothelial Growth Factor

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List of Original Papers

1. **Endothelial cells stimulate growth of normal and cancerous breast epithelial cells in 3D culture.**

   Ingthorsson, S., Sigurdsson, V., Fridriksdottir, A., Jonasson, JG., Kjartansson, J., Magnusson, MK., Gudjonsson, T.
   BMC Res Notes. 2010 Jul 7;3(1):184.

2. **Selection for EGFR gene amplification in a breast epithelial cell line with basal-like phenotype and hereditary background.**

   Ingthorsson, S., Halldorsson, T., Sigurdsson, V., Friðriksdottir, AJ., Bodvarsdottir, SK., Steinarsdottir, M., Johannsson, O., Magnusson, MK., Ogmundsdottir, HM., Gudjonsson, T.
   In Vitro Cell Dev Biol Anim. 2011 Feb;47(2):139-48.

3. **Expression and functional role of sprouty-2 in breast morphogenesis.**

   Sigurdsson, V., Ingthorsson, S., Hilmarsdottir, B., Gustafsdottir, SM., Franzdottir, SR., Arason, AJ., Steingrimsson, E., Magnusson, MK., Gudjonsson, T.
   PLoS One. 2013;8(4):e60798.

4. **EGFR overexpression inhibits ErbB2 induced EMT in epithelial cells with stem cell properties via myoepithelial differentiation.**

   Ingthorsson, S., Andersen, K., Hilmarsdottir, B., Maelandsmo, GM., Magnusson, MK., Gudjonsson, T.
   Manuscript in Preparation.

Declaration of contribution

Paper #1 Based on research performed by a previous Ph.D. student, Valgarður Sigurðsson (VS), I conducted co-culture of primary endothelial and epithelial cells in matrigel. I performed statistical analysis and immunocytochemistry. I performed co-cultures with established cell lines and Breast endothelial cells (BRENCs). Additionally, I devised experimental strategies and performed experiments involving transwell assays and the gradient concentration co-culture experiments. I wrote the paper along with my supervisors.

Paper #2 Using the A163 and A163-S1 cell lines I performed characterization by immunocytochemistry. I performed both 3D and transwell filter cultures and did all the immunocharacterization. I analysed the proliferation rates of the two cell lines and wrote the paper along with my supervisors.

Paper #3. The third paper is co-authored by myself and VS, which established the knockdown sub-lines. My role in this paper focused on three dimensional cell culture experiments and immunohistochemistry staining of mouse mammary glands. I performed data analysis and participated in writing the paper.

Paper #4. In the fourth paper I established all the D492 sub-lines, I performed all experiments except the mouse injections that were handled by Kristin Andersen our collaborator in Oslo, and miR analysis that was performed by Bylgja Hilmarsdóttir. I performed data analysis and wrote the paper along with my supervisors.

1 Introduction

1.1 The breast gland

The human mammary gland is a highly dynamic organ. Its basic structure is a branching epithelium composed of collecting ducts and terminal milk producing apocrine acinar structures commonly referred to as the terminal end lobular units (TDLUs, Figure 1). This epithelial compartment is separated from the collagenous stroma by a basement membrane. The breast stroma contains a variety of cellular populations, including fibroblasts, lymphocytes and endothelial cells.

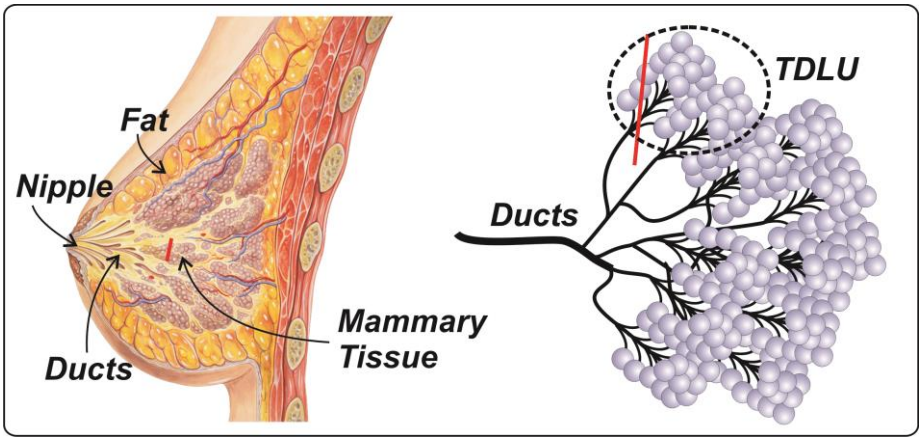

Figure 1. Schematic representation of the human breast gland.
The breast gland is composed of branching epithelial ducts that are embedded in collagen and fat rich stroma. These ducts terminate in the TDLU, the functional, milk producing unit of the breast gland. *Breast image on the left adapted from Patrick J. Lynch (CC BY 3.0). Red line depicts a virtual cut shown in figure 2.*

1.2 Breast gland development

Developmentally, the breast gland has been suggested to be a modified sweat gland. From an evolutionary standpoint, the mammary gland is one of the defining features of the class *mammalia*; all living mammalian species have mammary glands used to feed the young (Oftedal, 2002). Development of the mammary gland takes place in three distinct phases; embryonic formation of rudimentary ducts, high levels of branching morphogenesis during puberty and finally during pregnancy and lactation, where the gland fully matures and reaches its final functional state.

During embryonic development, rudimentary ductal structures are formed on the underside of the embryo along the milk line ridges in the epidermis. This developmental process is directed by the mesenchyme, through several factors, including Wnt signaling (Chu et al., 2004) and receptor tyrosine kinase signaling (EGFR and FGFR families) (Veltmaat et al., 2006). In humans, development along the milk line is repressed except for a single pair that further develops into the mammary gland. In mice, embryonic development results in five pairs of mammary glands. The embryonic mammary development appears to be guided through cell-cell interaction and signaling from the mesenchyme, independent of estrogen and progesterone signals while development during puberty and lactation is driven by hormonal signaling stemming from the pituitary gland and the ovaries. (Cowin & Wysolmerski, 2010; Hennighausen & Robinson, 2001). At birth both female mice (male mice do not develop mammary glands) and humans display a branching ductal tree originating from the nipple. The epithelial tissue consists of rudimentary but fully functional ducts; as occasionally evidenced by neonatal milk production. In mice the ducts terminate in the terminal end bud (TEB) and in humans in the TDLU, emphasizing the difference in the histoarchitecture of mouse and human mammary glands (Parmar & Cunha, 2004). These structures remain quiescent until the onset of puberty.

At the onset of puberty, marked increase in hormonal signaling from the ovaries and pituitary gland lead to a phase of increased proliferation and branching morphogenesis. In mice, the quiescent mammary ductal tree that formed in the embryonic phase branches and fills the fat pad with an elaborate ductal tree. In humans the branching network follows a similar development, growing in size, complexity and volume. Proliferation is driven in part by growth hormone (GH) from the pituitary gland, which in turn induces production of insulin-like growth factor (IGF1) in the liver. This signaling along with ovarian estrogen are the main drivers of proliferation in the mammary gland. Branching morphogenesis is driven by receptor tyrosine kinase signaling, especially through the EGF and FGF receptor families (Pond et al., 2013; Varner & Nelson, 2014; Zhang et al., 2014). Epithelial-stromal interactions are of great importance, where FGF derived from the stroma induces formation of branching points along the ductal tree. Amphiregulin (AREG), a ligand of EGFR has been shown to be of importance, as AREG knockout mice show abnormal stunted mammary development (Ciarloni et al., 2007). There are several signaling molecules that negatively regulate branching morphogenesis; among them are TGFβ, which when produced by epithelial cells can auto-inhibit further branching (Ingman & Robertson, 2008; Nelson et al., 2006). In both mice and humans, the post-pubertal mammary gland is ready to achieve the final developmental stage that takes place during pregnancy and lactation.

During pregnancy, the mammary gland undergoes the third and final stage of development. The alveolar population, which produces the milk in the lactating gland, expands greatly (Lydon et al., 1995). This is induced predominantly by hormonal changes, including increased levels of progesterone and prolactin that leads to a phase of expansion and branching. Progesterone drives the formation of branches from existing ducts. Prolactin, another hormone from the pituitary gland drives the differentiation of alveoli into a functional lactating gland (Oakes et al., 2008).

After weaning of the young, the mammary gland undergoes a dramatic transformation, where the ductal and alveolar network reverts back; nearly to its post-pubertal stage. This involution of the mammary tree is orchestrated by several factors; first from local factors where changes in the mammary gland itself triggers remodeling and apoptosis, and secondly through hormonal signaling, where further apoptosis and involution takes place. The end product of this involution is a mammary gland in resting state, able to undergo another phase of proliferation during subsequent pregnancies. Additionally, each menstrual cycle induces a mild phase of proliferation, similar to what happens in pregnancy, due to changes in hormonal balance. (Macias & Hinck, 2012)..

1.3 Cellular populations of the breast gland

The epithelial tissue is composed of two main cell types, the luminal epithelial cells (LEPs) and myoepithelial cells (MEPs) (Figure 2). These cell types arrange themselves in a double layer, the LEPs line the inside of ducts and acini and appear as cuboidal or columnar. LEPs in the acini are the milk producing cell population often referred to as alveolar cells while the ductal LEPs are referred to as ductal cells. LEPs are tightly bound together, display baso-lateral polarization, with apical tight junctions such as Zonula occludens, and a basally located nucleus. LEPs express a variety of cytokeratins (CK), such as CK18 and CK19. A subpopulation of LEPs express hormonal receptors, including Estrogen receptor α, (ER-α) which plays a pivotal role during development (Mallepell et al., 2006). Myoepithelial cells comprise the outer part of the epithelial structure and form the contractile component of the breast gland. In ducts, they form a dense squamous layer, isolating LEPs from the surrounding stroma, and sit on the basement membrane (Gusterson et al., 1982). In acini, MEPs form a basket-like mesh around LEPs, and in some areas, LEPs come into direct contact with the basement membrane. MEPs express basal cytokeratins such as CK5, 6, 14, and 17 in addition to vimentin. Additionally, MEPs express α-smooth muscle actin, which upon stimulation contracts and causes acini and lumens to contract, pumping the milk towards the nipple.

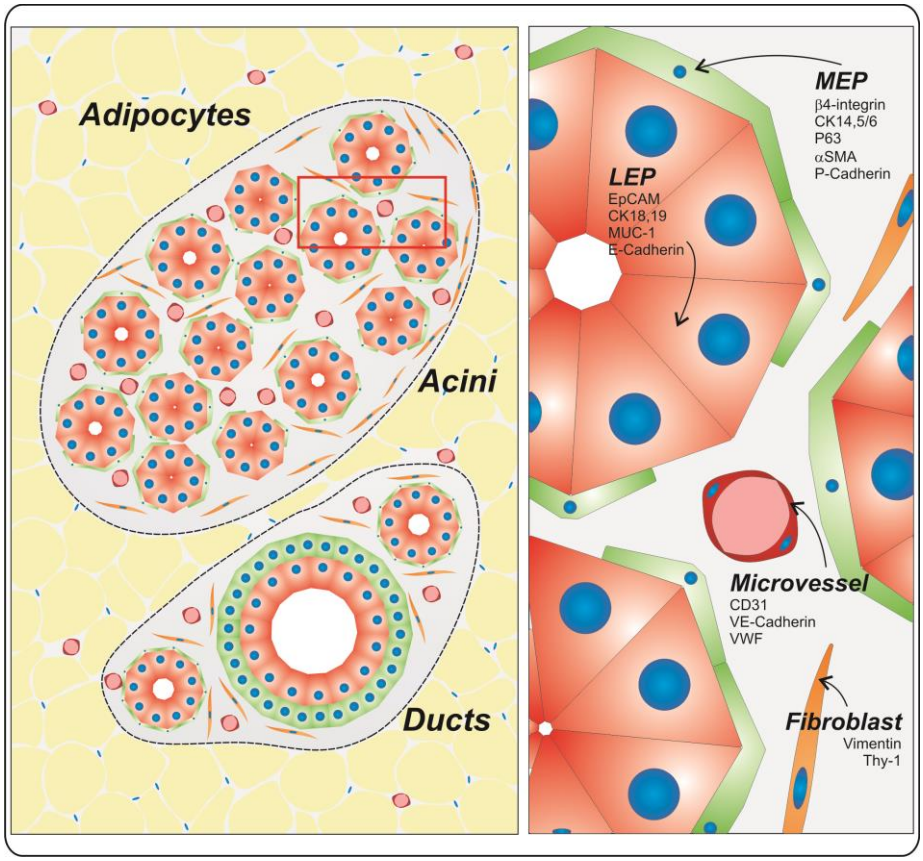

Figure 2. *Schematic histoarchitecture of the TDLU.*
The mammary acini and ducts are arranged in double layered structures. LEPs are situated towards the lumen, MEPs line the outside. Different cellular components within the breast gland can be characterized using identifying markers.

In contrast to human breast tissue, the epithelial structure of mice is embedded in a fatty stroma, rich in adipocytes, but relatively poor in fibroblasts. In humans the TDLU is surrounded by loose intralobular stroma rich in mesenchymal cells; which in turn is embedded in denser interlobular stroma (Fleming et al., 2008). The stroma is rich in collagen I and contains fibroblasts, immune cells, nerves, lymphatic- and blood vessels. The intralobular stroma is rich in microvasculature that supplies nutrients and growth factors to the breast tissue, both during development and through pregnancy and lactation (Djonov et al., 2001; Sigurdsson et al., 2006). In the mammary gland there has to be a resident pool of cells that supply each phase of proliferation and differentiation with new cells. It is generally accepted that these reserve cells are the actual stem cells within the mammary gland (Smith & Medina, 1988). In the next chapter I will discuss recent research detailing the similarities and differences between the murine and human mammary gland stem cell populations

1.4 Stem cells of the breast

Knowledge of the development and the cellular hierarchy of the mouse mammary gland is significantly more advanced than in the human gland. In the pre-pubescent mouse, the location of the mammary stem cells is within the TEB. These cells give rise to transit-amplifying progenitor cells by asymmetric divisions, which in turn differentiate into the terminally differentiated luminal and myoepithelial cells. The identity of murine mammary stem cells has been demonstrated in several research papers (Asselin-Labat et al., 2008; Visvader et al., 2014). Transplantation assays have significantly contributed to our understanding of the stem cell population in the mouse mammary gland (Smith & Medina, 2008). In these assays, the native mammary gland tissue in pre-pubescent mice is removed from the fat pad, resulting in a cleared mammary fat pad that can then be implanted with exogenous tissue or cell populations. If the injected cells have the ability to generate a new mammary tree they are referred to as repopulating cells. Studies by Shackleton, Stingl and colleagues (Shackleton et al., 2006; Stingl et al., 2006) showed that cells expressing β1-integrin and CD24, both markers used to identify stem cells in many organs were able to regenerate a functional mammary gland with high efficiency. As little as a single cell injected was sufficient to generate and repopulate the cleared mammary fat pad. Serial transplantations, where cells from reconstituted glands were re-sorted and again transplanted demonstrated that these cells harbored self-renewal abilities, one of the key hallmarks of stem cells. The exact differentiation hierarchy from stem cell to terminally differentiated cells is less defined, but of great importance due to the link between stem cell biology and breast cancer. One key question is whether bi-potent stem cells exist in the adult breast gland, or whether differentiation is driven by unipotent progenitors that each give rise to either myoepithelial or luminal cells. In a recent paper, Rios and colleagues (Rios et al., 2014) used lineage tracing in the mouse mammary gland to demonstrate that long lived stem-cells do not reside within the luminal compartment, but rather within the basal compartment. They tracked the progeny of LEPs by inducing fluorescent protein expression in cells expressing elf5, a LEP specific transcription factor and found that after 20 weeks the labeled population had for the most part been depleted. Using CK5, a basal cell marker present in myoepithelial and basal cells in the breast, they found that labeled basal cells gave off luminal progeny, demonstrating that within the adult gland, long lived bipotent stem cells reside within the basal compartment.

To identify the adult human stem cells, researchers have among other things used tissue from reduction mammoplasties, cell sorting, immunostainings and stem cell assays to identify candidate stem cell population. It has been established that bipotent stem cell candidates reside within ducts of the human mammary tree (Villadsen et al., 2007), and that those cells harbor characteristics of both luminal and myoepithelial cells for instance expression of both CK14 and

CK19. Expression of Aldehyde dehydrogenase (ALDH) has been demonstrated to be linked with breast stem cell biology, where the progenitor cells express ALDH, but not Estrogen receptor (Honeth et al., 2014). Other groups have shown that cells expressing α6-Integrin, but low/negative expression of EpCAM are able to form acini similar to those found within the normal breast gland (Eirew et al., 2008). By using low-attachment cultures, Dontu and colleagues were able to induce symmetric divisions in normal mammary stem cells by culturing them in a low attachment environment. They also demonstrated that by activating Notch signaling they could further increase the stem cell population in their assays (Dontu et al., 2003; Dontu et al., 2004). Candidate stem cells have been isolated using other markers. In 2002 a study by Gudjonsson and colleagues showed that cells exhibiting a supra-basal location, i.e. residing above the basal compartment, but not reaching the lumen of the gland, could differentiate into both luminal and myoepithelial cells (Gudjonsson, Villadsen, et al., 2002). They did this by enriching for cells expressing EpCAM, a marker for luminal epithelial cells, but not sialomucin-1, which is expressed at the apical side of cells reaching the lumen. In culture, these cells had a phenotype resembling both luminal and myoepithelial cells and in three dimensional culture were able to form branching colonies resembling the TDLU (Gudjonsson, Villadsen, et al., 2002). To be able to further demonstrate the stem cell nature of human breast stem cells, researchers have sought to recapitulate the breast gland *in vivo*. For obvious reasons, transplantation experiments are not available to study the human mammary gland *in situ*. To compensate, the use of mouse models has been tested. As stated earlier, the histology of the human and mouse gland differs considerably, especially with regard to the surrounding stroma. To address these differences, researchers have tried compensating by introducing human stromal cells alongside the epithelial cells (Kuperwasser et al., 2004). Kuperwasser and colleagues isolated human epithelial organoids and implanted into murine cleared mammary fat pads that had first been “humanized” by introduction of human fibroblasts. In this model, injected organoids were able to proliferate and form outgrowths resembling the human acini and in addition the epithelial structure was able to respond to hormonal changes during pregnancy.

1.5 Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are a large family of cell surface receptors for growth factors and cytokines. RTKs are composed of an extracellular receptor domain and an intracellular kinase domain connected by a transmembrane region. Upon binding of a ligand the RTKs form a dimer, which leads to intracellular phosphorylation and downstream signaling, including JNK, ERK and AKT pathways. In the breast gland several RTK families play a role in normal development, most prominently the EGFR and FGFR families. They play a role in directing branching morphogenesis, as

well as during cellular remodeling and maintenance of the breast gland. In cancer, changes in expression and signaling intensity of RTKs are very common. Point mutations in RTKs are not common in sporadic breast cancer (Generali et al., 2007; Tilch et al., 2014), but are found within tumors harboring mutations in either BRCA 1 or 2 (Weber et al., 2005).

1.5.1 Epidermal growth factor receptors and ligands.

The EGF receptor family encompasses four genes, EGFR (HER1), EGFR2, EGFR3 and EGFR4 (HER2-4, ErbB2-4). These receptors are structurally similar, but vary greatly with regard to ligand affinity and kinase activity. Several ligands have been shown to bind with the EGF receptor family, with different affinities to each RTK (Figure 3). All four RTKs demonstrate a high level of homology in their kinase domain, while the extracellular domain is more varied. They can form both homodimers and heterodimers within the receptor family (detailed in Figure 4). While all four receptors are of importance for normal development, I will focus on EGFR and ErbB2 due to their relevance to breast development and cancer. The mechanism of activation of all four RTKs follows a similar pattern, all four require an active conformation in order to dimerize. ErbB2 has no known ligand, but can bind other EGF receptor family partners. ErbB2 has been shown to persist in an active conformation, meaning it does not need to bind a ligand to become available for dimerization, making it the preferred binding partner within the family. The remaining three receptors adapt an active conformation upon ligand binding (Figure 4, far left). Once the extracellular domains come together in a stable dimer, the intracellular kinase domains form an asymmetric dimer and the receptors are endocytosed followed by phosphorylation of several possible tyrosine and serine residues (Figure 4). The exact phosphorylation site depends on the dimerization partner and the ligand, as the kinase domains of different homo/heterodimers have been shown to result in slight variation with the kinase dimer shape (Mi et al., 2011; Muthuswamy et al., 1999). Individual phosphorylation sites can be either autophosphorylated (by the individual receptors themselves) or transphosphorylated (phosphorylated by the other dimer partner). Phosphorylation then leads to activation of downstream signaling pathways. In EGFR, activation through protein kinases B and C are most prominent, activating MAP kinase and Akt related pathways, resulting in cell proliferation, increased motility and survival.

EGFR has several known ligands. Most relevant in breast biology are EGF, TGFα and amphiregulin (AREG) (Figure 3), all of which have important function in the normal gland development. The EGFR ligands exist as cell-bound pro-forms, which can act in a paracrine fashion. When they are released via MMP cleavage, (e.g. ADAM17) they can activate pathways in distant cells. EGFR in the normal breast gland is located predominately in the basal/myoepithelial cells, fibroblasts and endothelial cells (Gompel et al.,

1996). EGFR and its ligands play an important role during embryonic and pubertal development and maintenance of the mammary gland. AREG acts as a mediator between estrogen and progesterone signaling during puberty, by activating EGFR. AREG-induced EGFR signaling is required for normal TEB formation in pubertal mice (Aupperlee et al., 2013; Ciarloni et al., 2007). In recent years, EGFR has emerged as a potential therapeutic target for Triple Negative breast cancer (TNBC), as overexpression of EGFR has been shown to be common, and associated with poor prognosis (H. S. Park et al., 2014). EGFR is used as one of the markers to distinguish basal-like tumors from other TNBC (Bosch et al., 2010; Burness et al., 2010).

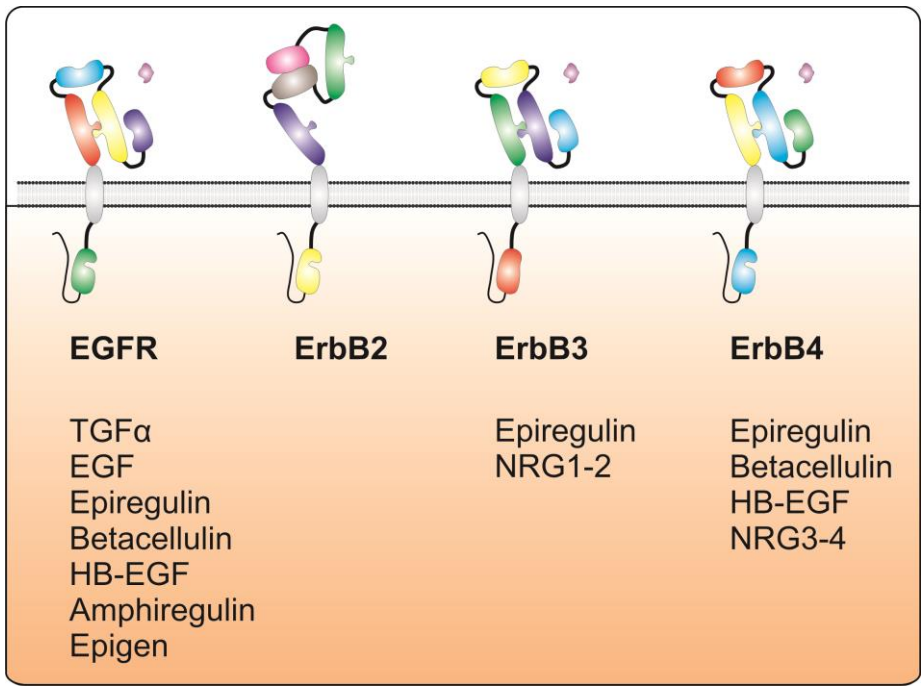

Figure 3. *EGFR family and ligands.*
The four EGFR family proteins share high levels of homology. Notable differences are the constitutively active conformation of ErbB2 extracellular domain. The four receptors have different affinities to ligands. ErbB2 has no known ligand. Adapted from (Clasadonte et al., 2011; Ferguson, 2008; S. Li et al., 2005).

ErbB2 is expressed within luminal cells of the breast gland (Gompel et al., 1996). During pregnancy and lactation, ErbB2 is required, as knockdown of ErbB2 has been demonstrated to lead to abnormal ductal branching (Sternlicht et al., 2006). ErbB2 amplification can lead to ligand independent EGFR activation and downstream signaling. ErbB2 is amplified in the HER-2 breast cancer subgroup. ErbB3 and -4 play a role during pregnancy and lactation, ErbB3 as a dimerization partner to ErbB2, and ErbB4 appears to act predominately in an

intracellular manner, in the cytosol and nuclei (Ishibashi et al., 2013). As has been described earlier, mammary epithelial cells exist in a highly dynamic, but controlled environment; aberrant changes to signaling can thus have profound effects on cellular behavior. Both EGFR and ErbB2 affect cell polarity, a key step towards increased motility (Chatterjee et al., 2012; Lu et al., 2003). A paper by Seton-Rogers and colleagues showed that over-expression of ErbB2, in addition to TGFβ induced EMT in MCF10a cells. They demonstrated that in three-dimensional culture, cells could change phenotype from being epithelial, to highly invasive and mobile (Seton-Rogers et al., 2004).

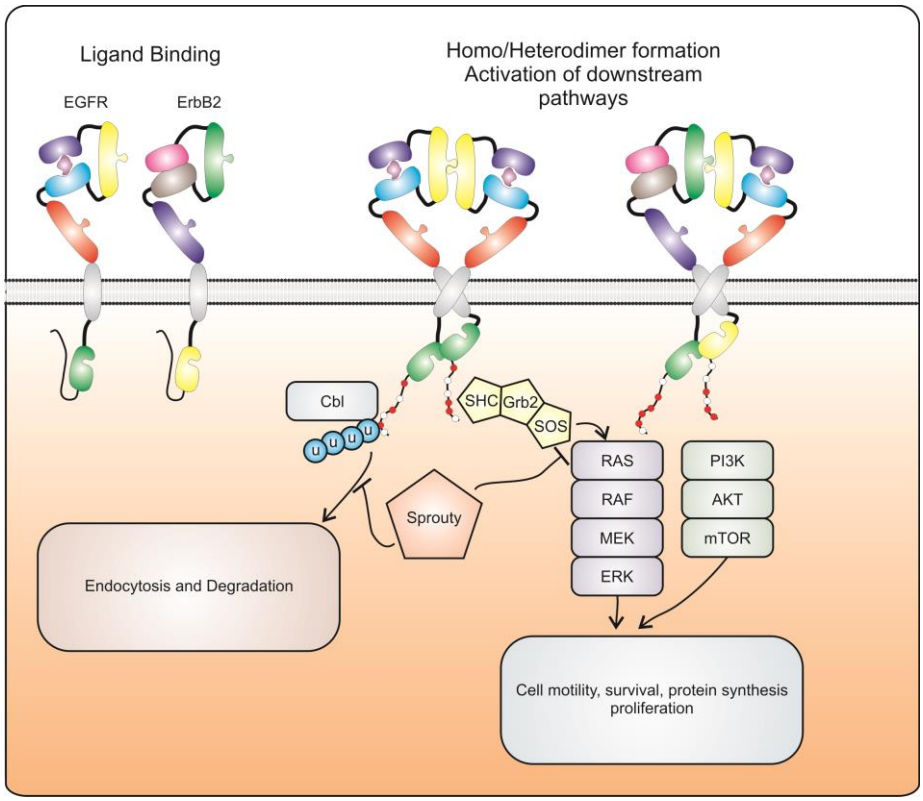

Figure 4. *Dimerization and activation of EGF receptor family.*
When EGFR binds a ligand, it adops an active conformation enabling dimerization. Dimerization is followed by phosphorylation of intracellular residues (red) and activation of downstream pathways. Sprouty can regulate EGFR activation by bowth inhibiting the RAS pathway as well as reducing ubiqutylation of the EGF receptor, thereby reducing degradation. Adapted from (Ferguson, 2008; Gross et al., 2001; Red Brewer et al., 2009; Stang et al., 2004).

There are numerous pathways that modulate the EGFR signaling cascade, including protein tyrosine phosphatases which dephosphorylate target proteins within the cascade or the receptor themselves, other methods include the control over whether the receptor is recycled to the cell surface or degraded. Certain

residues such as tyr1045 on EGFR have been shown to be associated with receptor degradation by proteasomes via Cbl induced ubiquitination of the receptor (Figure 4) (Stang et al., 2004). Studies have shown that different ligands cause differential activation at this residue, where amphiregulin does not readily lead to tyr1045 activation; but induced recycling of the receptor back to the cell surface while EGF stimulation causes strong tyr1045 phosphorylation and increased EGFR degradation (Stern et al., 2008). One highly interesting group of phosphorylation modulator proteins are the sprouty proteins which are RTK antagonists and have been shown to affect branching morphogenesis in several organs and organisms.

The sprouty protein family derives its name from its discovery in Drosophila, where loss of sprouty led to increased tracheal branching during development (Hacohen et al., 1998). In humans, the sprouty proteins are four, (sprouty 1-4) and have been shown to affect phosphorylation of several RTKs, most prominently the FGFR. The mechanism in which sprouty antagonizes RTK signaling has not been fully elucidated, but several mechanisms have been demonstrated, ranging from dephosphorylation of key proteins such as RAF or inhibition of protein binding to RTKs, such as interference with Grb2, a key protein in initiation of MAPK pathways (Gross et al., 2001). In the breast gland, sprouty-2 has been most studied. Sprouty-2 contains a domain with strong similarity with the tyr1045 site of EGFR, and studies have shown that sprouty-2 can interfere with Cbl-induced ubiquitination of EGFR via competition, thus stabilizing EGFR and preventing degradation – in addition to other mechanisms described above – thus both prolonging and dampening the signal (Rubin et al., 2003). The diverse function of sprouty in RTK signaling has led to great interest in the protein family, especially with regard to cancer. Sprouty 1 and 2 have been demonstrated to be downregulated in breast cancer (T. L. Lo et al., 2004). In other tissues, for example in colon, liver and lung cancer, sprouty proteins have been shown to be similarly down-regulated (Barbachano et al., 2010; Fong et al., 2006; Sutterluty et al., 2007). Knockdown studies have demonstated that sprouty inhibits tumor growth, as loss of sprouty has led to increased tumor formation in mouse models. Conversely, over-expression of sprouty has inhibited tumor growth, showing the role of Sprouty as tumor suppressors (Faratian et al., 2011; C. C. Lee et al., 2004; Masoumi-Moghaddam et al., 2014).

1.6 Cellular crosstalk in the breast gland

The epithelial glandular structure undergoes constant remodeling and as mentioned previously this is regulated by both paracrine and endocrine factors, and through interplay between both the stroma and both of the epithelial lineages. In this chapter I will briefly discuss recent research in epithelial stromal interaction in the breast gland and aim to describe how these signals are regulated.

As mentioned before, both luminal and myoepithelial progenitor cells are believed to reside within the basal/myoepithelial population. These cells then give rise to transit amplifying cells that eventually terminally differentiate into LEPs and MEPs (Rios et al., 2014). Controlling the ratio between LEP and MEP populations is highly important to maintain normal breast gland structure and function. Myoepithelial cells have been shown to have antiproliferative effects on both normal and malignant breast cells. A study by Pasic and colleagues showed that a stronger EGFR signaling cascade through Erk1/2 by increased EGF concentration led to increased myoepithelial differentiation (Pasic et al., 2011). In ducts MEPs effectively surround LEPs, whereas in the acini they form a more basket-like pattern (Moumen et al., 2011). This means that systemic signals from the stroma - for example during puberty, pregnancy and lactation, when branching morphogenesis occurs - affecting the luminal cells must be mediated through the myoepithelial compartment. MEPs have been demonstrated to reduce Matrix Metalloprotease (MMP) production in cancer cells (Jones et al., 2003). Knocking down proteins that direct myoepithelial differentiation has been shown to induce proliferation in the luminal compartment, and lead to generation of alveoli without systemic signals (Radice et al., 1997). Additionally, myoepithelial cells maintain correct polarity of luminal epithelial cells in 3D culture (Grudzien-Nogalska et al., 2014; Gudjonsson, Ronnov-Jessen, et al., 2002). In the study by Gudjonsson and colleagues, they showed that myoepithelial cells that were derived from tumors affected the luminal cells differently, as they were not able to correct the polarity of the luminal cells in culture, indicating that tumor derived myoepithelial cells had lost some of the regulatory properties, including the production of laminin-1. During cancer initiation, cells initially lose polarity and fill the lumen of ducts, forming ductal carcinoma *in situ* (DCIS). These initial tumors are encapsulated by a layer of myoepithelial cells. This is one of the diagnostic factors used to discriminate between *in situ* and invasive disease. During later progression steps, myoepithelial cells are progressively lost, and in invasive disease, differentiated myoepithelial cells are sparse and discontinuous (Polyak & Hu, 2005). Whether the loss of myoepithelial cells is due to increased apoptosis, reduced differentiation from basal progenitors, or simply dilution within the tumor is still unclear. During cancer progression, and the loss of the myoepithelium, the cancer cells are increasingly exposed to the stromal environment, which plays a big role in the function of both the normal and malignant breast gland.

1.6.1 Epithelial-stromal interaction

As stated previously, the epithelial compartment in the normal breast gland is embedded in collagen rich stroma. Individual components of the breast stroma are believed to play a strong role in maintaining tissue homeostasis and normal development. The initiating signal of branching morphogenesis is thought to originate within the stroma, where signals from fibroblasts, including FGF and

EGF induce proliferation of the epithelial ductal tree, and migration/invasion into the surrounding extracellular matrix (ECM) by induction of MMP expression (Wiseman et al., 2003). In mouse-human xenografts the presence of human fibroblasts was crucial for the implantation and proliferation of the epithelial organoids (Kuperwasser et al., 2004). During branching morphogenesis, the epithelial cells also self-regulate the density of branching, in part through TGFβ signaling (Ingman et al., 2008; Nelson et al., 2006). Endothelial cells, especially the breast microvasculature play a pivotal role in the normal development of the breast gland, for the most part through delivery of nutrients and endocrine signals (Djonov et al., 2001). In the normal gland, the myoepithelial cells produce components of the basement membrane, isolating the epithelial compartment from the stroma (Gudjonsson, Ronnov-Jessen, et al., 2002). In addition, myoepithelial cells orchestrate both the generation and inhibition of new blood vessels (Barsky & Karlin, 2005; Rossiter et al., 2007). During puberty, pregnancy and lactation the microvasculature undergoes drastic changes to accommodate for increased metabolism in the breast gland. A study performed by Rossiter and colleagues showed that knocking out VEGF-A produced by myoepithelial cells had a strong antiangiogenic effect in the mouse mammary gland, which led to an abnormal and poorly functional mammary glandular tissue (Rossiter et al., 2007). Previous studies have demonstrated that normal myoepithelial cells also inhibit angiogenesis, in part by production of maspin (Pandey et al., 2010).

Aside from the role of transporting nutrients and oxygen, microvessels have been shown to play a direct regulatory role in the development of several organs, including the prostate. In castrated rats that were given testosterone, the vasculature of the prostate expanded before the glandular tissue, indicating a regulatory role of the endothelial network (Franck-Lissbrant et al., 1998). In the breast gland, research on the role of endothelial cells has focused on the part played during cancer progression and metastasis. Few studies have focused on endothelium in normal tissue or the direct effects of endothelial cells aside from systemic transport. Shekhar and colleagues published a paper describing how endothelial cells in co-culture with human breast epithelial cells induce branching morphogenesis in three-dimensional culture (Shekhar et al., 2000). Our laboratory has focused on endothelial-epithelial interaction. Using tissue acquired from reduction mammoplasties, we have described a method for isolating primary human breast endothelial cells (BRENCs) (Sigurdsson et al., 2006). Using these BRENCs we have devised a co-culture model that can recapitulate aspects of the normal breast gland *in vitro* (Paper #1). This model will be discussed in further detail in a later chapter in this thesis.

During cancer progression, the stroma undergoes drastic reprogramming. In response to dysregulation of the epithelial structure, signaling from the

mesenchyme shifts to a pattern more similar to wound healing (Byun & Gardner, 2013). Wound healing is a complex response involving several different factors, many of which are the same as those used during branching morphogenesis, as well as in tumor progression. Growing tumors require vast amounts of nutrients and oxygen and this leads to hypoxic conditions within the tumor, leading to increased production of VEGF (Hendriksen et al., 2009). VEGF is a key component in the formation of new blood vessels. In addition to blood vessel formation, the ECM undergoes drastic changes. Monocytes infiltrate the tissue and differentiate into macrophages. These tumor associated macrophages (TAM) cause native fibroblasts to transform into myofibroblasts, an activated form of fibroblasts that play a key role in wound healing and undergo apoptosis once healing is finished (Mao et al., 2013). During wound healing, TAMs secrete a variety of growth signals and ECM molecules, including EGF, FGF, PDGF and TGFβ, all of which subside when healing is done. In the tumor environment however, these signals persist due to the constant remodeling of the tumor surroundings. Myofibroblasts secrete a plethora of matrix remodeling proteins and growth factors all of which influence the tumor, and in connection with microvessel growth can lead to metastasis formation, the final stage of cancer progression. It is of great importance to understand the interaction between stroma and epithelium, given its importance in breast cancer progression.

1.7 Breast cancer

Breast cancer is a pervasive disease that originates in the epithelial breast tissue. It is estimated that one in eight women will develop breast cancer (DeSantis et al., 2014). Breast cancer progresses through several disease stages, ranging from ductal carcinoma *in situ* to distant metastatic disease. Prognosis is determined by several factors, including stage, grade and marker expression pattern.

1.7.1 Breast cancer classification

In order to classify tumors for better treatment options, biopsies and tumor samples are generally first checked for expression of the main hormone receptors involved in breast biology; estrogen receptor (ER), progesterone receptor (PR) and overexpression of HER2. Using these markers, pathologists group breast cancer into three main types: Hormone receptor positive cancers, these tumors are usually of low grade, i.e. more differentiated. These tumors in general have better prognosis than the other groups. Tumors exhibiting HER2 amplification have worse prognosis than the hormone receptor positive tumors. In general, HER2 amplified tumors are hormone receptor negative. Cancers not expressing hormone receptor, and no amplification of *HER2* are referred to as Triple Negative Breast Cancer (TNBC) (Rakha & Chan, 2011; Schmadeka et al., 2014). These tumors

usually have high grade, and have the worst prognosis of the tumor subtypes described here. TNBC tumors are often very heterotypic, while the ER/PR positive cancers resemble the luminal compartment of the normal breast, TNBC exhibit traits of basal or undifferentiated cells.

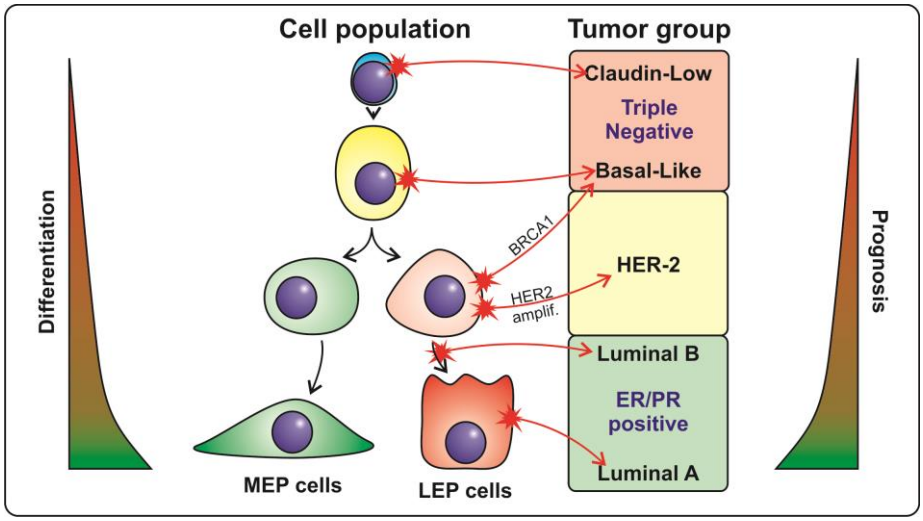

Figure 5. *Cellular hierarchy and breast cancer subgroups.*
Stem and progenitor cells exist within the breast gland, responsible for repopulating the breast gland during each consecutive pregnancy. Different cancer subgroups are believed to arise from within this hierarchy, the least differentiated cells are thought to give rise to the Claudin-Low and Basal-Like tumors. *BRCA1* mutations have been demonstrated to arise from luminal progenitors that revert back to a basal like state. Luminal A and Luminal B tumors derive from more differentiated luminal cells. Based on (Oakes et al., 2014; Perou, 2011; Visvader & Stingl, 2014).

Using cDNA microarrays derived from primary tumor samples, Perou, Sorlie and colleagues were able to group the tumors using hierachial clustering into distinct subgroups (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003) The most prominent groups that emerged from the clustering were Luminal A and Luminal B; both estrogen receptor positive, Basal-like; poorly differentiated tumors resembling the basal progenitor population of the breast gland, HER2 enriched; phenotype associated with the HER2 amplified group described earlier, and Normal-like, a group resembling in signature to that of normal breast gland. When disease outcomes were compared with tumor subtype the data coincided, to a large extent, with the previous prognostic data of ER/PR positive, HER2 amplified and TNBC groups (Sorlie et al., 2003). The distinction beween Luminal A and Luminal B did demonstrate that not all estrogen receptor positive cancers might respond to treatment the same way. Additionally, the overlap between TNBC and Basal-like tumors is not complete, as basal-like cancers are highly heterogeneous (Lehmann & Pietenpol, 2014). Within the basal-like tumors, a population of highly invasive, poorly

differentiated tumors, claudin-low tumors have relatively recently been described. Often triple negative, these tumors are characterized by low expression of epithelial cadherins and keratin expression, and are believed to originate in the least differentiated progenitor populations of the breast (Perou, 2011). These data made it clear that expression profiling of tumors could give real prognostic values, and it seems obvious that a more detailed analysis of tumors, compared with looking at hormone receptor status, has the potential to be of benefit for the patient when developing treatment strategies. In the following chapters I will refer to tumor groups based on the expression profiling, (e.g. basal-like, instead of TNBC)

The similarities between tumor subgroups and the normal cell populations of the breast posed the question whether the tumor groups had their origins within their respective epithelial compartment (Oakes et al., 2014; Perou, 2011; Visvader, 2009). Additionally, research groups have been heavily focused on identifying potential therapeutic targets within the basal-like cancer group, which has the worst prognosis, in addition to being more common in younger, pre-menopausal women. Expression profiling of tumors demonstrated that individual tumor groups resembled the hierarchical organization of the breast gland. Luminal A and B resemble the luminal epithelial cells, are driven by hormonal signals, and can be treated using tamoxifen and aromatase inhibitors (Hammond et al., 2010; Schiavon & Smith, 2014). HER2 enriched tumors are usually ER negative, they are also more heterogeneous, and tend to be more prone to form metastasis. They can be treated using Trastuzumab, (Herceptin) a monoclonal antibody that inhibits the function of the HER2 receptor (Baselga et al., 1998). Trastuzumab treatment has drastically increased disease free survival of HER2 breast cancer patients. Basal-like breast cancer (BLBC) has until now had no targetable treatment options, and hence has the worst prognosis. BLBC is more common in younger women, and has high rates of metastasis, cells in BLBC are more invasive, and often have acquired mesenchymal traits, referred to as epithelial to mesenchymal transition (EMT). Tumors harboring BRCA1 mutations are often classified within the Basal-like group, as they demonstrate high levels of heterogeneity and high grade (Bai et al., 2013; Lim et al., 2009).

1.7.2 Cancer Stem Cells and EMT

The link between stem cells and cancer has been known for decades. The earliest evidences are reported in germ cell tumors, where teratomas exhibit an expression pattern of multiple germ layers (reviewed by (Bustamante-Marin et al., 2013)). In addition, origin of leukemias from disruption in hemapoietic stem cell differentiation has been known for a long time (reviewed by (Huntly & Gilliland, 2005)). Cancer stem cells (CSC) is a term

often used to describe cells within tumors that drive tumor growth, in a similar manner to the normal progenitor cells of a given organ during normal development. During treatment these cells have increased survival odds, through survival pathways that in the normal breast protects stem cells from damage during their lifetime and also due to slow proliferation, meaning that after treatment they re-initiate the tumor (Kakarala & Wicha, 2008). This feature has led to these cells being sometimes referred to as tumor initiating cells (TIC). In 2003, Al-Hajj and colleagues described a tumorigenic cell population able to initiate tumors through serial transplantations into mice. These cells were identified to be $CD44^{Hi}/CD24^{Lo/neg}$, and injecting only a few of these cells was sufficient to initiate new tumors, demonstrating their malignancy and self-renewal capabilities, while other cancer cell populations were unable to regenerate tumors *in-vivo* (Al-Hajj et al., 2003). This indicated that only a small ratio of cells within a given tumor could both self renew, and fuel tumor growth via production of tumor cells with limited self-renewal abilities. Studies have shown that within tumors there exist tumor initiating cells, that have low proliferative rates, ability to self-renew and increased plasticity (Blagosklonny, 2005). The use of CD44 and CD24 as markers of stem cell populations in breast cancer has since become widespread within the literature, along with $ALDH1^{+}$, which further selects for stemness (Ginestier et al., 2007). The origin of these cells is still under debate, two main competing hypotheses exist; firstly, that the TICs originate from normal mammary progenitor cells, through a series of mutations and secondly that tumor cells through a series of mutations and epigenetic changes transdifferentiate into a stem-like state (Marjanovic et al., 2013). The mechanism by which these cells multiply has been proposed to be dysregulation of asymmetric division which in the normal breast places tight constrictions on the number of progenitor cells (Huebner et al., 2014; Oakes et al., 2014). Another hallmark of CSCs is their shift from predominately epithelial phenotype to a more mesenchymal phenotype, referred to as epithelial to mesenchymal transition (EMT).

EMT is a natural developmental process where epithelial cells undergo transition into a mesenchymal state. This is associated with downregulation of cytokeratins and epithelial cadherins, and upregulation of mesenchymal proteins such as vimentin and N-cadherin (Scheel & Weinberg, 2012). During embryonic development, EMT takes place during gastrulation and neural crest formation. Partial EMT takes place during wound healing, where organized epithelial tissue dissociates in response to repair signals (Yan et al., 2010). In the tumor microenvironment signaling from the stroma induces drastic changes to the epithelial cells, as discussed earlier. As a consequence, the EMT process is hijacked in the most invasive cancers (Mani et al., 2007). EMT induction leads to cells acquiring a CSC phenotype,

as evidence by increased survival and motility, $CD44^{Hi}/CD24^{Lo/neg}$ expression phenotype, they have low levels of E-cadherin and keratins and are highly invasive (Singh & Settleman, 2010). EMT and the CSC phenotype have been shown to be closely linked through several signaling pathways. In a paper from 2008, Mani and colleagues demonstrated that cells that have undergone EMT also harbor many characteristics of stem cells. Additionally, stem cells isolated from both mouse and human mammary tissue were shown to have high expression of mesenchymal markers such as snail and twist (Mani et al., 2008).

MicroRNAs (miR), small interfering RNA molecules have been shown to play an important role in EMT and CSC biology (reviewed by (Nickel & Stadler, 2014)). miR-200 expression in breast tumors is often downregulated, and has been shown to lead to EMT (Korpal et al., 2008). miR clusters are often methylated in tumor samples, leading to reduced expression and widespread effects, including EMT (Vrba et al., 2010). Other pathways involved in EMT include TGFβ and canonical wnt signaling. TGFβ and wnt signals in the normal breast have a significant role during puberty and pregnancy during branching morphogenesis, mostly by inhibiting proliferation and migration. In tumors however, TGFβ has been shown to enhance tumorigenicity and mobility of cells by promoting EMT (de Kruijf et al., 2013). Data from our laboratory and others have shown that endothelial cells can induce EMT in epithelial cells, in part through HGF and TGFβ signaling (Kimura et al., 2013; Sigurdsson et al., 2011). Both EGFR and ErbB2 have been demonstrated to be important for cancer stem cell maintenance, where ErbB2 appears to prevent differentiation pathways (Farnie et al., 2007; Kakarala et al., 2008; X. Li et al., 2008). To conclude, EMT and CSC phenotypes are closely linked, and stem from the deregulation of several response pathways induced by the tumor-stroma interaction, similar to wound healing and developmental processes in normal tissues (Micalizzi et al., 2010).

1.8 Modeling breast development and cancer progression

In previous chapters I have cited several research papers that have used different methods to elucidate the mechanisms in which the normal breast develops and progresses to cancer. I have listed the findings, but not the main methods used to obtain the results presented. In this chapter I will compare several different methods used in both *in vitro* and *in vivo* research.

1.8.1 Human breast tissue

For basic understanding of the architecture of the normal and malignant human breast, access to tissue acquired through a variety of methods, including reduction mammoplasties and mastectomies, and established cell lines is highly important. Tissue can be both fresh frozen and cryosectioned,

or paraffin embedded and sectioned in a microtome. Staining using antibodies against specific antigens have revealed the protein expression pattern of the breast gland. Western blotting, flow cytometry and quantitative PCR have supplied additional information and helped researchers to elucidate the architecture and function of the breast gland. While these approaches have given vast amounts of data they represent merely snapshots of a breast gland at the time of sectioning. Furthermore, samples of tumors demonstrate only the final phenotype of a given tumor, while tumor progression often takes years (Mardis, 2014). In order to be able to track changes over time, the use of *in vivo* models has given invaluable insights into the development of both the normal and cancerous breast.

1.8.2 *In vivo* models

Animal research models have given a wealth of data detailing the normal development of organs as well as serving as disease models. With regard to the mammary gland, the mouse gland has been extensively studied. Similar to the human breast gland the mouse gland undergoes three distinct phases of differentiation, during embryonic development, where rudimentary ducts are formed, during puberty, where the gland expands, and then during pregnancy when the gland undergoes differentiation. The use of mouse models has proven invaluable when establishing differentiation pathways and stem cell hierarchy in the mammary gland (reviewed by (Visvader et al., 2014)). The mouse as a model for mammary gland development stems from several factors, including short generations, ease of access and breeding, and a vast repertoire of knockdown strains; almost any gene in the mouse genome can be knocked out, and the effects monitored. Another method commonly utilized is fusing a given protein with a fluorescent protein, such as GFP or RFP, and monitor protein localization during development. The mouse gland has also served as a recipient for xenografts, where cells or pieces of tissue are injected into the mammary fat pad. This approach has been widely used to measure tumorigenicity and cancer progression, but also to analyze normal development. Xenografts are often performed after first clearing the fat pad of native mammary tissue; before puberty, the mouse mammary tissue is localized to one edge of the fat pad, and if removed, the fat pad does not become infiltrated by native mammary tissue during puberty. While the mouse mammary gland is similar to the human breast gland in many ways, there are striking differences. Aside from the obvious size difference, the human mammary gland tree terminates in the TDLU, while the mouse gland terminates in the TEB. Additionally, the stroma surrounding the mouse mammary network is not as collagenous as the human gland; glandular tissue is more found within fatty tissue. To address these differences the use of *in vitro* modeling is of great importance.

1.8.3 *In vitro* models

There are several important approaches that are widely used for modeling normal and malignant mammary gland biology. Culture of established cell lines over culture of primary cells is widely used both due to ease of access and increased stability, as primary cells have a limited culture time due to replicative senescence. Cell lines established from tumor tissue have been shown to represent to an extent the phenotype of the tumor they originate from (Kenny et al., 2007). Cell lines representing the normal mammary gland are in lower supply. The environment of the cells matters a great deal based on the context of the research. For modeling signaling, proliferation, survival and drug tolerance conventional monolayer culture can provide valuable data. To study more complex processes, such as anchorage independent growth, soft agar, or mammosphere assays are widely used, both approaches build on the premise of preventing cells from attaching to a substrate either by embedding in agarose, or using specially treated culture plates that prevent attachment.

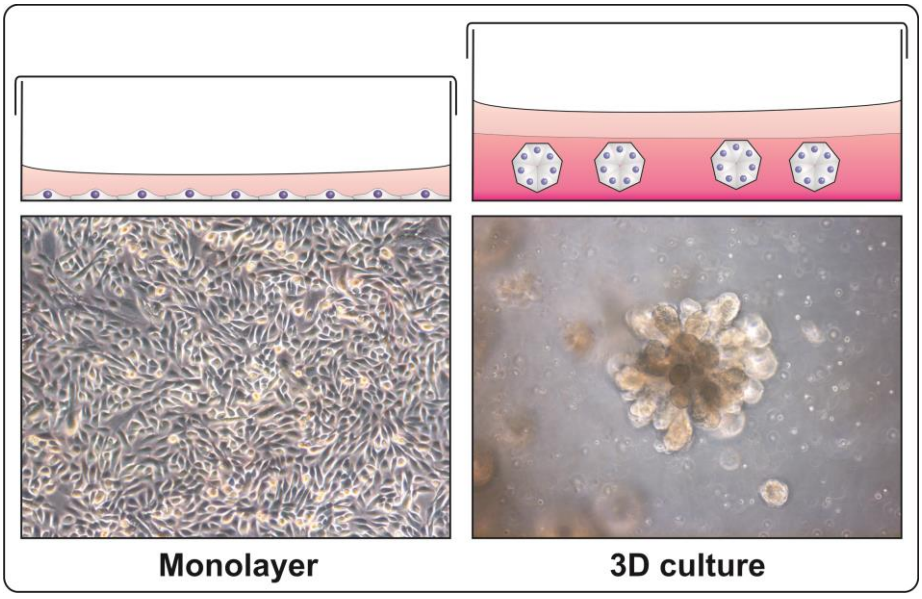

Figure 6. *Differences between 2D an 3D culture.*
Conventional culture in monolayer techniques can supply vital information regarding cellular processes, while 3D culture adds an extra dimension with regard to morphogenic studies. *D492 stem cell line, grown on monolayer (left) and embedded in matrigel (right).*

To study polarization, lumen formation and branching morphogenesis *in vitro,* three dimensional culture models have grown in popularity in the last two decades. In three dimensional culture, cells are cultured within extracellular matrix proteins most often collagen or laminin rich reconstituted

basement membrane molecules (matrigel) (Benton et al., 2014). The two most commonly used cell lines for normal breast gland research are MCF10a and HMT3522, both derived from normal breast tissue (Debnath & Brugge, 2005; Weaver & Bissell, 1999). These cell lines have given valuable insight into several developmental processes, including lumen formation and polarization (Muthuswamy et al., 2001; Reginato et al., 2005; F. Wang et al., 2002). These cells have been manipulated in order to coax them towards a more malignant phenotype, including loss of polarity, and tumorigenicity when injected into mice. This enables cells to proliferate and differentiate in a manner more similar to that found in the body, especially when compared with conventional monolayer culture. There are very few cell lines able to differentiate into both main epithelial lineages of the breast. The D492 cell line was established from tissue from reduction mammoplasty. The cell line was immortalized and once established was shown to be able to differentiate into both luminal and myoepithelial cells. When embedded into matrigel, the cell line is able to form elaborate branching structures with both myoepithelial and luminal cells present with the correct histoarchitecture, enabling the *in vitro* modeling of branching morphogenesis (Gudjonsson, Villadsen, et al., 2002; Sigurdsson et al., 2011). Heterotypic interaction of different mammary gland cells has mostly been limited to how fibroblasts affect the remodeling of the breast gland during development and cancer progression, the other cell types, including the endothelial cells have not received as much focus.

2 Aim of the Thesis

The role of stem cells in the normal breast is to provide a source of cells to enable cycles of cell proliferation and differentiation during each menstruation cycle and more prominently during pregnancy, lactation and involution. Due to their longevity in tissues, stem cells in the breast are also suspected to be candidate tumor initiating cells and thus studying their role in normal development and cancer is of great importance. In recent years, microvessels have been shown to be important regulators of stem cell niche in distinct organs. To be able to analyze *in vitro* the role of stem cells in breast morphogenesis and cancer, a culture model is needed that captures to a certain extent the natural environment of the breast gland, including interactability with the surrounding stroma. The use of mouse models has greatly enhanced our knowledge of normal mammary gland development and breast cancer; but to be able to model stem cell biology in humans, greater control over experimental variables are needed, calling for an *in vitro* approach. *In vitro* modeling is complementary to *in vivo* modeling, providing the possibility to control all environmental factors. Furthermore, cell lines demonstrate greater flexibility with regard to knockdown and over-expression approaches. The role of ErbB2 amplification in breast cancer has been analysed extensively, especially with regards to therapeutic options. Still there is much to be learned, in particular regarding crosstalk between ErbB2 and other EGFR RTK family members, and the role of ErbB2 and EGFR in stem cell biology.

The general aim of this thesis was to develop a three dimensional culture model that recapitulated aspects of the breast gland environment, and to use this model to analyze the role of the EGFR receptor family and its downstream signaling molecules in stem cell biology and cancer progression The general aim was followed in the four papers published or in preparation:

1. Establish an *in vitro* culture model which can be used to analyze the effect of breast endothelial cells on different epithelial cells (paper 1).

2. Analyze the role of EGF signaling in established breast cell lines (paper 2).

3. Analyze the effect of knocking down sprouty-2, an EGFR signaling modulator, and determine the role of EGFR signaling in branching morphogenesis and mammary gland integrity (paper 3).

4. Analyze the molecular and morphogenic effects of EGFR and ErbB2 overexpression in a breast epithelial derived stem cell line (paper 4).

3 Materials and Methods

Detailed materials and methods can be found in each paper, I will use this chapter to summarize the main methods, and in some cases discuss differences between assays.

Table 1. *Media formulations.*

Additive	*Final concentration*		
	H14	**CDM3**	**CDM4**
EGF	10ng/ml	100ng/ml	20ng/ml
Insulin	250ng/ml	3µg/ml	3µg/ml
Transferrin	10µg/ml	25µg/ml	25µg/ml
Hydrocortisone	500ng/ml	500ng/ml	500ng/ml
Na-Selenite	2.6ng/ml	2.6ng/ml	2.6ng/ml
Estradiol	0.1nM	0.1nM	0.1nM
Prolactin	0.15IU	--	--
Cyclic AMP	--	10nM	10µM
Ascorbic Acid	--	10µg/ml	10µg/ml
Bovine Serum Albumin	--	0.01% w/v	0.01% w/v
Ethanolamine	--	100µM	100µM
Fetuin	--	20µg/ml	20µg/ml
Fibronectin	--	100ng/ml	100ng/ml
Phosphoethanolamine	--	100µM	100µM
Triiodotyronine	--	10nM	10nM
Cholera Toxin	--	--	10ng/ml

Cell culture

Primary epithelial cells

Primary epithelial cells were isolated from normal tissue derived from reduction mammoplasty. The tissue was finely chopped using scalpels and digested overnight at 37°C in 900U/ml Collagenase III (Worthington). After digest, the solution was centrifuged at 1000rpm for 40 seconds to settle epithelial organoids. Organoids were retrieved, reduced in size by passing through a needle, and washed four times in PBS, allowing them to settle under gravity between washes. After rinsing, organoids were seeded on Collagen I coated tissue culture flasks (BD), and maintained on chemically defined media 3 (CDM3) (see table 1). For purification of luminal and myoepithelial cells, cells were selected based on EpCAM+MUC-1 and β4-integrin+Ty-1 expression, respectively. Cells were sorted using magnetically

labeled secondary antibodies and equipment from Miltenyi Biotech. Purified luminal epithelial cells were cultured on CDM3 media, while myoepithelial cells were maintained on CDM4 media for up to 8 passages (table 1). Primary endothelial cells were isolated as described by (Sigurdsson et al., 2006), microvascular organoids were selected using CD31 labelled magnetic beads and cultured on EBM-2 culture media (Lonza) supplemented with 30% Fetal bovine serum for the first passage, reduced to 5% for subsequent passages (referred to as EGM5). In paper #2 we immortalized the A163 cell line using retroviral vectors containing HPV16 E6/E7 oncogenes. These genes help immortalize cells trough dysregulation of the gatekeeping proteins Retinoblastoma and P53, thereby enabling cells to evade growth arrest and increasing the likelihood of activation of other pathways, including hTERT which enables longer proliferation as telomeres are maintained instead of shortening through each cellular division (Band et al., 1990).

All values represent final concentrations in culture media (DMEM:F12 1:1)

Cell lines

MCF10a, D382, A163, D492 cells and their derived sub-lines were maintained on Serum free H14 media (table 1), A163-S1 was cultured without EGF. The cancer cell lines MCF7, MDA-MB-231 and T47-D were cultured based on ATCC recommendations.

3D culture assays

In general, 300µl Matrigel in a 24 well plate setup was used; the stated amount of cells was embedded in matrigel, which was then seeded on a plate and incubated at 37°C for 30 minutes. Cells were then supplemented with media and maintained for up to three weeks, changing media three times per week.

Monoculture

For primary epithelial 3D culture, 10^4 and 10^5 cells were seeded alone and maintained on either CDM3/4 or EGM5 culture media for two weeks. D492 cells and sub-lines were seeded at a density of 10^4 or $2.5x10^4$ cells and maintained on H14 media.

Co-culture

Most co-culture experiments conducted in the thesis were based on co-culture between breast endothelial cells (BRENCs) and breast epithelial cells. Within Matrigel, BRENCs stay quiescent, but viable. When cells were placed in co-culture with endothelial cells, EGM5 media was used. For co-culture of primary cells, 10^4 epithelial cells were seeded along with $2x10^5$ endothelial cells. For certain experiments, the density of endothelial cells was reduced to 1,000 cells per well. When co-culturing cell lines, 500 epithelial cells were seeded with $2x10^5$ endothelial cells. Co-culture experiments involving transwell filters were setup where epithelial cells were embedded into

matrigel in the bottom chamber (500 cells per well), and a transwell filter containing endothelial cells in monolayer was transferred on top. In one experiment, a gradient of densities was achieved by seeding endothelial and epithelial cells into separate gels, allowing the gels to merge in the center. This gave a gel with only endothelial cells on one end, a mixture of both cell types in the center, and then only epithelial cells at the opposite end.

Mammosphere assays

To assay cell survival under low attachment conditions, cells were seeded on plates coated with Poly(2-hydroxyethylmethactylate, poly-HEMA) to prevent cell attachment. Cells were seeded at a concentration of 1,000 cells per 6-well tray, and maintained for two weeks, after which colonies were counted and size measured.

Immunohisto- and cytochemistry

In the thesis, expressional analysis was performed on both human and mouse tissues, as well as cell staining from monolayer and 3D assays. Cells in monolayer culture were fixed using either methanol or formaldehyde based on the recommendations for the antibody being used. In general, tissue samples from human or mouse sources were either frozen in dry ice chilled n-hexan and subsequently cryosectioned; or dehydrated and embedded in paraffin for microtome sectioning. Both conventional DAB staining and Immunofluorescence staining using secondary fluorescent Alexa Fluor antibodies from Invitrogen were used. For visualization of immunofluorescence, Zeiss LSM5 Pascal and Olympus Fluoview 1200 confocal microscopes were used.

Western blotting

For Western blots, protein lysates were made using RIPA lysis buffer. Protein concentrations were estimated using Bradford reagent and in general 5µg of denatured protein was loaded into each well. Westerns were performed using pre-cast Nupage gels (Invitrogen) and PVDF membranes. In papers #1-3, Western blotting was visualized using HRP antibodies and ECL bioluminescence. These blots were exposed to film, and developed. In paper #4, Western blots were visualized using near-infrared immunofluorescence and scanned using equipment from Li-Cor (Odyssey). For standardization of signals, actin, Histone H3 and GAPDH were used as loading controls.

Real-Time PCR

RNA was extracted from cells and pulverized tissue samples using Trizol. RNA was reverse transcribed into cDNA using random Hex primers. Primer pairs and probes were acquired from Applied Biosystems and PCR was performed on 7500 Real Time PCR system from Applied Biosystems. GAPDH was used for normalization.

Knockdown using shRNA

We used commercially available short hairpin lentiviral vectors to knock down sprouty-2. As our experiments in 3D culture take up to three weeks, transient transfection of vectors was deemed unfeasible, as transient vector expression is reduced over time, while the use of lentiviral vectors leads to prolonged expression of the DNA. Cells were selected based on puromycin selection. In addition, the shRNA vectors expressed GFP, for easier visualization.

Viral transduction of EGFR and ErbB2 vectors

In order to overexpress EGFR and ErbB2, the same considerations had to be made regarding the length of experiments. Additionally, having cells stably expressing the introduced genes was beneficial, as cells could be passaged without having to repeatedly transfect. For EGFR, ready-made retroviral vectors were available from the plasmid repository Addgene. These plasmids were transfected into a packaging cell line, yielding retroviral vectors which were then used to transduce into D492 cells. Transduced D492 cells were selected based on puromycin selection. ErbB2 containing vectors were also acquired from Addgene, and cloned into a lentiviral expression plasmid. Similar techniques were used to transduce D492, as with EGFR. ErbB2 expressing cells were based on selection for eGFP expression, which was transcribed downstream of ErbB2, with an internal ribosomal entry site (IRES) between the two. The main difference between lentiviral and retroviral transduction is that retroviruses require cells to be in s-phase for successful transduction, while lentiviral vectors have no such requirement.

Mouse xenografts

To assess tumorigenicity of the D492 sublines, cells were injected into fat pads of NOD/SCID mice at a concentration of $5x10^5$ cells per injection site. Cells were injected in 100µl of 1:1 mixture of PBS and Matrigel. Tumor growth was measured three times per week, and after the experimental period, tumor tissue was formalin fixed and paraffin embedded

Statistical Analysis

Data are presented as means of triplicates, error bars represent standard deviation, unless stated otherwise. For statistical analysis, two-tailed T-test was used. For significance, P values below 0.05 were considered significant.

4 Results and Discussion

In this chapter I will discuss the data presented in the published papers and manuscript that form the basis of my thesis. All papers are enclosed with the thesis. I will discuss each paper individually and put the results in perspective to the existing literature. I will provide additional data that has not been published, but is relevant for discussion of the context of the thesis.

4.1 Paper #1 - Endothelial cells stimulate growth of normal and cancerous breast epithelial cells in 3D culture.

Epithelial-stromal interactions in mammary gland development and remodeling have been described in a number of research articles and reviews (reviewed by (Macias et al., 2012; Parmar et al., 2004)). However, the role of endothelial cells in the context of normal breast development and morphogenesis hasn't received much attention. The main focus with regard to endothelial cells has been on angiogenesis during breast cancer progression, as this is a pivotal concept for metastasis formation. It has however been noted in several papers that endothelial cells also play a role in normal development in distinct organs. This has been shown among others in the pancreas and prostate (Franck-Lissbrant et al., 1998; Lammert et al., 2001). A lactating breast gland is highly active and requires an efficient supply of oxygen and nutrients. In the normal breast gland, the TDLUs are surrounded by a dense microvascular structure (Djonov et al., 2001; Macias et al., 2012; Sigurdsson et al., 2006). In the mouse mammary gland and prostate, endothelial cell expansion has been shown to precede epithelial cell expansion, suggesting a possible regulatory role in development (Bates et al., 2008). Based on these publications, we wanted to see if microvascular endothelial cells played a role in breast morphogenesis.

Monitoring tissue development *in vitro* requires the appropriate experimental assay. The focus of our laboratory has been on researching cell biology in a three dimensional environment, with culture assays based on reconstituted basement membrane matrix (such as matrigel) or collagen I gels. Valgardur Sigurdsson, a former Ph.D. student at our laboratory established methods for isolating and expanding normal breast endothelial cells (BRENCs) from reduction mammoplastic tissue (Sigurdsson et al., 2006). Using D492, a breast epithelial cell line with stem cell properties, Sigurdsson was able to demonstrate that BRENCs enhanced growth and branching morphogenesis of D492 cells (Sigurdsson et al., 2011).

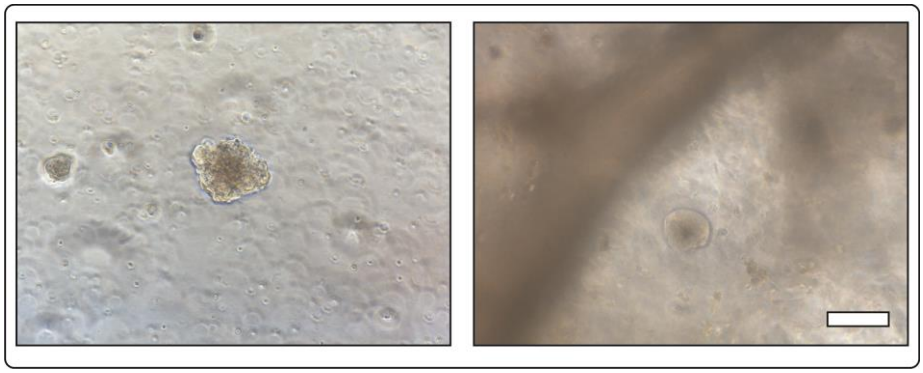

Figure 7. *Benefits of BRENCs over fibroblasts in co-culture.*
When embedded in matrigel BRENCs stay quiecent but viable, while fibroblasts quickly overgrow the culture obstructing visibility.

Based on the effect seen with co-culturing BRENCs with D492, I wanted to see if comparable effects would occur with distinct epithelial cells from the breast, both normal primary cells and normal-derived and malignant cell lines. I started out by isolating both luminal- and myoepithelial cells from the same tissue samples and setup co-cultures with BRENCs. When endothelial cells are seeded on top of matrigel, they form a network of elongated structures, reminiscent of microvasculature. These structures however dissociate within 72 hours. For our morphogenic studies, three days was too short a time period. We therefore attempted to embed the endothelial cells within the matrigel. Figure 1 in paper #1 (P#1 Fig. 1) shows that even after two weeks, viable and metabolically active BRENCs can be seen within the matrigel, based on their uptake of acLDL. Additionally, the cells do not proliferate in matrigel, but stay quiescent. This enabled us to setup co-culture experiments where BRENCs would act as three-dimensional feeder cells. Admittedly, endothelial cells arranged as single cells within a volume does not represent the physiological situation within tissues. Inducing BRENCs to form capillary-like structures within the gel would have been more representative, but the setup we used gave us increased spatial resolution, as the only proliferating cells in our assays would be the epithelial population. The great benefit of co-culturing with endothelial cells is the fact that they do not proliferate in the gel, but stay quiescent. We also performed co-culture experiments with primary fibroblasts. The fibroblasts proliferated and to a large extent overgrew the epithelial population, preventing observation (Figure 7). This could be prevented by treating the fibroblasts with either radiation or Mitomycin C, to stop them from proliferating in a similar manner as when embryonic stem cells are cultured (Ding et al., 2012), but that did not fall within the scope of this paper.

Primary breast epithelial cells can be isolated from reduction mammoplasties in a similar manner as BRENCs. After establishment and

initial culturing, sub-populations of luminal- (LEP) and myoepithelial (MEP) cells can be isolated using various sorting methods, including flow cytometry and magnetic cell sorting. When LEPs were seeded into matrigel they formed characteristic small colonies with a central lumen, a phenotype that has been well characterized in other papers (Gudjonsson, Ronnov-Jessen, et al., 2002; Petersen et al., 1992). These acinar colonies were polarized, as evidenced by their basal expression of β4-integrin and apical expression of ZO-1 (P#1, Fig. 2 and Figure 8). When we co-cultured endothelial cells along with LEPs, we noticed an increase in overall colony number and size. A number of large colonies with a large central lumen were seen. These colonies were polarized in a similar manner as the smaller colonies, but the lumen was greatly expanded. When we cultured myoepithelial cells in this co-culture system, we saw a significant increase in colony size and variation compared with control without BRENCs. MEPs do not form central lumens, but rather a mass of cells, expressing basal keratins.

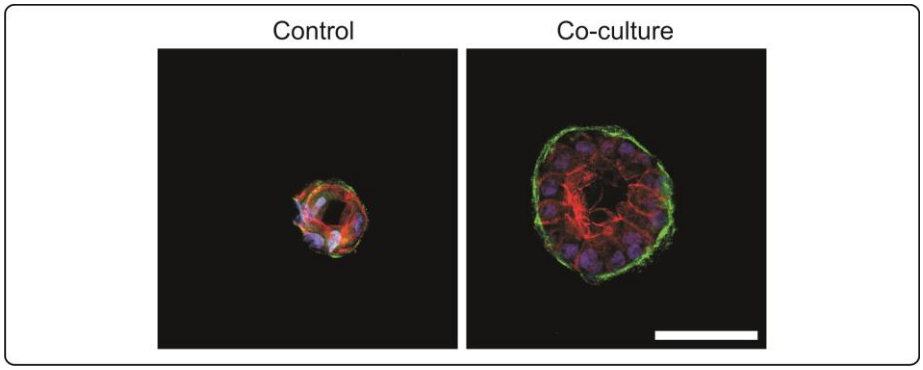

Figure 8. *Luminal epithelial cells in co-culture.*
Alone, LEPs form small polarized acini with a central lumen (left) In co-culture with BRENCs, LEPS formed large spherical colonies with a central lumen (right), much larger than in monoculture ***Green staining: β4-integrin, Red staining: F-actin. Bar: 50µm. Image adapted from (Ingthorsson et al., 2010).***

This increase in colony size indicated that endothelial cells were conferring some proliferative signals to the epithelial cells. Epithelial cells normally do not proliferate when seeded at low density in matrigel. To further characterize this effect we utilized several published cell lines, both normal and cancer derived. Cells were seeded at a clonal dilution, 500 cells per 300µl of matrigel. At these concentrations, none of the epithelial cell lines were able to proliferate and form colonies in monoculture. However, when we co-cultured with BRENCs, all cell lines responded by forming large colonies. The phenotype of the cell lines became evident in this assay, where for example the normal derived cell line MCF10a formed multiacinar structures with basal β4-integrin expression, whereas the malignant MDA-MB-231 formed stellate, mesenchymal-like colonies (P#1 Fig. 3). This multiacinar

structure of MCF10a has been previously reported but in that context the cells were expressing exogenous ErbB2 protein (Muthuswamy et al., 2001; Seton-Rogers et al., 2004). Cloning efficiency (colony number in each gel) was also dependent on the endothelial density, with greater colony formation as BRENCs density increased (P#1, Fig. 4).

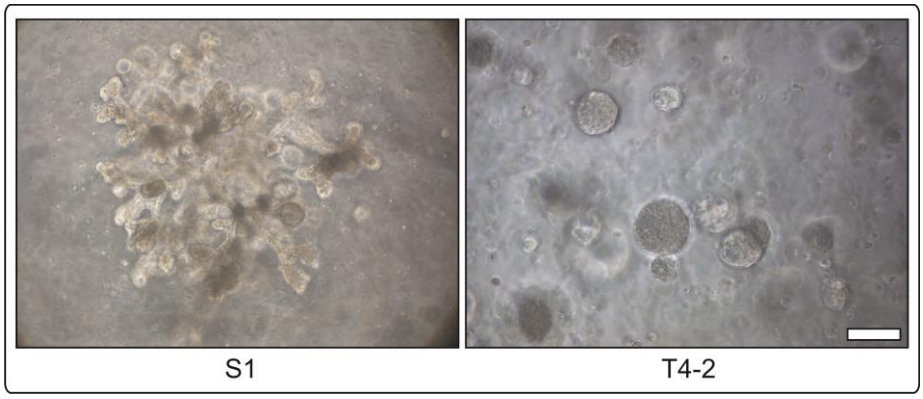

Figure 9. *HMT-3522-S1 forms branching colonies in co-culture.*
When co-cultured with BRENCs, both the S1 and T4-2 cell lines experience a strong proliferative boost. In addition, S1 undergoes branching morphogenesis, forming large colonies with hollow ducts and acini. *Bar: 100µm.*

When frozen gels were stained after the culture period, BRENCs could be seen dispersed throughout the gel but were not seen within epithelial colonies. Co-culture of endothelial cells and breast epithelial cells had been performed previously by other research groups (Shekhar et al., 2001; Shekhar et al., 2000), where endothelial cells were seeded in close contact with epithelial cells on top of matrigel as opposed to within. We wanted to determine whether in our model the effects seen were due to cell-cell contact, or derived from soluble factors. We therefore seeded endothelial cells in a separate transwell chamber, with the epithelial cells in matrigel directly below (P#1, Fig. 5). Interestingly, in this assay the proliferative signal was more pronounced in the normal derived cell lines than the cancer derived ones. This could be due to the cancer cell lines' greater survival or clonal ability; as in this assay the initial cellular density was slightly higher than in the previous co-culture experiments. To further analyze the proliferative effect of endothelial cells we devised a co-culture system where the two cell types form a gradient of densities. Ranging from 100% endothelial cells down to 100% epithelial cells, with a mixture of the two in between (P#1, Fig. 6). In this setup, the proliferative effect on MCF10a was most pronounced in the proximal area, where both cell types were in close contact. In the distal area a proliferative effect was also seen, but to a lesser degree than in the proximal. The malignant MDA-MB-231 showed a different phenotype, with limited differences between proximal and distal, and both significantly lower

than regular co-culture. This result can in part be explained by the increased appearance of mesenchymal colonies in co-culture gels.

Taken together, endothelial cells confer a strong proliferative boost to epithelial cells, and act as a three dimensional feeder layer, drawing out the phenotype of the different cell types, they do this by secreting soluble factors into the gel/media. The value of endothelial cells in co-culture has been well established in our laboratory, we have published papers demonstrating how endothelial cells affect morphogenesis in breast, lung and prostate models (Bergthorsson et al., 2013; Franzdottir et al., 2010; Sigurdsson et al., 2011)

The identity of the soluble factor conferring the proliferative and morphogenic signals was not verified in this paper; this work is currently ongoing at the laboratory. Within the breast gland the crosstalk between fibroblasts, macrophages and epithelial cells has been shown to be driven by matrix metalloproteases (MMPs) and growth factors, such as amphiregulin, HGF and EGF (Allen et al., 2014; Jones et al., 2003; Page-McCaw et al., 2007). Partial inhibition of endothelial-induced effects was seen by inhibition of HGF as reported by (Sigurdsson et al., 2011).

Not included in the paper are data involving co-culture of the normal derived epithelial cell line HMT3522-S1 (S1) with BRENCs. When this cell line is cultured in matrigel it forms tight spheres similar to LEPs, with apical and basolateral polarization (G. Y. Lee et al., 2007). A subline of S1, T4-2 forms tumours in mice and has lost this polarization in 3D culture. When we co-cultured these two cell lines with BRENCs we saw striking differences in morphology. In co-culture, S1 cells generate large branching colonies with hollow lumens while T4-2 form disorganized filled colonies (Figure 9). One of the main differences between these two cell lines is that T4-2 has greatly increased EGFR expression levels due to an amplification of the EGFR gene. These observations led us to wanting to re-create this model, and study the role of EGFR expression in 3D culture, polarization and morphogenesis, which is the context of paper #2.

4.2 Paper #2 - Selection for EGFR gene amplification in a breast epithelial cell line with basal-like phenotype and hereditary background

The role of EGFR in breast cancer has been under increased focus in recent years. EGFR expression has been linked to worse prognosis in triple negative cancers (Masuda et al., 2012), and amplifications on chromosome 7 (where EGFR is located) have been described in both breast cancer and cell culture models (Bhargava et al., 2005; Nielsen et al., 1994). During cancer progression, the microenvironment of the breast undergoes a dramatic disruption and the lumen fills up with proliferating cells forming ductal carcinoma in situ (DCIS). Within DCIS normal signaling is disrupted, which

results in selective pressure on the cells, often leading to malignant transformation over a long time period. Mina Bissell and colleagues have described how long term selective pressure can lead to transformation of cell lines (F. Wang et al., 1998; Weaver et al., 1995). In their work they established a normal-derived epithelial cell line HMT3522-S1. EGF was removed from the culture media and through selection, cells that were EGF independent appeared. Initially, these cells were nontumorigenic (S2), but after several passages, a tumorigenic cell line was established (T4-2). This cancer progression model has been used extensively in recent years to unravel cellular and molecular mechanisms of breast morphogenesis and cancer progression.

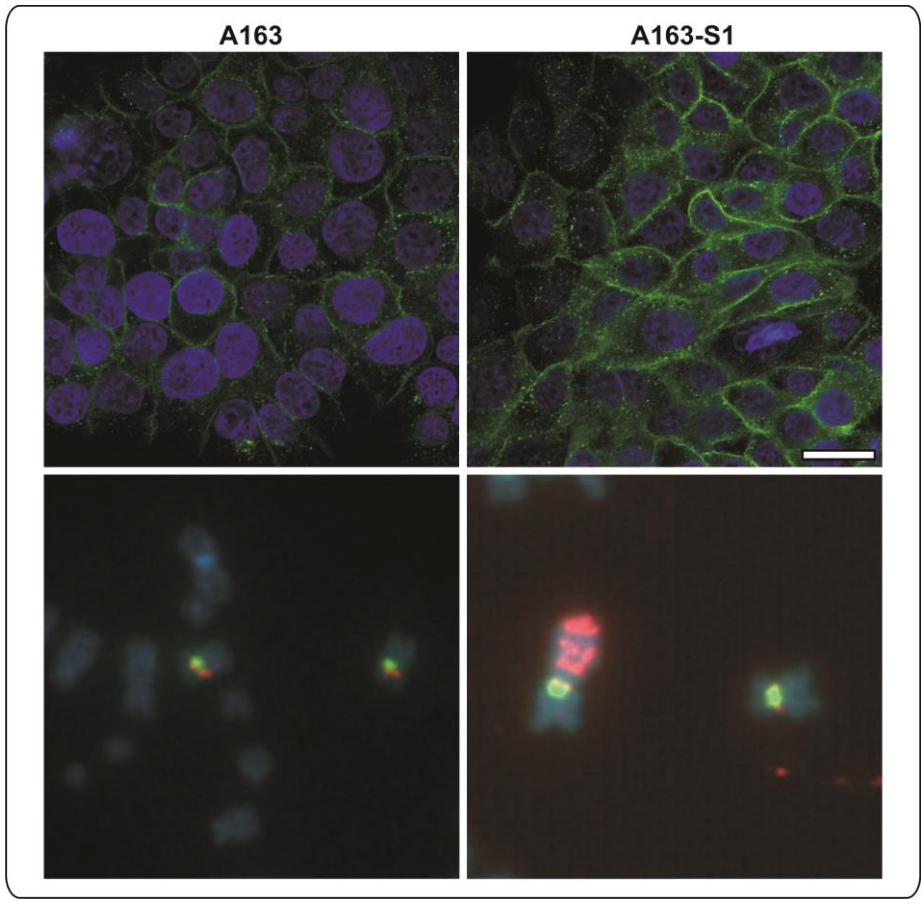

Figure 10. *EGFR is amplified in A163-S1.*
EGF removal from culture medium resulted in strong upregulation of EGFR in A163-S1 compared with A163 (top panel). This upregulation was the result of amplification of a region within chromosome 7q (lower panel).
Top panel: EGFR green. Bar: 50µm, bottom panel: Centromere 7 green, EGFR region red. Adapted from (Ingthorsson et al., 2011).

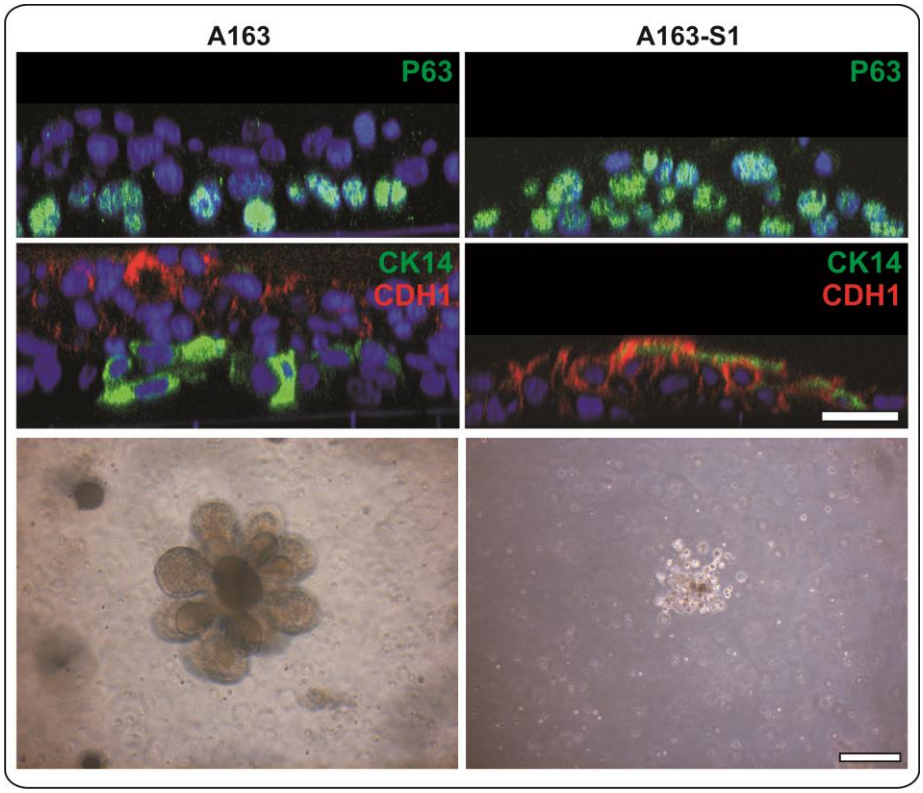

Figure 11. *A163-S1 shows disrupted polarization and 3D phenotype.*
When cultured on transwell filters, A163 demonstrates basal expression of p63 and CK14, while A163-S1 shows disruption in p63 and CK14 expression. *Bar:25µm*
In 3D co-culture with BRENCs, A163 forms branching colonies, while A163-S1 forms dissociated grape-like colonies. *Bar: 200µm.*

In this paper we generated a cell line from a unique source, namely breast epithelial tissue from a cancer patient with strong family history of breast cancer but with no known germline mutations. Cells were isolated from a tumor sample; the isolated cells were then immortalized using E6/E7 oncogenes from Human Papilloma Virus 16. This cell line, referred to as A163, had a mixed basal and luminal phenotype. This was evidenced by expression pattern of EGFR, CK5/6 and CK17, EpCAM, and a heterotypic expression of p63 (P#2, Fig. 1). Additionally, the cell line showed expression of CK19, a luminal cell marker. The A163 cell line was maintained on serum free H14 medium containing EGF. EGF is a strong inducer of proliferation and when EGF was removed from the culture media, the cell line entered a long-term quiescent phase. After several weeks showing no proliferation, proliferative foci were observed, and over time a new EGF independent sub-line was established, A163-S1. (P#2, Fig. 2). EGFR expression in A163-S1 was significantly higher compared with the mother line, based on both western blotting and immune-

fluorescence. Additionally, phosphorylation of EGFR independent of EGF was observed. We then looked at whether expression of ErbB2, another member of the EGF receptor family was increased. Compared with the cell line Skbr3, which harbors an amplification of ErbB2, very limited expression was seen in either A163 or A163-S1 cell lines.

The A163 cell line was originally established from tumor tissue. But whether the cell line arose from tumor cells or adjacent normal epithelium is not known. Karyotypic analysis revealed some chromosomal changes, which are common in established cell lines (Stepanenko & Kavsan, 2012). Interestingly, A163-S1 was shown to have an elongated 7q chromosome. FISH analysis revealed that 7q harbored multiple copies of the EGFR gene, most likely the reason for the upregluated EGFR levels in the cell line. Interestingly, the HMT-derived cell line T4-2 also had amplification of chromosome 7, in the form of trisomy (Briand et al., 1996; Nielsen et al., 1994). The fact that a similar change occurred in our system delineates how important EGFR signaling can be during cancer progression, and how strong selective forces, such as growth factor depletion can lead to similar genetic changes in different systems.

Immunostaining of A163-S1 showed that the cell line had shifted to a more basal/myoepithelial phenotype compared to the parental cell line, shown by uniform expression of p63 and increased expression of cytokeratin 14. In the normal gland, EGFR is associated with basal/myoepithelial populations and in cancer EGFR is often amplified in the basal-like subgroup (Shao et al., 2011). Additionally, CK19 and EpCAM expression was reduced. When the cells are allowed to reach confluency, A163 forms a uniform, dense cellular layer. In contrast, A163-S1 pile up in culture. Immunostaining of cells cultured for an extended period in transwell filters reveals a basal expression of p63 and CK14, and apical expression of E-cadherin, demonstrating a normal-like arrangement of LEP and MEP. This approach of using transwell filters to culture cells for long periods enabled us to fully visualize the polarization potential of the cell lines. Additionally, cells received media from the bottom, as well as the top, avoiding any cell stress in the basal layers. When A163-S1 was cultured in this setup, the cell line formed dense ridges and piles of cells interspersed by simple monolayer, demonstrating an increased ability to grow without strong cell-cell adhesion. Staining pattern of p63 is also changed, staining in nearly all cells, demonstrating a disruption in the tissue polarity. Additionally, CK14 expression is also modified, with expression being located at the apical side, rather than the basal side. These data show that aberrant expression of EGFR can cause changes to cells' spatial awareness, as cells no longer require strong cell-cell contact, and lose basal-apical tissue polarity. Additionally, an enrichment in cells expressing basal markers like p63 shows that EGFR, as a marker for basal-like tumors might not be simply a marker, but have a causal effect.

To look at how this amplification of chromosome 7q affected the 3D phenotype of A163-S1, we seeded both cell lines into matrigel. In 3D culture, A163 formed polarized acini with a lumen, sometimes filled with abnormal/apoptotic cells. This is in part due to it being immortalized using E6/E7, which causes increased proliferation and increased resistance to apoptosis. Overall, A163-S1 formed larger colonies, lumen formation was very rare, and colonies were significantly larger. In this paper, we did not include co-culture with endothelial cells. Unpublished data demonstrate that A163 formed branching structures, reminiscent of what can be seen in the breast gland. A163-S1 on the other hand did not form branching structures, rather, it formed grape-like and elongated structures, indicating a loss of cell cell adhesion (Figure 11). BRENCs stimulate branching morphogenesis ((Bergthorsson et al., 2013; Franzdottir et al., 2010; Sigurdsson et al., 2011), and unpublished data regarding branching morphogenesis in this chapter and in the previous chapter discussing paper #1). Additionally, due to the observation that EGF receptor signaling has been linked to breast morphogenesis during normal development (Pasic et al., 2011; Schroeder & Lee, 1998), I wanted to see how signaling pathways downstream of EGFR could affect branching morphogenesis in our model. As discussed in the introduction, the sprouty protein family plays an important role in modulating tyrosine kinase signaling in several organs, including the breast. The role of Sprouty during branching morphogenesis is the subject of paper #3, where we knocked down Sprouty-2 in the D492 cell line.

4.3 Paper #3 - Expression and Functional Role of Sprouty-2 in Breast Morphogenesis

Having seen how EGF receptor signaling affected the phenotype of A163 and A163-S1 cells and the disruption seen in branching morphogenesis in co-culture with endothelial cells, we wanted to analyze further the effects of increased EGF receptor activity. Both EGFR and ErbB2 have important functions in breast cancer progression. As mentioned in the introduction, there are several downstream regulators of EGF receptor signaling. One of those is Sprouty-2 (SPRY2), which has emerged as a potential tumor suppressor. Additionally, SPRY2 has been shown to play an important role in branching morphogenesis (Guy et al., 2009).

In the normal breast gland, SPRY2 is expressed predominately within the luminal epithelial compartment (P#3, Fig. 1A-B). We showed this via *in-situ* hybridization, immunofluorescence and western blotting. Three different biopsies were used for RT-PCR studies, all showing 15-58 fold greater SPRY2 levels in the luminal compartment compared to the myoepithelial compartment (P#3, Fig1B). SPRY2 has previously been shown to regulate branching morphogenesis in the kidney, lung and placenta, where deregulation of expression affected branch formation (L. Chi et al., 2004; Natanson-Yaron et al., 2007; Taniguchi et al., 2007). We analyzed the expression pattern of SPRY2 in mice

during pregnancy and lactation. In contrast to humans, the expression of SPRY2 was higher in the myoepithelial compartment. SPRY2 expression increased in pregnant mice compared with virgin, and reached the highest levels during lactation. The difference in SPRY2 localization between the human and mouse mammary gland is interesting. The reasons for this can be several; the difference in histoarchitecture of the two glands might be a key factor. The mouse epithelial tissue is mostly embedded in adipose tissue, while the human mammary epithelium is surrounded by collagenous stroma; different signaling pathways between the stroma and epithelium can affect SPRY2 expression patterns.

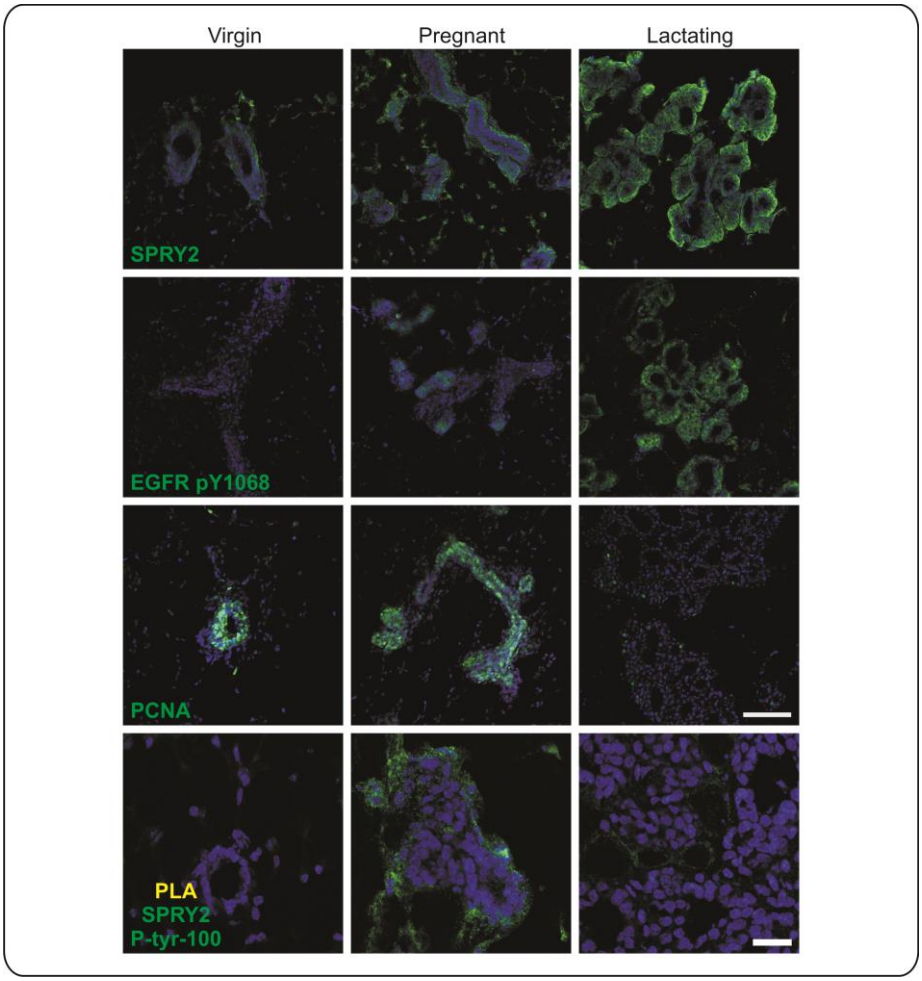

Figure 12. *SPRY2 and EGFR activation in the mouse mammary gland.*
SPRY2 levels increase during pregnancy and lactation, a similar pattern as seen with EGFR Y1068 activation. In the lactating gland, proliferation is minimal. SPRY2 phosphorylation is strongest during pregnancy, and is reduced during lactation, while SPRY2 levels remain high. *Bars: 100µm, upper and 25µm, lower. Adapted from (Sigurdsson et al., 2013).*

SPRY2 expression levels show a negative correlation with proliferation, but coincide with both total EGFR levels and phosphorylation of EGFR. SPRY2 has been shown to share structural homology at tyrosine Y55 with EGFR at tyrosine residue Y1045 (Sun et al., 2010). When phosphorylated, both EGFR and SPRY2 are ubiqutinated and degraded (Wong et al., 2001). When phosphorylated at this residue, SPRY2 can sustain EGFR activation by preventing endocytosis and degradation of the EGF receptor (Guy et al., 2003; Stang et al., 2004) (Figure 4). Due to the relatively recent interest in the sprouty family, antibodies that label phosphorylation specific sprouties, are not available, and researchers have used antibodies recognizing phosphorylated tyrosine to compensate (Friedmacher et al., 2013). To look at phosphorylation of SPRY2, we chose to use a proximity ligation assay (PLA) using antibodies against total SPRY2 and phosphorylated tyrosine. PLA assays are based on the premise that when two antibodies labeled with oligos are localized within a certain limit, the oligos can be annealed and amplified, enabling visualization of interaction (Gullberg et al., 2010). Using this assay we saw that activation of SPRY2 is strongest during pregnancy, when branching morphogenesis of the breast gland is at its highest point (P#3, Fig. 2C). During lactation, phosphorylation of SPRY2 is reduced again, while total SPRY2 levels remain high. These data might indicate that while branching morphogenesis is underway, SPRY2 maintains EGFR activation by sequestering c-Cbl ubiqutin ligase, while during lactation this role is reduced. These data and the changes to SPRY2 levels during branching prompted us to look in detail at the role of SPRY2 in branching morphogenesis using the D492 cell line.

During branching morphogenesis, D492 cells first form a mass, which then undergoes further proliferation, bifurcation and elongation of branching nodules. The final colony is highly branched, similar to *in-vivo* TDLUs (P#3, Fig. 3). The culture period is two-three weeks, where the branching morphogenesis takes place between days 8-16. D492 is able to undergo branching morphogenesis when seeded into matrigel without the need for additional factors, such as BRENCs; unlike other cell lines mentioned, like A163 and HMT-3522-S1, which require endothelial stimulation to branch. We looked at levels of SPRY2 transcription during branching morphogenesis and found that after day 8 in culture, transcription of SPRY2 drops during the time period when branching is taking place. Localization of EGFR and SPRY2 was demonstrated to be predominately at the branching tips, where cell proliferation was strongest. Similarly, activation of both EGFR (Y1068) and SPRY2 (via PLA) was localized mainly to the branching tips.

Because SPRY2 has been shown to reduce EGFR signaling, we decided to knock down SPRY2 in the D492 cell line. We achieved a good knockdown in one of three lentiviral vectors, and further increased knockdown by single cell cloning (KD3A) (P#3 Fig. 4A). We wanted to know whether SPRY2

knockdown led to changes in the mobility of D492 cells. Indeed, increased migration through transwell filters was seen, while proliferation of cells was not significantly changed (P#3 Fig. 4C-D). We next tested the cells for morphogenic changes in three-dimensional culture.

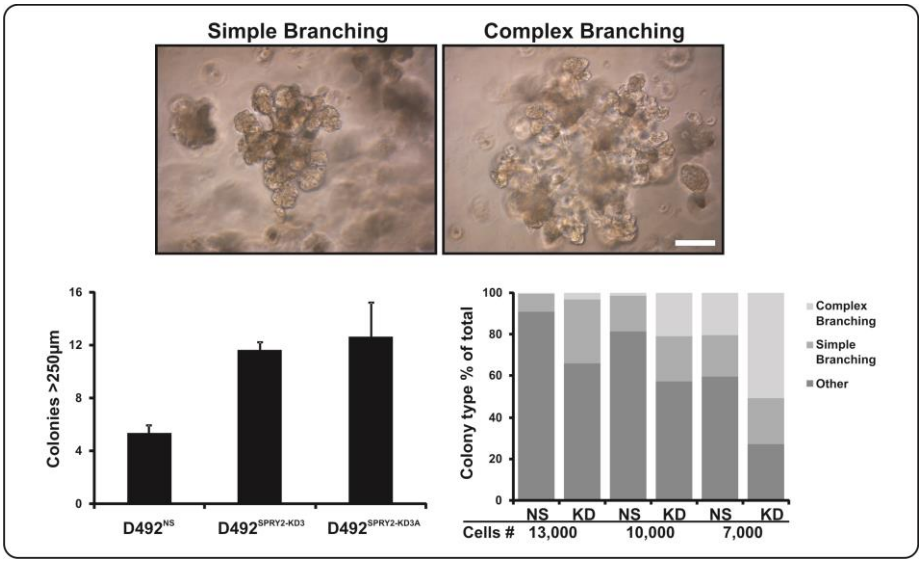

Figure 13. *SPRY2 knockdown increases branching complexity.*
When SPRY2 was knocked down, the number of large highly branched structures was increased. Knockdown cells were less sensitive to initial density of the culture and formed more numerous complex branching structures. *Bar: 100µm. Adapted from (Sigurdsson et al., 2013).*

Knockdown of SPRY2 led to an increase in highly branched colonies, referred to as complex branching in the paper (P#3 Figs 5A-D, Figure 13). The number of colonies with diameter greater than 250µm was also significantly increased. For D492 to form branching structures, the density of cells must not be too high. If density is too high, inhibition of branching in adjacent colonies takes place. This phenomena has been described by Celeste Nelson and colleagues (Nelson et al., 2006) In that paper, they showed that cells' invasive ability was controlled in part via autocrine factors such as TGF-β. In our system, SPRY2 knockdown led to reduced sensitivity to cell density, with knockdown cells forming more branching colonies at each density point tested (P#3 Fig. 5E).

We theorized that by decreasing initial density further we could get more complex brancing colonies. There was however a lower limit to seeding density, less than 5000 cells per gel resulted in nearly no cell proliferation, a similar pattern as seen in paper #1. We then setup a co-culture of D492 with BRENCs. In that assay, epithelial density could be brought down to 100 cells per gel. We have previously published a paper showing how co-culture with endothelial cells

led to epithelial-to-mesenchymal transition in D492 cells (Sigurdsson et al., 2011). In co-culture with endothelial cells, a drastic increase in the appearance of D492 derived mesenchymal colonies was seen in the SPRY2 knockdown cells. These colonies are characterized by the switch from E-cadherin to N-cadherin expression, and a loss of keratin expression. This effect was most prominent in the KD3A clone where SPRY2 was most effectively knocked down, where nearly all colonies had a spindle phenotype.

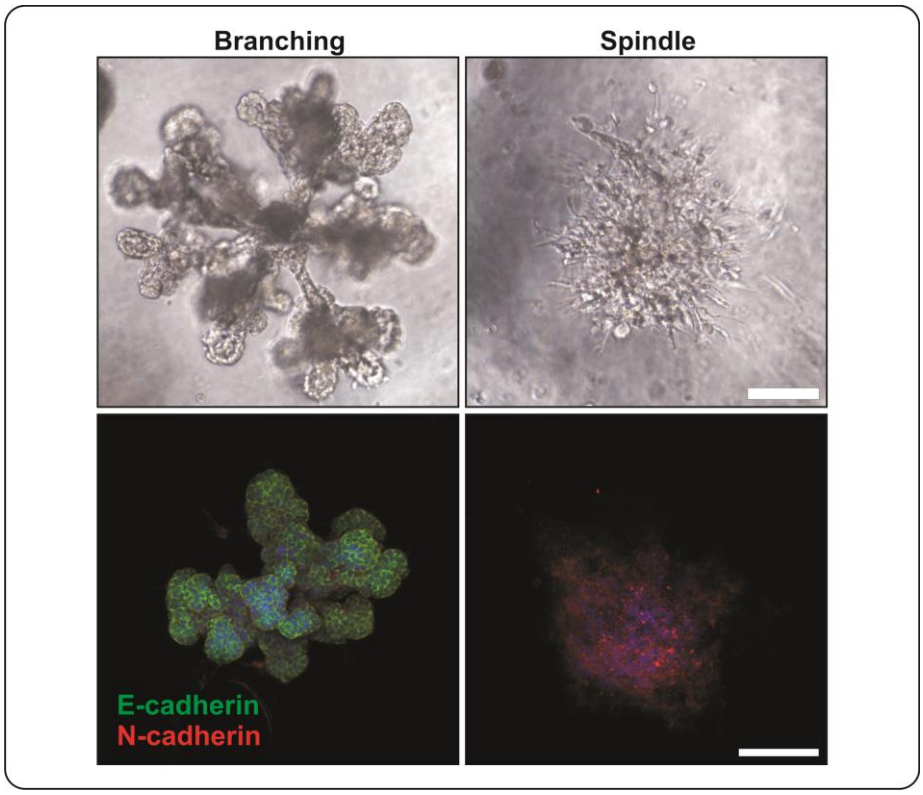

Figure 14. *In co-culture, SPRY2 knockdown leads to EMT.*
Knockdown of SPRY2 resulted in the appearance of spindle-like colonies in co-culture with BRENCs. These colonies had lost E-cadherin expression and gained N-cadherin expression, demonstrating an EMT phenotype. Colonies presented are representative branching and spindle colonies from co-culture experiments. *Bar=100µm.*

Branching morphogenesis requires cells to temporarily adapt a more mobile, invasive phenotype (Andrew & Ewald, 2010; Micalizzi et al., 2010). During that time, the branching process is choreographed via activation of receptor tyrosine kinases, such as EGFR and FGFR. The role of sprouty in this process is in part to dampen the mitogenic RTK signals. When SPRY2 was knocked down, this dampening effect was lost, resulting in a stronger activation of RTK signaling. These changes to signaling might be the cause of increased spindle-like colony

formation, as a loss of spatial sensing can result in uncontrolled branching leading to the phenotype seen in Paper #3 Fig. 5C.

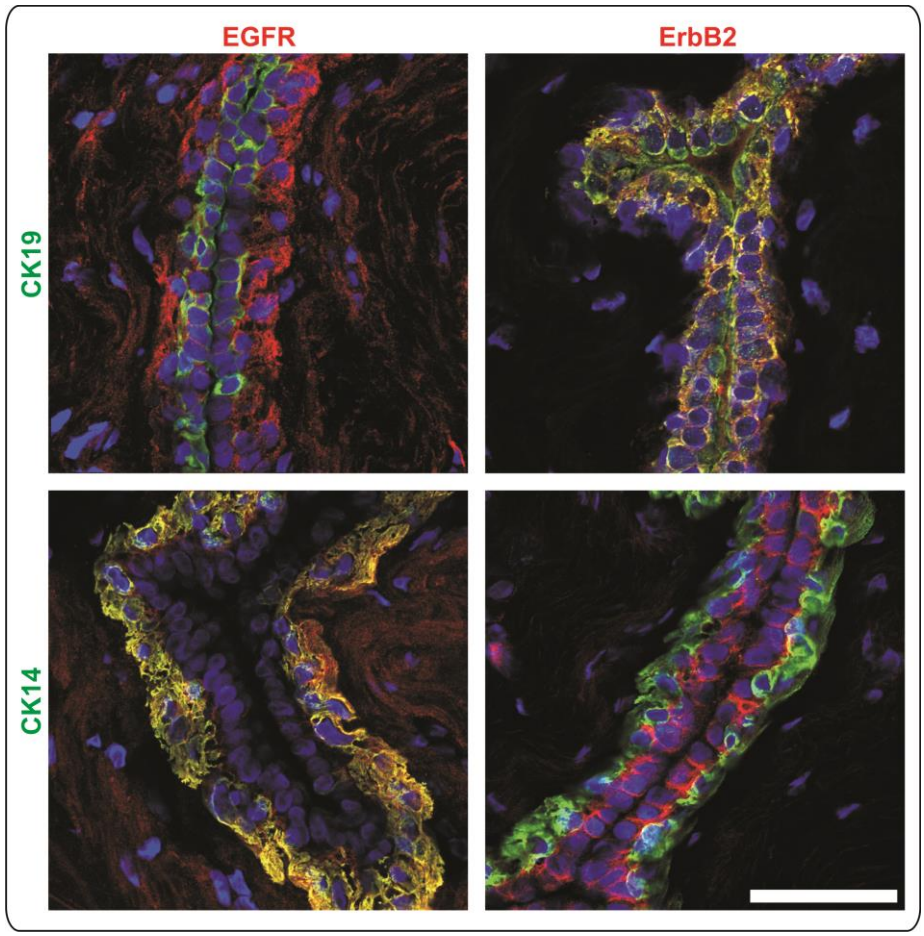

Figure 15. *EGFR and ErbB2 staining in the normal breast.*
In normal breast tissue, EGFR co-stains predominately with CK14, indicating a basal/myoepithelial localization. ErbB2 co-stains with CK19, indicating a luminal expression pattern. *Bar=50µm.*

It is necessary to mention that SPRY2 is a regulator of several RTKs in addition to EGFR, so knocking down SPRY2 affects several other pathways, including FGFR, c-met and other EGF receptor family members. As sprouty acts on such a broad range of pathways that are often dysregulated during cancer progression it has emerged as a prognostic factor that might help with determining treatment options. SPRY2 overexpression in the context of ErbB2 amplification has shown that it can sensitize ErbB2 amplified cells to Trastuzumab treatment (Faratian et al., 2011). In colon cancer, SPRY2 has been demonstrated to negatively control c-met related pathways (Holgren et al., 2010).

Based on the data presented in this paper, along with the EGFR induced effects seen in paper #2 we wanted to see how changes in expression of both EGFR and ErbB2 affected the behavior of D492 cells.

4.4 Paper #4 - EGFR inhibits ErbB2-induced EMT and reduces tumor growth in breast epithelial cells with stem cell properties by maintaining epithelial integrity

The EGF receptor family has been extensively studied in the subject of breast morphogenesis and cancer. There is however, a lack of studies showing how these proteins influence breast epithelial stem cells and branching morphogenesis. Building on our previously published papers we wanted to see how overexpression of EGFR and ErbB2 affected the phenotype of D492 in both monolayer and 3D culture.

We began by exploring how EGFR and ErbB2 are expressed in the normal breast gland. We cryosectioned normal breast tissue acquired from reduction mammoplasties and stained for EGFR and ErbB2 along with luminal marker CK19 and basal/myoepithelial marker CK14 (P#4, Fig. 1, and Figure 15). These stainings showed that EGFR is predominately located in the basal layer, as expression was co-localized with CK14, but not CK19. The opposite pattern was seen when staining for ErbB2, which localized with CK19. Previous papers have shown a similar staining pattern, with some cells expressing both EGFR and ErbB2 (Gompel et al., 1996).

Natively, D492 cells express EGFR, but have very limited ErbB2 expression (P#4, Fig. 2 and Figure 16). We induced ErbB2 expression using lentiviral transduction. Doing so, we achieved high expression of ErbB2, as evidenced by immunofluorescence and qRT-PCR. The upregulation of ErbB2 was more than 30 fold compared with control, which was expected due to the low expression in wild-type D492 cells. Interestingly, when we looked at EGFR levels in these cells we noticed a large drop in EGFR expression, both at protein and mRNA levels, suggesting that ErbB2 expression was in some way affecting EGFR transcription. RTK signaling is a tightly controlled mechanism, and strong over-expression of one receptor has been shown to be able to negatively regulate another (Flageng et al., 2013). We therefore decided to induce EGFR expression using retroviral transduction. These lentiviral and retroviral transductions yielded three sub-lines of D492, $D492^{EGFR}$, $D492^{ErbB2}$ and $D492^{EGFR/ErbB2}$. Compared with control D492 cells - transduced with empty vector plasmids - EGFR levels did not increase drastically, only around twofold, but as previously stated, D492 has an already relatively high expression level of EGFR, which can explain the apparently low increase in expression. Double expression of EGFR with ErbB2 led to mRNA levels comparable with control cells. At the protein level, EGFR expression was increased in $D492^{EGFR}$ cells, compared with $D492^{control}$, but was not greatly increased in $D492^{EGFR/ErbB2}$ compared with $D492^{ErbB2}$. This could be

explained by increased levels of endocytosis and degradation of the receptor (Henriksen et al., 2013) As shown in the introduction, ErbB2 has no known ligand, but is statistically the preferred dimerization partner of other EGF receptor family members, due to it being constitutively in an active conformation (Graus-Porta et al., 1997; Karunagaran et al., 1996). We analysed how ErbB2 overexpression changed cellular response to EGF stimulation. Cells were starved, and subsequently treated with EGF containing media. In starved cells phosphorylation of EGFR (Y1068) was seen in ErbB2 expressing cells only, being more pronounced in $D492^{EGFR/ErbB2}$ cells. Perhaps surprisingly, phosphorylation of ErbB2 was limited unless co-expressed with EGFR. This might be due to the strong downregulation of EGFR in $D492^{ErbB2}$, limiting EGF binding (Macdonald-Obermann & Pike, 2014).

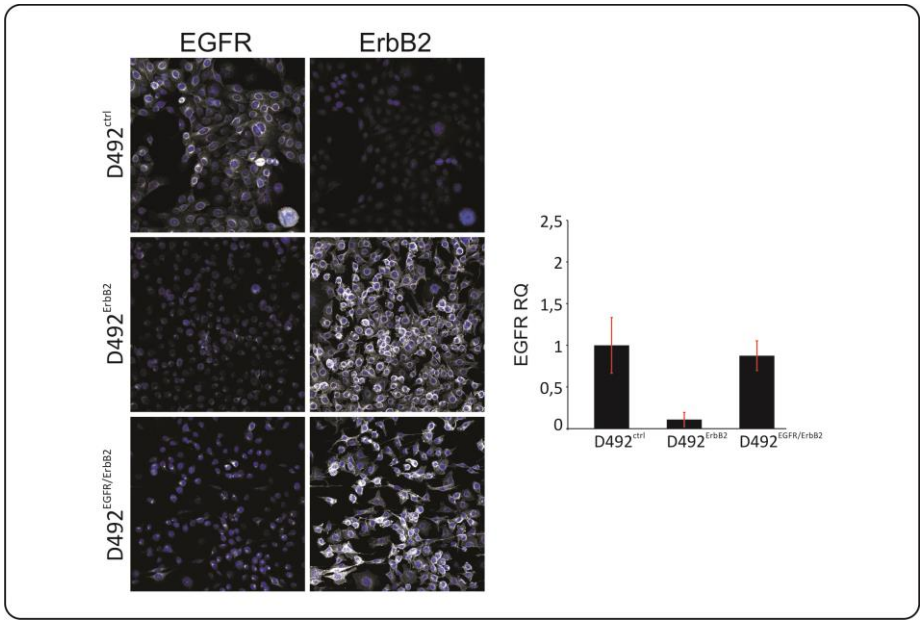

Figure 16. *ErbB2 induced EGFR downregulation in D492 cells.*
Lentiviral transduction of ErbB2 led to strong ErbB2 expression. ErbB2 overexpression in turn resulted in greatly reduced EGFR levels in D492 cells. Dual transduction of EGFR and ErbB2 recovered EGFR transcription levels.

D492 cells exhibit a heterotypic expression pattern of both luminal and myoepithelial markers, as shown in Figure 17, where both luminal CK19 and E-cadherin (CDH1), and myoepithelial CK14 and P-cadherin (CDH3) can be seen (P#4, Fig. 3). When we looked at keratin and cadherin expression levels in ErbB2 expressing cells we saw that ErbB2 overexpression resulted in near complete loss of CK14, CK19 and E/P cadherins. To determine whether this stemmed from ErbB2 upregulation or loss of EGFR, we stained $D492^{EGFR/ErbB2}$ cells, and noticed a partial recovery of cytokeratins and E-

cadherin, but not P-cadherin. Loss of keratins and epithelial cadherins can be an indicator of EMT. Western blots confirmed loss of keratin and cadherin expression in D492^{ErbB2} cells. Additionally, gain of mesenchymal markers N-cadherin (CDH2) and Axl were seen. Axl expression has previously been associated with both EMT in triple negative cancers as well as resistance to EGF receptor based therapies (Asiedu et al., 2014; Byers et al., 2013; Meyer et al., 2013). EGFR overexpression alone led to a shift towards a stronger myoepithelial expression pattern, as shown by increased p63, CK14 and P-cadherin, and reduced CK19. Increased EGFR signaling has previously been shown to direct myoepithelial differentiation (Pasic et al., 2011).

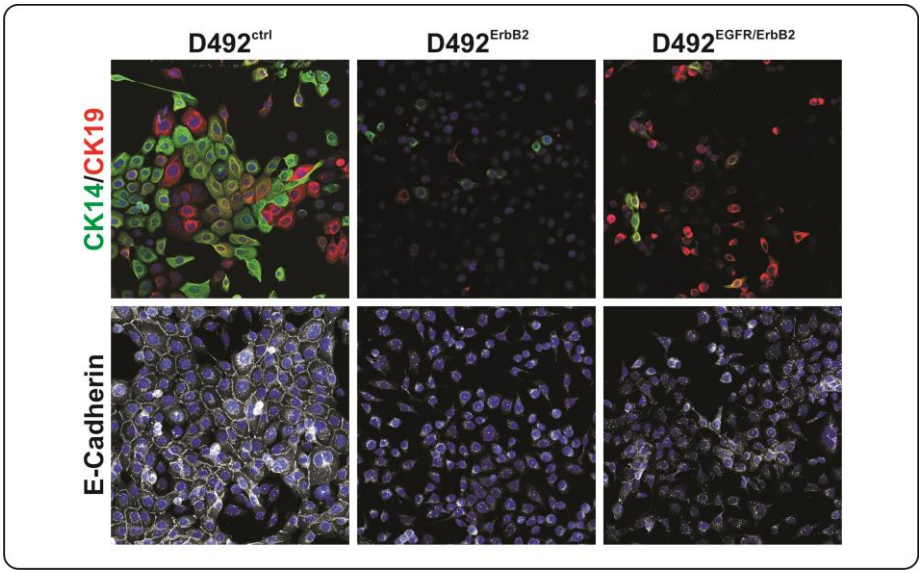

Figure 17. *Keratin and cadherin loss by ErbB2 overexpression.*
D492 cells exhibit a mixture of CK14 and CK19 expression levels. ErbB2 overexpression leads to loss of keratin expression, which is partially reversed by double expressing EGFR with ErbB2. E-cadherin expression is lost in ErbB2 overexpressing cells and partially recovered by addition of EGFR expression.

When allowed to grow beyond confluency, ErbB2 expressing cells formed pil es and ridges in monolayer, while control cells and EGFR expressing cells entered growth arrest. EGFR overexpression did lead to cells proliferating to a higher density than control cells, but did not cause pile-ups in culture. In low attachment culture, both EGFR and ErbB2 overexpression led to cells forming larger colonies. Colony count however was only increased in cells expressing ErbB2, demonstrating increased resistance to anoikis as has been previously described (Reginato et al., 2003; Whelan et al., 2013). In addition, cells expressing ErbB2 formed colonies with reduced adherence, as colonies no longer appeared as tightly bound solid round colonies as with D492^{ctrl} or D492^{EGFR}, but rather appeared as grape-like clusters with reduced adherence. This grape-appearance was in line with the observed loss of cadherin expression, and suggested that cells had become more mobile. We therefore proceeded with seeding the cells in matrigel to test the phenotype

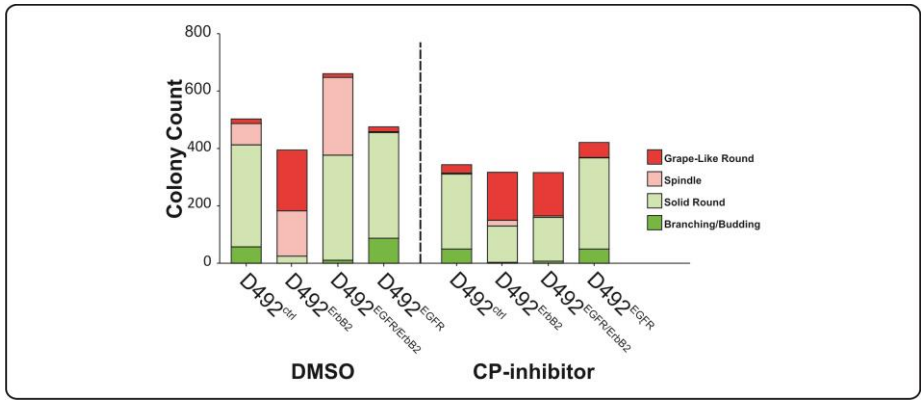

Figure 18. *ErbB2 inhibition reduces spindle colony formation.*
In 3D culture, D492 cells form predominately cells with an epithelial solid and branching phenotype. ErbB2 overexpression resulted in loss of epithelial colonies and increase in grape-like and spindle colonies. Epithelial colony formation could be rescued by overexpression of EGFR, and inhibition of ErbB2 by CP-724,7214, a small molecule inhibitor.

When we seeded ErbB2 overexpressing cells into matrigel, we noticed that while control cells formed predominately solid round and branching colonies, ErbB2 cells mostly formed Grape-like and spindle-like colonies. Grape-like colonies formed by ErbB2 expressing cells have previously been shown by Kenny and colleagues, where cell lines with ErbB2 amplifications showed a predisposition to forming colonies with a grape-like phenotype (Kenny et al., 2007). Spindle colonies in D492 have been previously reported, associated with endothelial co-culture, and associated with loss of epithelial markers, and gain of mesenchymal markers (Sigurdsson et al., 2011; Sigurdsson et al., 2013). Indeed, these colonies demonstrate a loss of e-cadherin, gain of n-cadherin, and loss of keratin expression, showing a characteristic EMT phenotype. Re-introduction of EGFR into ErbB2 cells resulted in both a re-appearance of solid colonies as well as increased expression of CK14 and CK19, and E-cadherin. To confirm that the EMT was caused by ErbB2 overexpression, we inhibited ErbB2 using the small molecule inhibitor CP-724,714, that has high specificity to ErbB2 over EGFR. Using the inhibitor, we showed that the appearance of spindle colonies was drastically reduced, confirming that ErbB2 function is required for spindle formation (Figure 18).

EMT is one of the key steps in cancer progression, where cells gain the ability to infiltrate tissues and form metastasis. EMT has also been linked with increased survival, and gain of stemcell properties (Cieply et al., 2013; Hardy et al., 2010; Scheel et al., 2012; Singh et al., 2010). All of these factors

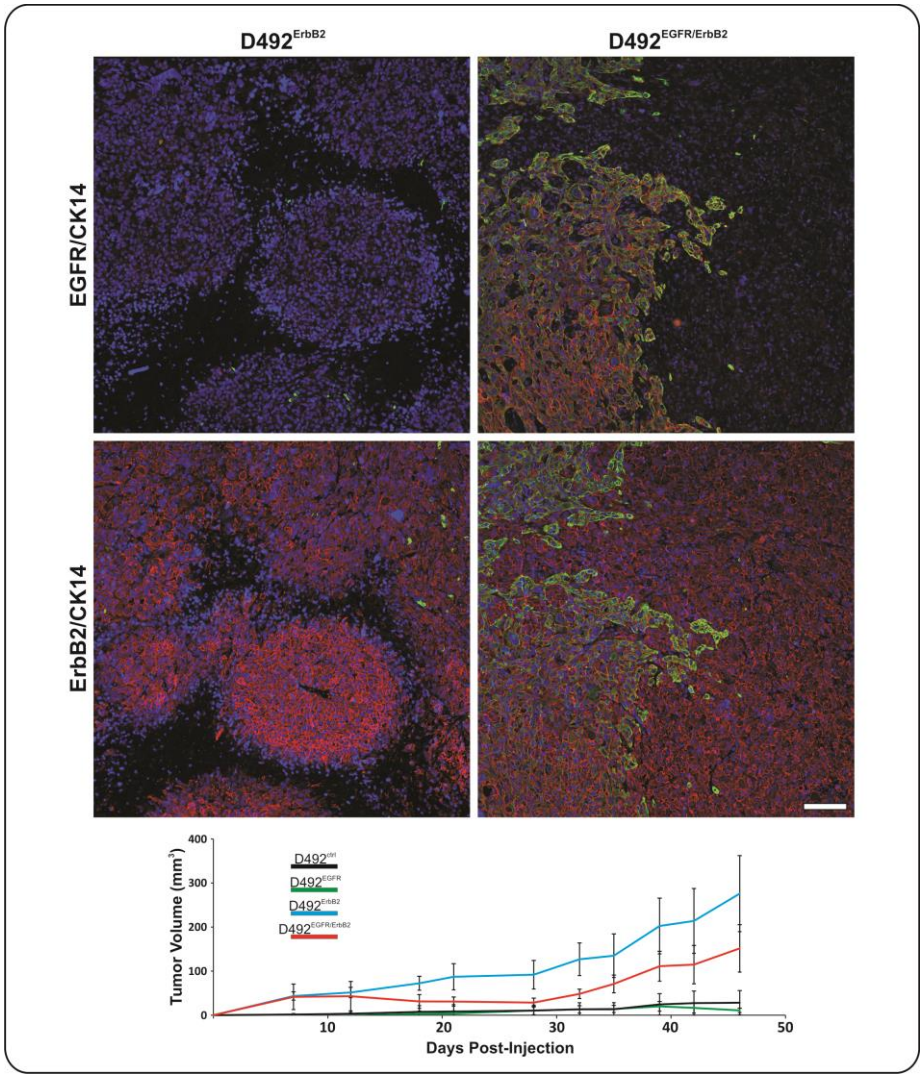

Figure 19. *EGFR induces keratin expression and reduces tumor size.*
In D492^{ErbbB2} cells, minimal EGFR and CK14 staining is seen, while all cells express ErbB2. In D492$^{EGFR/ErbB2}$ cells, CK14 expression is seen in those cells that also express EGFR. Tumor growth in D492$^{EGFR/ErbB2}$ is reduced compared with D492^{ErbB2}. *Bar=50μm.*

contribute to increased malignancy. We wanted to see whether ErbB2-induced EMT caused increased tumor formation in mice. Fat pads of NOD/SCID mice were injected with D492 cells expressing EGFR and ErbB2 alone or in combination, and compared with control. After 7 days, palpable tumors were found in all injections in ErbB2 expressing cells, while control and EGFR cells had 3/6 and 2/5 injection sites showing palpable tumors, respectively. Over time, lesions appeared in all injections, except for one control. Overall, control and

EGFR lesions grew very slowly compared with ErbB2 tumors (Figure 19). Tumors expressing both ErbB2 and EGFR grew slower than tumors expressing ErbB2 alone. After the experimental period, tumors were harvested and stained for marker expression. ErbB2 alone yielded tumors that ubiquitously expressed ErbB2, but had very limited EGFR, CK14, CK19 and E-cadherin expression. Double expression however showed that EGFR was able to reverse or inhibit loss of keratin expression (Figure 19), as while ErbB2 was again seen all over, EGFR appeared in patches, always accommodated with CK19, CK14 and E-cadherin expression.

In $D492^{ErbB2}$ cells a marked downregulation of miRNAs 200c and 141 were observed. Downregulation of these two miRNAs has been demonstrated to cause increased expression of ZEB1 and ZEB2, that lead to downregulation of E-cadherin (W. L. Lo et al., 2011; Neves et al., 2010; X. Wang et al., 2013). ZEB1, but not ZEB2 was shown to be upregulated in $D492^{ErbB2}$, showing that this EMT pathway is involved in ErbB2 induced EMT. In $D492^{EGFR/ErbB2}$, neither downregulation of miR-200c-141 nor upregulation of ZEB1 was seen, suggesting that EGFR inhibits the ErbB2 driven EMT pathway.

EGFR has emerged as a potential therapeutic target in breast cancer, especially in basal-like breast cancer, where EGFR has been shown to be associated with poor prognosis. Small molecule inhibitors of EGFR, such as gefitinib, erlotinib and lapatinib have been shown as potentially being beneficial to patients (Jackman et al., 2009). Tumors originally responding well to EGFR treatment often develop resistance to EGFR targeted therapy (Chong & Janne, 2013).The data presented in this manuscript show that when deciding upon targeted therapeutic options, the context of the tumor must be taken into account. These data suggest that in the context of ErbB2 expressing tumors, EGFR could act as a tumor suppressor, by maintaining epithelial integrity via miRNA expression. Loss of EGFR inhibition could then activate alternate pathways, including ErbB2 driven EMT, leading to metastasis and therapeutic resistance. The mechanisms behind the interplay between EGFR, ErbB2 and EMT factors remain to be elucidated. In cells expressing EGFR along with ErbB2, downregulation of miR-200c-141 was not observed, suggesting that EGFR is maintaining transcription of these miRNAs in our culture system.

5 Summary and Conclusion

In this thesis I have shown that breast microvascular endothelial cells contribute to proliferation and morphogenesis of both primary breast epithelial cells and established normal and malignant breast derived epithelial cell lines (paper #1). I have also demonstrated that changes to cell culture microenvironment, such as ablation of EGF in culture of A163 cell line can lead to clonal selection of EGFR amplified subpopulation. The EGFR amplified cells – referred to as A163-S1 - showed increased basal cell phenotype. Furthermore, A163-S1 lose polarity and become independent of density dependent growth arrest (paper #2). I have also demonstrated that EGFR interacting pathways affect branching morphogenesis and may predispose breast epithelial cells to EMT as evidenced by knocking down SPRY2 (paper #3). In paper #4 I demonstrate that ErbB2 overexpression can cause D492 breast epithelial stem cell line to undergo EMT. The EMT phenotype could then be partially reversed by inducing EGFR expression. Additionally, EGFR reduced ErbB2 induced tumor growth in NOD/SCID mice. In papers #2-4, increased EGFR expression caused a stronger basal/myoepithelial phenotype, suggesting that EGFR might negatively affect tumor growth in certain circumstances by maintaining epithelial marker expression. These data emphasize the need to think about tumor biology in a greater context, where certain proteins can have a dual effect; behaving as an oncogene or a tumor suppressor, depending on expression of other proteins in the tumor.

5.1 Creation of endothelial rich three-dimensional microenvironment for breast morphogensis

Three-dimensional cell cultures have contributed extensively to our knowledge regarding normal morphogenesis and also how normal morphogenesis goes awry during cancer progression. This is in particular evidenced in breast biology and breast cancer research. Through all my work in this thesis I have used 3D reconstituted basement membrane matrix (3D-rBM (purchased as Matrigel) rich in laminins, collagen-IV and extracellular matrix proteins simulating the basement membrane matrix. The method we mostly used was to embed epithelial cells alone, or in co-culture with endothelial cells, as single cells, which were then monitored for up to three weeks for proliferation and morphogenesis. To gain better control over the three-dimensional environment for certain experimental setups, other gel substances, such as pure collagen-I that can then be supplemented with predetermined concentrations of growth factors can also be used (J. H. Lee et al., 2011).

In many of the experimental setups, we used co-culture of endothelial cells with breast epithelial cells of various origins to stimulate morphogenesis and proliferation. By co-culturing endothelial cells in a separate compartment from epithelial cells both physically in a transwell assay as well as spatially using gradient gels as in paper #1 we demonstrated that proliferative signals are at least to some degree derived via soluble factors, and not dependent on cell-cell contact. Good candidates for such soluble cell-derived factors would be growth factors or metalloproteases. Epithelial cells are highly dependent on cellular density for proliferation. Primary cancer cells are notoriously difficult to culture ex-vivo, and undergo strong selection where only a small population is able to proliferate (Dimri et al., 2005). The use of endothelial cells as facilitators of proliferation might be useful to establish cultures of primary cancer cells. Endothelial cells greatly enhance the proliferative ability of epithelial cells at limiting concentrations, meaning that epithelial cells are able to proliferate and undergo morphogenesis without being limited by proximity to other epithelial structures as shown by Celeste Nelson and colleagues in 2006 (Nelson et al., 2006). In that study they showed that inhibition of sprouting due to spatial geometry was in part controlled by TGFβ.

We have not been able to fully identify the proliferative and morphogenic factors secreted by endothelial cells. That project is currently ongoing at the laboratory, where we have built on the published papers, especially paper #1 presented earlier in addition to other co-culture papers we have published in recent years, detailing the morphogenic effect of endothelial cells in breast, lung and prostate culture systems (Bergthorsson et al., 2013; Franzdottir et al., 2010; Ingthorsson et al., 2010; Sigurdsson et al., 2011). Stromal cells like fibroblasts and macrophages have been demonstrated to secrete a wide variety of factors that act in a paracrine fashion on the epithelial tissue of the breast (Fleming et al., 2008; Hynes & Watson, 2010; Kuperwasser et al., 2004; Pannuti et al., 2010; Sternlicht et al., 2006). We are currently in the process of defining endothelial derived proteins, and have begun preliminary tests focusing on Amphiregulin and CCL5 based on results showing that these factors were upregulated in co-culture, using commercially available cytokine arrays (Ray Biotech). Amphiregulin has been shown to have a role in mammary gland development through the action of matrix metalloproteases that release cell-bound amphiregulin (Aupperlee et al., 2013; Ciarloni et al., 2007; Page-McCaw et al., 2007).

We demonstrated the ability of endothelial cells to stimulate morphogenesis at limiting concentrations first in paper #1 with established normal and cancer cell lines. When we co-culture normal derived D492 and HMT-3522-S1 cells with endothelial cells we get increased branching morphogenesis. HMT-3522-S1 forms branching colonies with hollowed lumens, a phenotype that to my best knowledge has not been seen before

with that cell line and warrants further exploration. In the case of D492 cells, the branching structure does not form hollow lumens, presumably owing to the immortalization technique used when the cell line was established (Gudjonsson, Villadsen, et al., 2002). Co-culturing MCF10a in this assay gave us a phenotype reminiscent of the phenotype reported by the Brugge lab in 2001, where overexpression of ErbB2 resulted in the formation of multiacinar structures (Muthuswamy et al., 2001). Addition of TGFβ to that setup caused colonies to become highly invasive, a phenotype that in ways contradicts the outcome previously discussed from Nelson's paper (Nelson et al., 2006), but in part represents the dual role of TGFβ as a proliferative inhibitor in normal tissue, but inducer of invasion during cancer progression (reviewed by (Seoane, 2006)). Currently, work is ongoing at the laboratory aiming towards fully characterizing the proliferative signals between endothelial cells and epithelial cells.

5.2 EGFR and ErbB2 in the normal and malignant breast gland

There are four EGF receptors expressed in mice and humans, they can all dimerize, and there are several known ligands. Different members of the EGF receptor family are upregulated at distinct time points during breast development (Schroeder et al., 1998). The normal function of EGFR and ErbB2 related signaling in the breast gland is to facilitate branching morphogenesis during embryonic development, puberty and pregnancy. In paper #2 we monitored the effects of EGF ablation in the media on the cell line A163. This cell line was established from tumor tissue, immortalized using the E6/E7 HPV16 oncogenes and cultured in a chemically defined media. Establishing cell lines from tumor tissue can be very problematic, as tumor cells are highly dependent on their environment for proliferation, and go easily into senescence in vitro (reviewed by (Kuilman et al., 2010)). Due to the relatively normal-like nature of the A163 cell line, it is uncertain whether the cell line arose from the tumor tissue, or the adjacent healthy tissue that can be found within tumor samples. The phenotype of the A163 cell line could be described as a mixture of basal and luminal properties. A163 depends on EGF supplementation for proliferation and expresses EGFR, but not ErbB2. When we removed EGF, the cells stopped proliferating and became quiescent. After several weeks proliferative foci were seen and the growing cells were independent of EGF signaling. This EGF independent cell line was then determined to harbor an amplification of the EGFR gene. In addition to increased EGFR expression, the sub-line (A163-S1) demonstrated a stronger basal/myoepithelial phenotype. This shift towards a basal/myoepithelial expression profile was also seen in paper #4 when we induced EGFR overexpression in the stemcell line D492. This suggests that EGFR drives basal/myoepithelial differentiation in the normal gland, and changes in EGFR expression in cancer might affect the tumor phenotype. Two recent

studies have demonstrated the ability of EGFR to induce differentiation of cells into either luminal or myoepithelial direction (Mukhopadhyay et al., 2013; Pasic et al., 2011). In these studies, amphiregulin supported luminal differentiation, while EGF or TGFα supported myoepithelial differentiation. Upregulation of EGFR expression or amplification of the gene has been described in the literature (H. S. Park et al., 2014; Secq et al., 2014), and has been strongly associated with basal-like breast cancer.

ErbB2 has been shown to promote luminal cell proliferation (Muthuswamy et al., 2001) and invasion via interaction with desmosomes, hemidesmosomes and STAT3 (Guo et al., 2006; Huang et al., 2011). Upregulation of ErbB2 in D492 cells resulted in cells obtaining an EMT phenotype, both in monolayer and in 3D culture. Additionally, we noticed a significant drop in EGFR, both at the RNA and protein level. The reduced EGFR at the protein level could be explained by increased receptor degradation, as one mechanism for modulating EGFR signaling is internalization of the receptor followed by degradation (Umebayashi et al., 2008). ErbB2 can dimerize with EGFR, which leads to a stronger activation of the Akt pathway over erk activation (Y. Chi et al., 2013). The reasons for reduced EGFR transcription are currently unclear, and in fact previous studies have shown that ErbB2 helps to maintain EGFR levels when co-expressed (Grassian et al., 2011). The EGFR gene has been shown to have a highly complex promoter region, in addition to an enhancer region within the first intron, making transcriptional control decidedly multifaceted (Brandt et al., 2006).

The EMT phenotype of $D492^{ErbB2}$ cells could be partially reversed by inducing EGFR expression along with ErbB2. Partial recovery of keratin expression and E-cadherin was observed, along with lower expression of mesenchymal markers Axl and N-cadherin. Mouse xenografts were also shown to have slower growth when EGFR was expressed along with ErbB2. When we looked at possible mechanisms behind these tumor-suppressive properties of EGFR, we noticed that the miR-200-141 cluster was strongly downregulated in $D492^{ErbB2}$ cells, but not in $D492^{EGFR/ErbB2}$ cells. Downregulation of this microRNA cluster has been repeatedly associated with invasive phenotype and EMT in cancer (Banyard et al., 2013; Dykxhoorn et al., 2009; Howe et al., 2012; Radisky, 2011). In addition, the EMT associated transcription factor ZEB1 was upregulated in ErbB2. ZEB1 has been shown to negatively affect E-cadherin expression (Gregory et al., 2008; S. M. Park et al., 2008). EGFR expression along with ErbB2 reversed this phenotype, as mir200c levels remained high, and ZEB1 levels remained low. How EGFR affected miR-200 levels is currently unclear, but reports have shown that mechanisms within the tumor environment can cause cells to switch between epithelial and mesenchymal states (Liu et al., 2014). These data might suggest that EGFR expression along with ErbB2 could result in less invasive disease; that EGFR maintains epithelial phenotype while ErbB2

suppresses it. These observations are quite interesting, as while the oncogenic role of ErbB2 has been agreed upon for a long time, the observed role of EGFR has also been that of an oncogene. It is clear that deregulation of both of these receptors can cause drastic changes to the behavior of cells. The phenotype associated with overexpression of either EGFR or ErbB2 in tumor tissue is quite distinct; EGFR is associated with BLBC, while ErbB2 is associated with its own subgroup (HER2 enriched) that is associated with more luminal epithelial differentiation. Studies have shown that in DCIS, expression of both EGFR and ErbB2 is more common (Suo et al., 2001) than in invasive tumors (Giltnane et al., 2009). Our data might suggest that while dual positive EGFR/ErbB2 expression might be common in *in situ* cancer, EGFR downregulation might be needed for invasion to occur.

5.3 Concluding remarks and future perspectives

Breast morphogenesis and breast cancer progression are complex processes involving multiple parameters. It is becoming increasingly clear that heterotypic epithelial-stromal interactions are of great importance for both normal development and cancer. To recapitulate some of these events researchers have taken advantage of three-dimensional culture models. These models have proven very valuable in basic developmental and cancer research in the last two decades. While monolayer cultures are of importance in many biological aspects they do not capture *in vivo* like phenotype to the extent that 3D culture can do. Modeling heterotypic interactions between different cell populations has given new insight into critical steps during development and cancer progression. In this thesis I present data showing how endothelial cells can have proliferative and morphogenic effects on various epithelial cell lines. I also demonstrate the varied functions of the EGFR receptor family members EGFR and ErbB2, and downstream regulator Sprouty-2, and how aberrant expression of these proteins can drastically change the phenotype of normal and malignant mammary cell lines, both *in vitro* and *in vivo.* Increased understanding of how the EGFR family affects distinct cell populations in the breast is important and helps to understand the origin and progression of different subtypes of breast cancer.

The transition from DCIS to IDC represents a fundamental step in cancer progression. DCIS lesions are characterized by a surrounding layer of myoepithelial cells, which in IDC becomes progressively discontinuous or lost completely. It can be argued that the D492 three-dimensional culture model represents to some extent the phenotype seen in DCIS: The cell line forms branching ductules and acini that are surrounded by CK14 (myoepithelial) expressing cells, with lumens filled with CK19 (luminal) expressing cells. Upregulation of ErbB2 in D492 leads to loss of EGFR, and subsequent loss

of myoepithelial differentiation. This in turn causes cells to adopt a highly invasive, mesenchymal phenotype.

The intricate relationship between different EGFR family members emphasizes the need to study the receptors together, instead of considering them separate entities. Continued work at the laboratory aims towards further characterization of EGFR and ErbB2 interacting mechanisms, and how these models can be improved and further knowledge gained especially with regard to clinical relevance.

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Original papers

Paper I

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RESEARCH ARTICLE Open Access

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

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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

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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

1×10^4 primary epithelial cells were suspended in 300 μl rBM along with 2×10^5 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 2×10^5 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 -

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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 7×10^4 BRENCs embedded into 100 μl of rBM and seeded in a 4-well chamber slide. 3×10^3 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

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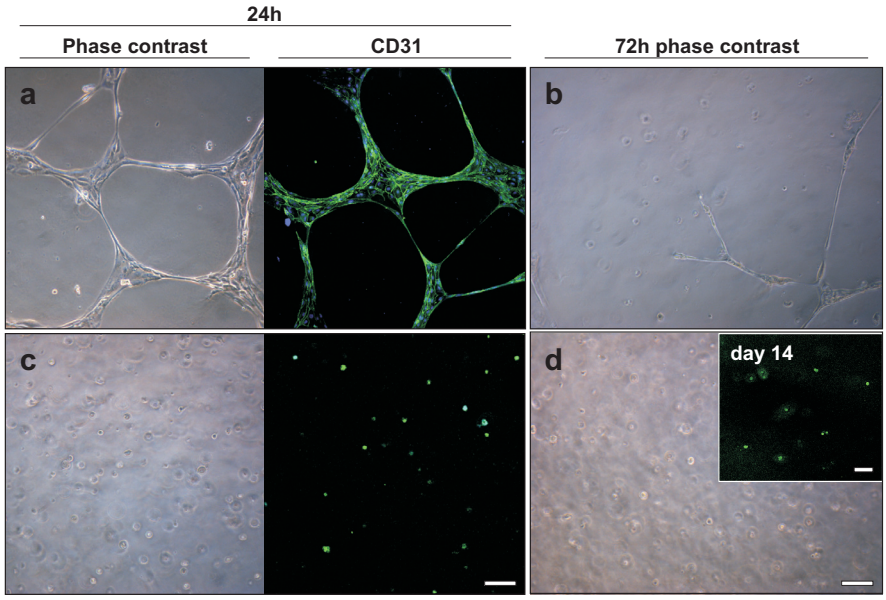

Figure 1 Endothelial cells stay quiescent but metabolically active when cultured within rBM. When BRENCs are seeded onto rBM they form network structures within a few hours that disassociate from the rBM within 72 hours (upper panel). In contrast BRENCS that are embedded into rBM (lower panel) stay quiescent 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-

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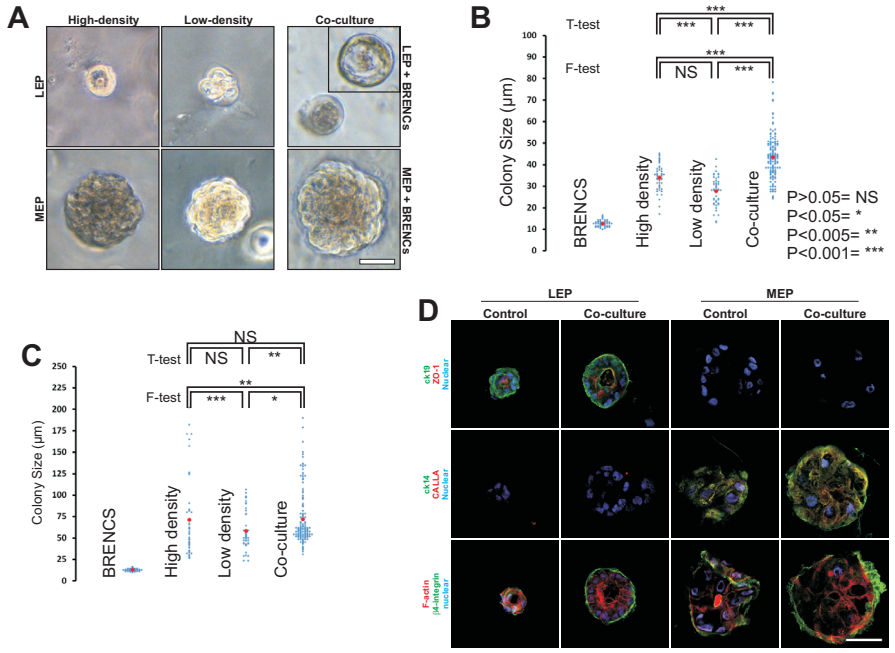

Figure 2 BRENCs stimulate growth and morphogenesis of primary breast epithelial cells. **A. BRENCs stimulate growth of both luminal epithelial and myoepithelial cells in rBM**. *Upper panel:* At high density, LEP cells form colonies with a small central lumen. At low density, no lumen formation is seen. 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

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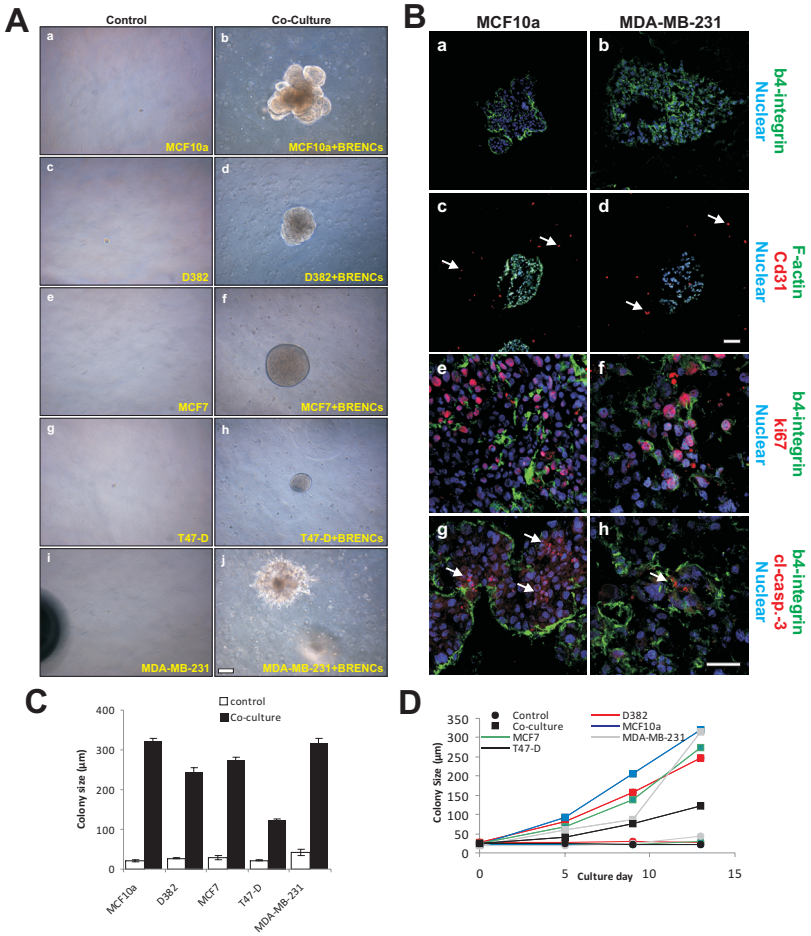

Figure 3 BRENCs induce clonal growth of normal and malignant breast epithelial cell lines. A) BRENCs generate positive environment for clonal growth of breast epithelial cells. MCF10A, D382, MCF7, T47-D and MDA-MB-231 cells were seeded at a clonal dilution, alone or in co-culture with BRENCs. When cultured 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).

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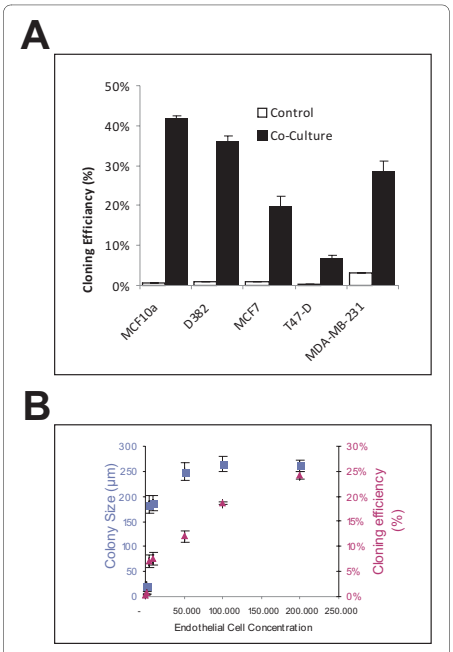

Figure 4 BRENCs enhance cloning efficiency of normal and malignant breast epithelial cell lines in co-culture. **A) Colony formation (cloning efficiency) is greatly increased in co-cultures**. MCF10A cells' colony forming ratio increased from 1% to 41.9%. MDA-MB-231 colony formation increased from 3.2% 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-

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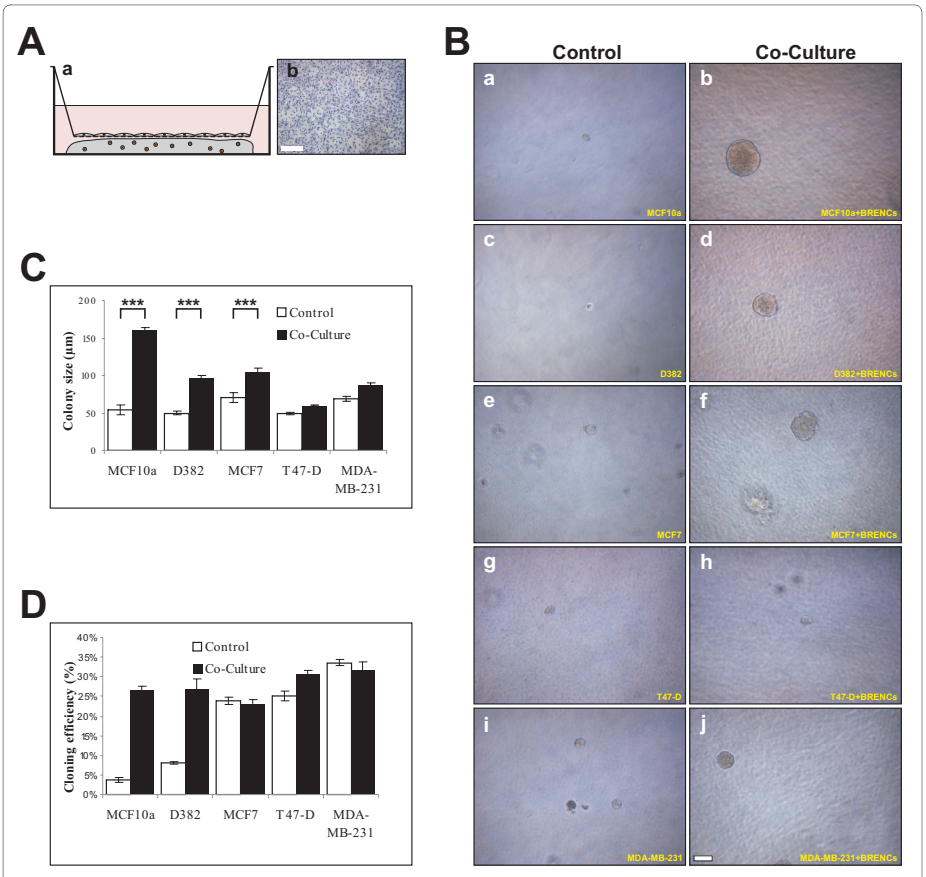

Figure 5 Endothelial-derived effects are mediated trough soluble factors. **A) Schematic figure of a Transwell co-culture assay**. BRENCs (grey) were seeded on Transwell (TW) filter inserts and allowed to reach confluency. Epithelial cells (orange) were embedded into Matrigel and seeded in the lower 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

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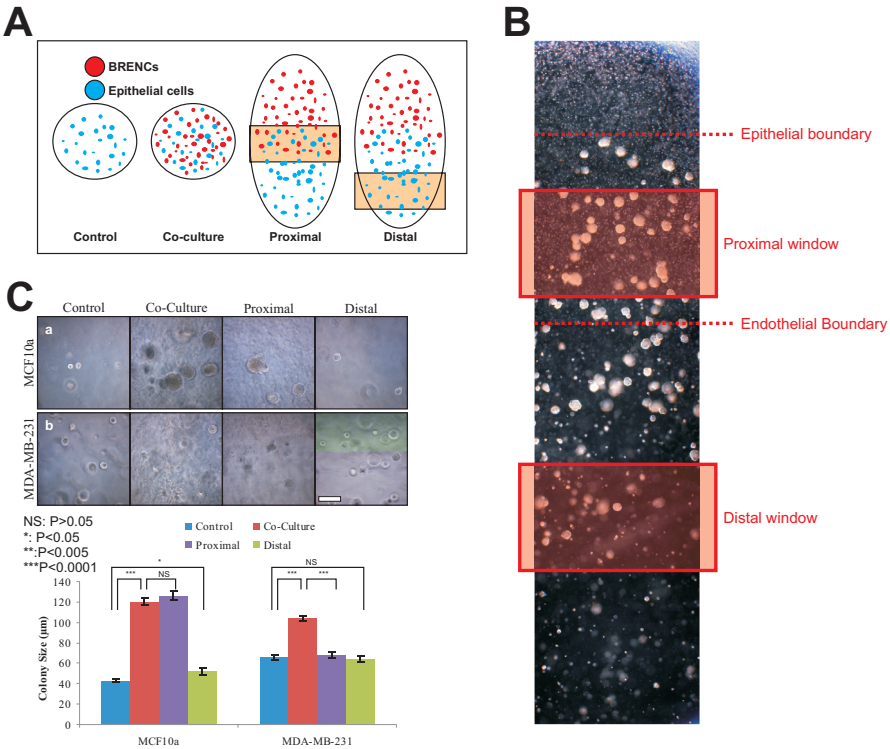

Figure 6 Proliferation effects decrease in relation with distance from endothelial cells. A) Schematic representation of BRENCs and epithelial cells in gradient co-culture. BRENCs and epithelial cells were embedded into separate gels on culture plates. The gels were allowed to fuse before culture medium 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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Selection for EGFR gene amplification in a breast epithelial cell line with basal-like phenotype and hereditary background

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Abstract An epithelial cell line, referred to as A163, was established from breast carcinoma derived from a patient with a strong family history of breast cancer but no known breast cancer susceptibility mutation. A163 was propagated in a serum-free culture medium including the epidermal growth factor. Immunophenotypic characterization demonstrated a mixed luminal and basal-like phenotype. When epidermal growth factor was excluded from the culture medium, A163 entered a quiescent period followed by a period of increased cell proliferation in a subpopulation of the cells. The epidermal growth factor-independent subpopulation retained the basal-like phenotype of the parental cell line. Karyotype and fluorescent in situ hybridization analysis showed an amplification of epidermal growth factor receptor on 7q in A163-S1 only, resulting in high expression of total and phosphorylated epidermal growth factor receptor. The A163-S1 sub-line piles up in culture, indicating a loss of contact inhibition. When grown on transwell filters, A163 shows basal expression of P63 and cytokeratin 14, whereas A163-S1 expresses P63 ubiquitously, and has lost the basal specific expression of cytokeratin 14, indicating a loss of polarity. Furthermore, when cultured in reconstituted basement membrane matrix, A163 form polarized normal like acini. In contrast, A163-S1 form large disorganized structures with lack of polarity. These cell lines may prove useful to understand molecular changes in breast cancer progression, in particular basal-like breast cancer subtype with bad prognosis and no current treatment options.

Keywords Cancer progression · EGFR amplification · Basal cancer · 3D culture · Culture models

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Introduction

Breast cancer is a prevalent disease affecting roughly 10% of women worldwide, where an estimated 5–10% of the cases are believed to be due to a hereditary component, such as mutations in the BRCA 1 and 2 genes (Fackenthal and Olopade 2007).

Breast cancer is a complex disease, as evidenced by the variety of phenotypes that are commonly used to predict clinical outcome and to guide towards appropriate therapy (Sainsbury et al. 2000; Sorlie et al. 2001). It is generally believed that breast cancer develops over a period of many years or even decades, progressing through several phases of increased tumorigenicity, making early diagnosis vital (Briand and Lykkesfeldt 1986).

To be able to develop novel treatment strategies in breast cancer it is also pivotal to recognize and understand the many steps involved in breast cancer formation and subsequent progression (Lacroix and Leclercq 2004). Tumor progression models that can recapitulate the in vivo-like environment of various breast cancer subtypes are thus critical.

Over the years, highly useful cell culture models have been developed to answer important questions in breast cancer biology, such as cancer progression as well as the general biology of the normal breast (reviewed in Gudjonsson et al. 2004; Lacroix and Leclercq 2004). When cultured in reconstituted basement membrane (rBM) matrix, normal luminal epithelial cells generate polarized acini mimicking epithelial cells in situ. In contrast, breast cancer cells fail to polarize correctly and continue to grow resulting in large spheres (Petersen et al. 1992). The rBM assay is thus a convenient assay to discriminate between normal and cancerous cells. The shortcoming of using primary cells in long term culture experiments is their limited life-span. This can be bypassed by using immortalized cell lines (Gudjonsson et al. 2004). The HMT-3522 cell line series is an example of how immortalized cells can serve as a model for normal morphogenesis and tumor progression and can be used to identify potential drug target and to screen for drugs that can halt or revert the malignant phenotype (Weaver et al. 1995). The HMT3522 cell line has progressed along the carcinogenic pathway generating normal (sub-line 1 (S1)) and pre-malignant (S2) sub-lines (Briand and Lykkesfeldt 2001). After removal of EGF from culture medium, prolonged passages in culture and transplantation into nude mice, S2 spontaneously transformed into malignant cells. The tumor cells were explanted and cultured as HMT3522-T4. Interestingly, the HMT3522-T4 has acquired trisomy 7q harboring the EGF receptor gene (Briand et al. 1996).

Basal-like breast cancers are characterized by heterogeneous expression of luminal and myoepithelial markers and thus are believed to originate in stem cells or cells with stem cell properties. Basal-like breast cancers express cytokeratins (CKs) such as CK5/6, 14 and 17 (myoepithelial) and CK 18 and 19 (luminal) and expression of the basal cell associated transcription factor p63. Furthermore, amplifications of the EGFR gene and overexpression of EGFR is also one of the characteristic features of basal-like breast cancer (Korsching et al. 2008). Gene expression and genomic profile studies have shown that hereditary breast cancer in particular those with mutant BRCA1 tend to fit the basal-like category (Toyama et al. 2008; Stefansson et al. 2009).

In this study, we have used a retroviral construct containing the E6 and E7 oncogenes to establish a new cell line from a breast cancer patient with a strong family history of breast cancer but no known mutations. The cell line, referred to as A163 shows basal-like phenotype as evidenced by immunocytochemistry. Based on the basal-like expression profile of A163, we theorized that this cell line could serve as a candidate cell line in a tumor progression assay to capture critical aspects in basal-like breast cancer progression. Withdrawal of EGF from culture medium resulted in new sub-line referred to as A163-S1. A163-S1 retains the basal-like phenotype of the parental cell line but has acquired extra copies of *EGFR*, and has lost contact inhibition polarization in 3D culture.

Materials and Methods

Cell culture. Primary epithelial cells from breast cancer tissue taken from a 51-yr-old woman with recognized family history of cancer but no known mutation (Thorlacius et al. 1996; Bergthorsson et al. 1998) were transduced with retroviral constructs containing pLXSN-E6E7. Transduced cells were selected with Geneticin (G418, Invitrogen, Carlsbad, CA), Cell lines were grown on Collagen I coated (Inamed, Gauting, Germany) culture flasks (BD Biosciences, Bedford MA) in H14 medium (Briand et al. 1987) consisting of DMEM/F12 basal medium (Invitrogen) supplemented with 200 μg/ml neomycin, 50 IU/ml penicillin, 50 μg/ml streptomycin, 10 ng/ml EGF, 0.1 nM estradiol, 0.5 μg/ml hydrocortisone, 250 ng/ml insulin, 2.6 ng/ml Na_2SeO_3, 5 μg/ml prolactin and 10 μg/ml transferrin (EGF was purchased from Peprotech (Rocky Hill, NJ), other growth factors purchased from Sigma (St. Louis, MO)). H14 medium without EGF was used for growth factor depletion culturing.

Proliferative assay. Proliferation of both cell lines was measured using crystal violet. Cells were seeded at an initial density of 10^4 cells per well in a 24-well tray. Each day, three wells from each culture were fixed with 3.5% formaldehyde for 10 min, then rinsed with PBS and stained with 0.1% crystal violet for 10 min. After staining, the cells were again rinsed with PBS. After the culture period, the crystal violet was released in acetic acid. Absorbance was measured at 590 nm.

Three-dimensional cell culture. Epithelial cells were suspended at 10^5 cells in 300 μl rBM (growth factor reduced

Matrigel, BD Biosciences) on ice and incubated at 37°C for 30 min. Cultures were supplemented with H14 medium and maintained for 2 wk.

Transwell filter culture. Cells were seeded at an initial density of 5×10^4 cells in a 12-well sized 0.4 μm pore size transwell filter (Corning Life Sciences, Lowell, MA) and allowed to grow in H14 medium for 4 wk. After the culture period, filters were removed using a scalpel. Filters were then fixed in 3.5% formaldehyde for 10 min and in 0.1% Triton-X100 for 10 min. Primary antibody was incubated overnight and secondary antibody for 60 min. Samples were counterstained with TO-PRO-3 and visualized using Zeiss LSM 5 Pascal laser scanning microscope (Carl Zeiss, Aalen, Germany). Z-stack projections were produced in Zeiss LSM Image Browser version 4.

Immunocytochemistry. Monolayer cell cultures were fixed in −20°C methanol for 10 min. Primary antibodies were directed against various proteins listed in Table 1. Rabbit anti-mouse immunoglobulins (Z0259, Dako, Glostrup, Denmark) were used as secondary antibodies and a peroxidase-conjugated anti-peroxidase mouse mAb was used as tertiary antibody (P850, Dako). The peroxidase reactions were performed using 3,3-diaminobenzidine (Dako) for 10 min. The cultures were counterstained with hematoxylin. Imaging was performed using a Leica DMI3000 microscope and a Leica 310FX imaging system (Leica, Wetzlar, Germany).

Three-dimensional cultures 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. Cryostat sections were fixed in methanol at −20°C for 10 min and incubated with primary antibodies (Table 1) mixed in PBS+10% FBS for 30 min. Slides were incubated with isotype-specific fluorescent antibodies (Alexa fluor (AF, 488 (green), 546 (red) Invitrogen) mixed in PBS+10% FBS for 30 min in the dark. The specimens were then incubated with a fluorescent nuclear counterstain (TO-PRO-3, Invitrogen) and mounted with coverslips using Vectashield (Vector Laboratories, Burlingame, CA). Immunofluorescence was visualized using Zeiss LSM 5-Pa laser scanning microscope.

Table 1. List of antibodies used for immunocytochemistry

Antibody	Clone	Species	Isotype	Company
ck14	LL002	Mouse	IgG3	Abcam
ck19	A53-B/A2	Mouse	IgG2a	Abcam
EpCAM	VU1D9	Mouse	IgG1	Novocastra
ZO-1	1A12	Mouse	IgG1	Zymed
E-Cadherin	HECD1	Mouse	IgG1	Zymed
Ck5/6	D5/16B4	Mouse	IgG1	Zymed
P63	7JUL	Mouse	IgG1	Novocastra
EGFR	D38B1	Rabbit	IgG	Cell Signaling
pEGFR	53A5	Rabbit	IgG	Cell Signaling
ErbB2	29D8	Rabbit	IgG	Cell Signaling

Western blot. Cells were washed twice with ice-cold PBS and subsequently lysed using RIPA lysis buffer. Equal amounts of proteins, as determined by Bradford analysis, were loaded and run on NuPAGE 10% Bis–Tris gel (Invitrogen) using MES running buffer (Invitrogen) for 35 min at a constant voltage of 200 V. Proteins were then transferred to a polyvinylidene difluoride membrane (Invitrogen) for 1 h at 30 V. The blots were then blocked in 5% BSA for 1 h and subsequently incubated with the primary antibody overnight. The blots were then washed and incubated with the secondary antibody, horseradish peroxidase-conjugated anti-mouse or rabbit (Amersham Biosciences UK Ltd., Little Chalfont, UK) at a dilution of 1:10,000 for 1 h. The protein bands were visualized using enhanced chemiluminesecene system (Amersham Biosciences). The chemiluminescent signal was detected using Hyperfilm (Amersham Biosciences).

Karyotypic analysis. Karyotyoic analysis was performed at the Chromosome Laboratory at the Department of Genetics and Molecular Medicine, Landspitali University Hospital, with standard cytogenetic procedures. Briefly, cells were incubated with metaphase arresting solution (Genial Genetic Solutions, Ltd.) for 3 h, followed by hypotonic treatment (0.0075 M KCl) for 20 min. at 37°C, fixed with methanol/acetic acid (1:3) and G-banded with trypsin solution and Leishman's stain. Thirty cells were analyzed from each culture.

Fluorescence in situ hybridization. Fluorescence in situ hybridization (FISH) analysis was performed with standard cytogenetic procedures. Briefly, for the detection of EGFR gene amplification, the clone PR5-1091E12, which spans the EGFR gene region was used. As a control for the ploidy level of chromosome 7, the clone pZ7.5 for centromere 7 was used. Both clones were courtesy of Dr. Mariano Rocchi's Web-based Resources for Molecular Cytogenetics (http://www.biologia.unibia.it/rmc). The PR5-1091E12 clone was labeled with SpectrumRed-dUTP (Vysis) and the pZ7.5 centromeric clone with fluorescein-12-dUTP (Enzo-Roche), by nick translation. Probes were diluted in c-DenHyb-2 hybridisation buffer (InSitus Biotechnologies, Albuquerque, NM) as described by the manufacturer. Chromosomes and probes were simultaneously denatured on a heated plate with lid placed on top of Perkin Elmer Cetus DNA Thermal Cycle heat-block at 85°C for 10 min followed by overnight hybridization at 37°C in a humid

chamber. Post-hybridization washes were performed three times in 0.1× SSC at 60°C for 5 min each. Slides were air dried and mounted with DAPI.

Results

Characterization of a novel basal-like epithelial cell line from patient with strong family history of breast cancer. The A163 cell line was established as described in material and methods. Immunophenotypic characterization demonstrated a mixed phenotype as evidenced by heterogeneous expression of luminal and myoepithelial/basal cell markers. EpCAM staining is seen in all cells (Fig. 1*A*), whereas staining of CK 5/6, 17 and 19 is heterogeneous in intensity (Fig. 1*B–D*). Limited staining of CK14 is seen (Fig. 1*E*). P63 staining is seen in subpopulations of the culture (Fig. 1*F*).

EGF ablation from culture medium results in EGFR gene amplification. When EGF was removed from the growth medium, the A163 cell line initially responded by growth arrest (Fig. 2*A* left). The cells remained quiescent for several wk. After this latency period, proliferating cell foci appeared and eventually the cells started to slowly proliferate again and finally reached a stable growth rate (Fig. 2*A–B*). This sub-line is referred to as A163-S1.

Immunostaining against EGFR revealed an increased expression of EGFR in A163-S1 compared to A163 (Fig. 2*C*). This was confirmed by western blot (Fig. 2*D*). Expression of EGFR in A163 responds to EGF activation, whereas ablation of EGF from the culture medium results in lowered expression of EGFR. This effect is not seen in A163-S1, where EGFR expression levels do not respond EGF stimulus (Fig. 2*D*). EGFR phosphorylation levels show a similar pattern, indicating that signaling through the receptor no longer requires exogenous EGF in A163-S1. ErbB2 is an important dimerization partner with EGFR. ErbB2 has no known ligand but high expression of ErbB2 can result in reduced sensitivity to growth factor stimuli. ErbB2 expression levels remained low in both cell lines (Fig. 2*D*).

Karyotype analysis on both cell lines, by G-banding of chromosomes was carried out at passages 39 and 44 for A163-S1 and A163, respectively, and from passage 78 from both cell lines (Fig. 3*A*). Both cell lines showed some chromosomal instability which is commonly associated with in vitro cultured cell lines (Lacroix and Leclercq 2004). A striking feature was an enlarged chromosome 7 that was seen in all A163-S1 cells in both passage 39 (data not shown) and in passage 78, but not in A163 (Fig. 3*A*). Chromosome 7 includes the gene for EGFR. To see if the large chromosome 7 had more than one copy of the EGFR gene, FISH analysis was conducted. Analysis of both cell lines showed consistently two copies of EGFR in every pair of chromosome 7 in A163, but in contrast A163-S1 contained multiple copies of EGFR on the enlarged chromosome 7 (Fig. 3*B*). Thus, withdrawal of EGF from the culture medium resulted in increased copy number of EGFR gene in A163-S1 followed by overexpression of the EGFR protein.

A163-S1 retains basal-like phenotype but has lost contact inhibition and show a transformed phenotype. A163-S1 showed a similar expression pattern of p63 and cytokeratins 5/6 and 17 as the mother cell line A163, whereas expression of CK19 was decreased. EpCAM and CK14 staining was more heterogeneous, suggesting a shift towards a more basal phenotype than in A163 (Fig. 4). Interestingly, in contrast to A163, A163-S1 shows a loss of

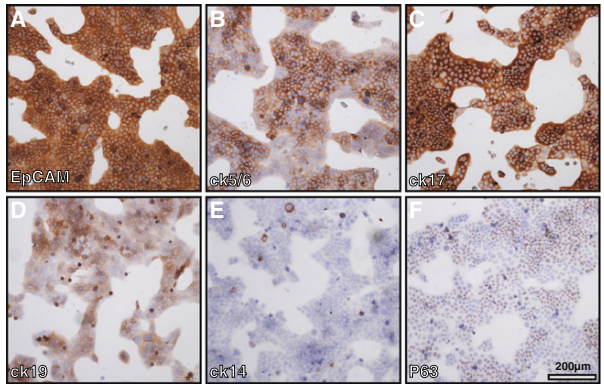

Figure 1. A163 show basal-like phenotype. Immunostaining shows expression of EpCAM (*A*), CK5/6 (*B*), CK17 (*C*), CK19 (*D*), CK14 (*E*) and P63 (*F*). Nuclear staining hematoxylin.

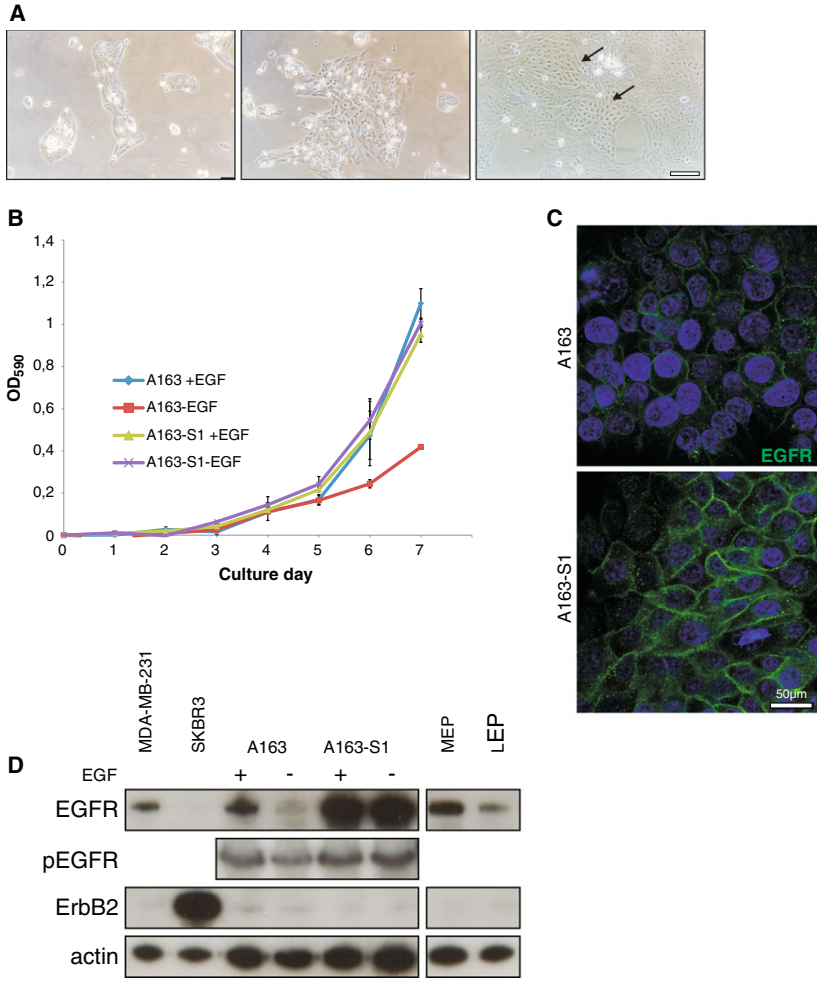

Figure 2. Ablation of EGF from A163 gives rise to new sub-line (A163-S1). (*A*) *EGF removal from A163.* After removal of EGF from culture medium the cells enter a quiescent state (*left*). After a few weeks, cell proliferation started focally (*middle*) in the culture flask and gradually a new cell line A163 sub-line1 (A163-S1) was established (*right*, *arrows* indicating proliferating islets). (*B*) *Proliferation of A163-S1 is independent on EGF stimulation.* Without EGF stimulation, proliferation of A163 is limited (*red line*). In contrast, no discernible difference in proliferation is seen with or without EGF stimulation in A163-S1 cultures (*green* and *purple lines*). (*C*) *EGFR expression is higher in A163-S1 than A163.* Immunofluorescent staining shows that expression of EGFR (*green*) is more prominent in A163-S1 than A163. *Blue*, nuclear staining. (*D*) *EGFR is overexpressed in A163-S1.* Western blot against EGFR shows increased levels of EGFR protein in A163-S1 compared to A163. Furthermore, A163-S1 does not respond to EGF stimulation in the same way as A163, as evidenced by blotting against both total EGFR as well as phosphorylated EGFR. EGFR level in A163-S1 is much higher than in the basal-like breast cancer cell line MDA-MB231 and in normal luminal and myoepithelial cells. ErbB2 expression is low in both A163 and A163-S1 cell lines but highly expressed in ErbB2 amplified cancer cell line SKBR3. Actin was used as a loading control.

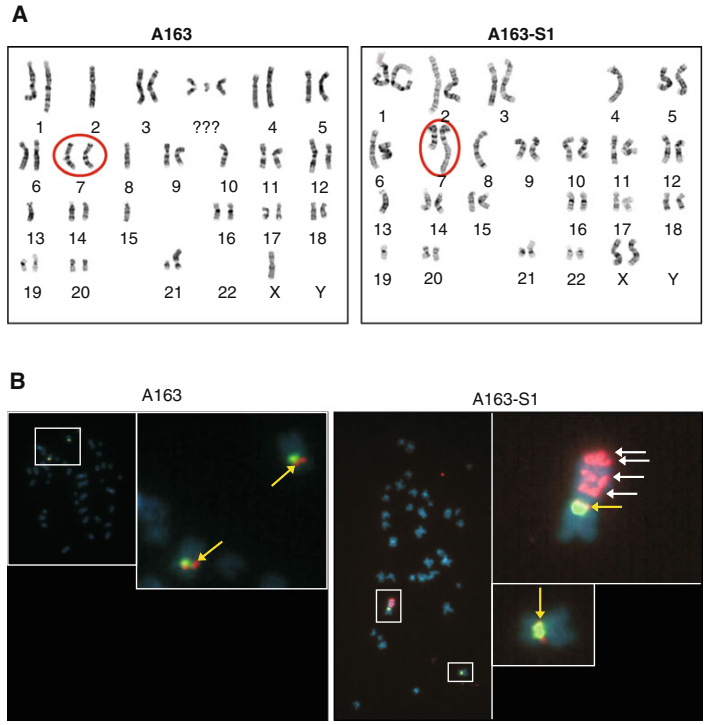

Figure 3. A163-S1 has gained extra copies of *EGFR1* on an enlarged chromosome 7. (*A*) *Karyotype comparison of A163 and A163-S1.* Karyotype analysis was carried out on 30 cells from each cell line in passage 78 for A163 and A163-S1. Both cell lines had some aberrant chromosomal structures which are commonly seen in cell lines. A163-S1 showed consistently an enlarged chromosome 7. (*B*) *A163-S1 has gained extra copies of EGFR.* Chromosomes were hybridized with fluorescein (*green*) and SpectrumRed-labeled probes specific for chromosome 7 centromeres and EGFR, respectively. A163 consistently shows two copies of EGFR on all chromosomes 7 (*left*) but A163-S1 has numerous copies on an enlarged chromosome 7 (*right*).

contact inhibition in monolayer and aberrant orientation of cells when cultured on transwell filters (Fig. 5). When A163 becomes superconfluent, it covers the surface of the culture dish and creates a dense layer of small cells. In contrast, A163-S1 forms islets of large flat cells surrounded by a population of smaller cells that have lost contact inhibition and form large piles and ridges surrounding these flat islets (Fig. 5*A*). When cultured on transwell filters and

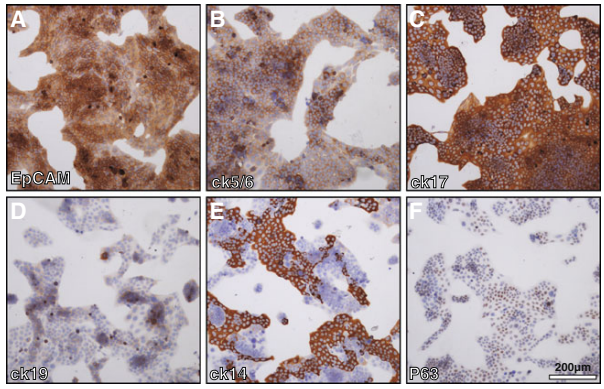

Figure 4. A163-S1 retains the basal-like phenotype but has lost contact inhibition. Immunophenotypic characterization demonstrates that A163-S1 has shifted towards a more basal phenotype as evidenced by the loss of CK19 expression and heterogeneous expression of CK14, P63, and EpCam. The cell line retains the heterogeneous expression pattern of CK5/6 and CK17.

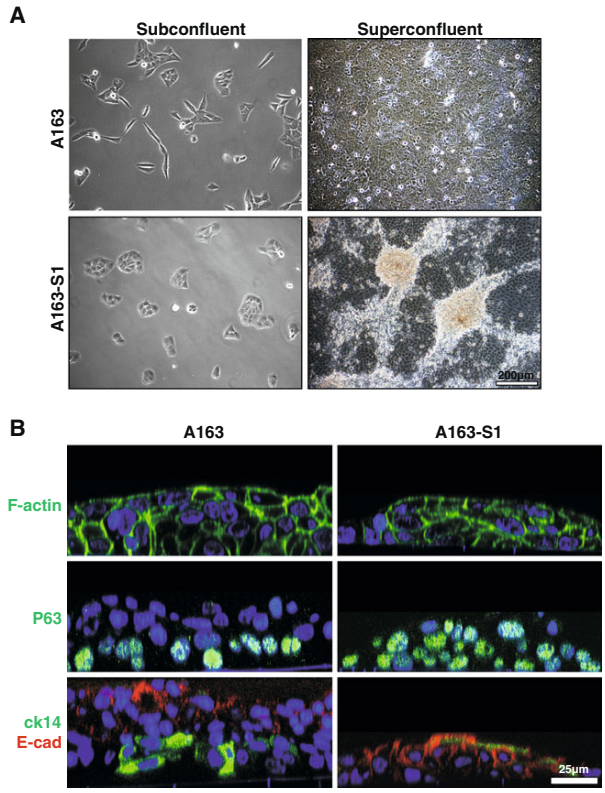

Figure 5. A163-S1 has lost contact inhibition and spatial recognition. (*A*) *A163-S shows a transformed phenotype.* When confluent in culture A163 cells maintain a dense layer of cells. In contrast, A163-S1 has lost contact inhibition and pile up in ridges within the culture flask. (*B*) *A163-S1 has lost spatial recognition.* Expression of P63 and CK14 are restricted to the basal layer of A163 cells when cultured transwell filter. In contrast, P63 expression in A163-S1 is not restricted to the basal cells, but spread throughout the cell layers. CK14 staining is seen apically. In both cell lines, E-cadherin expression is stronger towards the apical layer.

allowed to proliferate after reaching confluency, A163 cells form a polarized stratified epithelium with basolateral differentiation based on expression pattern of CK14 and p63 in the basal lying cells. In contrast, E-cadherin expression is stronger in the apical zone thus mimicking to some extent the spatial orientation of luminal epithelial and myoepithelial cells in situ (Fig. 5*B*). In contrast A163-S1 cells show a loss of spatial orientation as evidenced by irregular distribution of p63 and CK14 positive cells. P63 expression is seen in all levels of the culture, whereas CK14 expression is seen in the apical part but not the basal layer. E-cadherin expression is comparable to that of A163 (Fig. 5*B*). This indicates that A163-S1 has lost spatial recognition, resulting in aberrant expression pattern of the basal markers p63 and CK14.

A163 and A163-S1 show distinct growth pattern when cultured in 3D environment. We next cultured both cell lines in a rBM matrix. In this assay, normal breast epithelial cells have been shown to form polarized acinus-like structures whereas cancer cells form disorganized structures (Petersen et al. 1992). In this assay, A163 formed small polarized in vivo-like structures (Fig. 6) reminiscent of normal primary cells. In contrast, A163-S1 formed larger aberrant solid structures. A portion of the colonies formed grape-like colonies with disorganized, loosely connected cells (Fig. 6, insert). Expression of CK14 and E-cadherin was similar between both cell lines, whereas p63 expression was only seen in A163-S1 colonies. As before, expression of EGFR was stronger in A163-S1 colonies (Fig. 6).

Discussion

In this paper, we have established an in vitro model that captures the initial changes towards a malignant phenotype

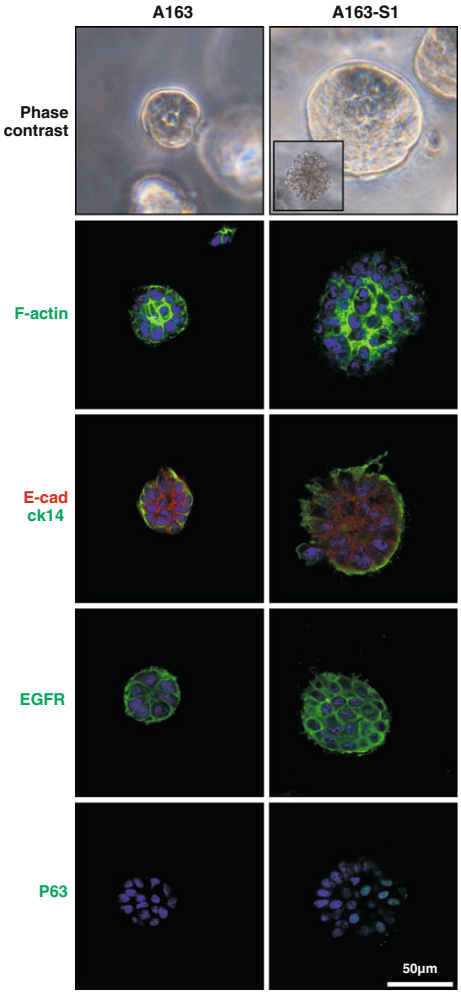

Figure 6. A163-S1 forms large disorganized colonies in 3D culture. When placed into three-dimensional matrigel, A163 forms small, polarized colonies with a central lumen, sometimes filled with abnormal/apoptotic cells, whereas A163-S1 forms much larger colonies without central lumen formation and lack of polarization (*top panel*). F-actin further demonstrates this difference. Interestingly, subpopulations of A163-S1 cells generate grape-like structures of cells indicating loss of cell–cell connection (*top*, *right* (*insert*)). Cytokeratin and E-cadherin expression is similar to that seen in monolayer and transwell cultures, whereas P63 expression is not seen in A163. As before, EGFR expression is stronger in A163-S1.

in a subgroup of breast cancer commonly referred to as a basal-like breast cancer. Based on a previously published breast cancer progression model (Briand et al. 1996), we created an EGF-independent sub-line from a basal-like cell line. The A163 cell line shows a bipotential phenotype as evidenced by marker expression for both luminal epithelial- and myoepithelial cells. Removing EGF from the cell culture medium forced the cells to enter a quiescent state for a few weeks before a small population of cells gradually re-entered a proliferative phase. This new sub-line, A163-S1 retains the basal phenotype of the parental cell line, but has undergone chromosomal and phenotypic changes that have previously been associated with changes in cancer cells including amplification of the EGFR gene.

Basal-like breast cancer. Transcriptional profiling of large cohorts of human breast cancers has resulted in the identification of five distinct subtypes (Perou et al. 2000). These studies indicate that between 17% and 37% of human breast tumors display a basal epithelial phenotype (Perou et al. 2000; Sorlie et al. 2001). The basal phenotype is associated with an early age of onset and short relapse time (Rakha and Reis-Filho 2009). In contrast to ER-positive luminal tumors and ErbB2 overexpressing tumors, for which specific therapeutic approaches are available, basal-like cancers are poorly differentiated, and lack molecular targets for drug design.

A large number of these tumors are detected during routine mammograph screening (Korsching et al. 2008), reflecting the aggressive nature of these tumors. For these reasons, the basal epithelial subtype contributes significantly to breast cancer mortality (Korsching et al. 2008). Due to the mixed phenotype of basal-like breast cancer, it has been suggested that such cancers may originate in stem cells or immediate progenitor cells of the breast (Foulkes 2004). The prominent characterization of basal-like breast cancer is the triple negative (ERneg, PRneg, ErbB2neg) phenotype. However, these tumors also express a mixed pattern of cytokeratins such as the luminal CKs 8, 18, and 19 and the myoepithelial CKs 5/6, 14, and 17 (Sarrio et al. 2008).

Recent studies have shown that basal-like breast cancer show low expression of BRCA1 and up to 30% exhibit increased EGFR expression (Toyama et al. 2008). This has connections to our data where A163-S1 has acquired increased copy number of EGFR.

EGFR amplification. It is well established that EGFR2 is amplified in about 25–30% breast cancers and furthermore, recent data suggests that EGFR is amplified or overexpressed in approx 15–20% of breast cancer (Burness et al. 2010). EGFR is also amplified in a number of breast cancer cell lines including BT-20 and MDA-MB-468 (deFazio et

al. 2000; Agelopoulos et al. 2010). In this study, we have shown that depriving A163 of EGF led to increased copy number of the EGFR gene on chromosome 7. Agelopoulos et al. demonstrated that chromosomal instability can contribute to selective regain of gene copy number, through breakage-fusion bridges, resulting in amplification on chromosome 7. (Agelopoulos et al. 2010). The A163 cell line was originally established using a retroviral construct disrupting the function of P53 and retinoblastoma, major checkpoint controllers in the cell cycle. The original tumor used to generate the cell lines had wild-type *P53*, *BRCA1*, and *BRCA2.* Checkpoint disruption enables chromosomal changes, thus representing the initial steps towards malignancy, increased cell proliferation and survival.

In vivo, selective pressure towards higher EGFR expression might arise in ductal carcinoma in situ, where disruption of the tumor microenvironment limits the access of centrally located tumor cell to EGF produced by the surrounding stroma. Indeed, high EGFR expression in early stage cancer has poor clinical prognosis (Burness et al. 2010).

High EGFR expression levels, due to either increased transcription or amplification are strongly correlated with basal-like breast cancer (Milanezi et al. 2008), thus becoming a possible target for basal-like cancer drug targeting. There are several mAb and TKI inhibitors targeting EGFR in different phases of clinical trials, including lapatinib (Burness et al. 2010).

Conclusion

In conclusion, this study showed that targeted stress on a specific GF-signaling pathway selects for outgrowth of subclones of cells that have become autonomous for this growth signal, thus confirming and supporting previous findings. This data has implications regarding the initial steps of cancer formation where disruption of homeostasis places selective pressure on subpopulation of cancer cells, pushing towards a malignant phenotype. EGFR-targeted drug therapies are thus an important field, where our cell lines could prove useful in drug response trials, for example as a model for escape mechanisms from EGFR-targeted drug treatment.

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Paper III

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PLOS ONE

Expression and Functional Role of Sprouty-2 in Breast Morphogenesis

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Abstract

Branching morphogenesis is a mechanism used by many species for organogenesis and tissue maintenance. Receptor tyrosine kinases (RTKs), including epidermal growth factor receptor (EGFR) and the sprouty protein family are believed to be critical regulators of branching morphogenesis. The aim of this study was to analyze the expression of Sprouty-2 (SPRY2) in the mammary gland and study its role in branching morphogenesis. Human breast epithelial cells, breast tissue and mouse mammary glands were used for expression studies using immunoblotting, real rime PCR and immunohistochemistry. Knockdown of SPRY2 in the breast epithelial stem cell line D492 was done by lentiviral transduction of shRNA constructs targeting *SPRY2*. Three dimensional culture of D492 with or without endothelial cells was done in reconstituted basement membrane matrix. We show that in the human breast, SPRY2 is predominantly expressed in the luminal epithelial cells of both ducts and lobuli. In the mouse mammary gland, SPRY2 expression is low or absent in the virgin state, while in the pregnant mammary gland SPRY2 is expressed at branching epithelial buds with increased expression during lactation. This expression pattern is closely associated with the activation of the EGFR pathway. Using D492 which generates branching structures in three-dimensional (3D) culture, we show that SPRY2 expression is low during initiation of branching with subsequent increase throughout the branching process. Immunostaining locates expression of phosphorylated SPRY2 and EGFR at the tip of lobular-like, branching ends. SPRY2 knockdown (KD) resulted in increased migration, increased pERK and larger and more complex branching structures indicating a loss of negative feedback control during branching morphogenesis. In D492 co-cultures with endothelial cells, D492 SPRY2 KD generates spindle-like colonies that bear hallmarks of epithelial to mesenchymal transition. These data indicate that SPRY2 is an important regulator of branching morphogenesis and epithelial to mesenchymal transition in the mammary gland.

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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 [1,2]. This process gives rise to the airways of the lungs [3], the urine collecting ducts [4], the prostate [5], salivary glands [6] and the mammary glands [7,8].

The molecular events that induce and regulate branching morphogenesis are highly conserved between different organs and between different species [9]. Receptor tyrosine kinases (RTKs), such as fibroblast growth factor receptors (FGFRs) and epidermal growth factor receptors (EGFRs) are key mediators of signals that regulate proliferation, differentiation and branching morphogenesis in the mammary gland [1,10]. Extracellular cues such as FGFs and EGFs act via their respective receptors to activate intracellular pathways, such as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI-3 kinase) pathways which are critical for proper development of branched organs [1]. In addition, aberrant expression and activation of RTKs such as the EGFR family is common in a number of cancers including breast cancer (reviewed in [11]). Molecular signaling in branching morphogenesis must be precisely regulated both spatially and temporally to ensure normal homeostasis. Recent studies have underscored the importance of negative feedback control of RTK signaling for ensuring correct cell fate and morphogenesis [12]. Sprouty, initially shown to be critical for tracheal development in Drosophila [13], is now known to act as a conserved negative feedback regulator of RTK signaling in higher eukaryotes [14–20]. There are four mammalian Sprouty proteins (SPRY1–4) and they have been proposed to participate in a classical negative feedback

loop of RTK signaling through the MAKP pathway [21]. However, detailed molecular mechanisms of action of the sprouty proteins have not been fully elucidated. The studies of sprouty in the mammals have thus far mostly focused on the regulation of FGFR and EGFR [21]. Sprouty proteins have been identified as regulators of FGFR, c-Met and EGFR signaling in lung, kidney and vasculogenesis but their role in the human breast gland morphogenesis has not been systematically analyzed [22]. Although, sprouty proteins are considered negative inhibitors of RTK signaling their role in maintaining signal activity has been reported. Thus, SPRY2 has been shown to delay EGFR breakdown in endosomes after internalization by binding the catalytic RING Finger of Casitas B-lineage lymphoma (c-Cbl), an E3 ubiquitin ligase that has been identified to target EGFR degradation. SPRY2 sequester c-Cbl molecules from activated EGFR and disregulate EGFR ubiquitination and downregulation, thereby potentiating the amplitude and longevity of intracellular signals [23,24].

In mouse mammary glands the branching ducts are embedded in fat-rich stroma whereas in humans, breast ducts are more elaborate and terminate in the lobuli commonly referred to as the terminal duct lobular units (TDLU) [25]. The TDLUs are composed of differentiated luminal- (LEP) and surrounding myoepithelial (MEP) cells, separated from the stroma by a 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 lose adhesion and acquire transient epithelial to mesenchymal transition (EMT) resulting in increased motility [7,26]. Temporal EMT phenotypes have also been linked to cancer progression and metastasis [26–28]. 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 a major factor seen in many cancer forms, including breast cancers [1]. Lo et al. [29] have shown that SPRY2 expression is suppressed in breast cancers suggesting that SPRY2 might function as a tumor suppressor. Interestingly, Faratian et al. [30] have recently shown that reduced expression of SPRY2 is an independent prognostic factor in HER2 positive breast cancer. These data link candidate morphogenic pathways to breast cancer progression.

Three-dimensional cultures have proven to be important tools for recapitulating an *in vivo* like context in the mammary gland [31,32]. We have previously shown that D492, an epithelial cell line with stem cell properties, generates TDLU-like structures in 3D culture [33,34]. D492 is thus a good model to dissect molecular mechanisms regulating branching morphogenesis. We have also shown that endothelial cells stimulate growth and morphogenesis of breast and lung epithelial cells [35,36]. Most recently, we demonstrated that endothelial cells facilitate branching morphogenesis of D492 in co-culture and furthermore induces a subpopulation of D492 to generate spindle-like colonies through an EMT conversion [37]. Here, we show that SPRY2 is predominantly expressed in luminal epithelial cells of duct and lobuli in human breast tissue. We also show that SPRY2 is highly expressed in the pregnant and lactating mouse mammary gland with phosphorylated SPRY2 peaking during pregnancy. Expression of SPRY2 is associated with expression of phosphorylated EGFR (pY1068) and activation of the downstream MAPK signaling pathway. Using D492, we show that SPRY2 is expressed at the branching tips and suppression of SPRY2 through shRNA gene knockdown increases branching morphogenesis and promotes epithelial to mesenchymal transition when cultured with endothelial cells.

Materials and Methods

Cell culture

The breast epithelial stem cell line D492 was maintained in H14 medium [38], 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 were maintained on CDM3 and CDM4 as previously described [35,39]. Primary human BRENCs were isolated from breast reduction mammoplasties as previously described [40] 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 (Becton Dickinson, BD, Falcon). 7×10^3, 1×10^4 and 1.3×10^4 D492 cells were suspended in 300 µl of reconstituted basement membrane (rBM) purchased as matrigel (BD). Co-culture experiments were carried out with 1×10^3 D492 mixed with 5×10^4 BRENCs. 100 µl of mixed cells / rBM were seeded in each well in a 96 well plate and cultured on H14 (Monoculture) or EGM5 (Co-culture) for 16 days.

Isolation and processing of mammary glands and 3D cell cultures

Human tissue from breast reductions was used for immunohistochemistry and for isolation of primary breast epithelial cells. Primary LEPs and MEPs were isolated by magnetic cell sorting (MACS) as previously described [39]. Murine mammary glands were dissected from C57BL/6 mice at the following stages: 6 week old virgins, day 15 of pregnancy and day 2 of lactation. Mammary glands were snap frozen in liquid nitrogen and preserved at −80°C. Isolation of colonies from 3D cell culture was done as previously described by gentle dissociation in PBS-EDTA buffer [41].

Immunochemistry

Formalin-fixed, paraffin embedded human tissue blocks from reduction mammoplasty biopsies 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 EDTA buffer for 15 minutes. Frozen mouse mammary glands were cryosectioned at 15 µm 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), ki67 (Abcam, ab833), E-Cadherin (BD Biosciences, cat. 610182), N-Cadherin (BD Biosciences, cat. 610921).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).

In situ Proximity Ligation assay

Protein phosphorylation of Spry2 was studied *in situ* by Proximity Ligation assay (PLA) using the Duolink(R) kit (Olink Bioscience, Uppsala, Sweden) [42]. Sections from mouse mammary glands and 3D cultures were fixed with PFA, blocked and incubated with primary antibodies, Sprouty-2 at 1:50 dilution

(#07-524, Upstate/Millipore), and P-Tyr-100 at 1:100 dilution (#9411S, Cell Signaling), overnight at 4°C. The remaining steps of the PLA were performed as suggested by the kit manufacturer. Cells were incubated with secondary anti-mouse PLUS and anti-rabbit MINUS probes. Pairwise binding to the target allowed free oligonucleotide ends of the probes to come into close proximity, and the free ends enabled formation of circular DNA molecules through ligation. The DNA circles were then amplified and detected by hybridization of fluorescently labeled oligonucleotides. Nuclei were counterstained with TO-PRO 3 (Invitrogen). Single phosphorylated Spry2 molecules were visualized using LSM 5 Pascal confocal microscope (Carl Zeiss, Jena).

In Situ Hybridization

2981bp segment of Spry2 DNA was amplified from human blood cells, with forward (CTAAGCCTGCTGGAGTGACC) and reverse (GGAACTTTGAAAAACCAACA) primers generated with online Biology Workbench (http://workbench.sdsc.edu). DIG-labeled RNA probe synthesis was performed according to the manufacturers instructions (DIG RNA Labeling Mix, Roche). Paraffin embedded normal breast tissue slides were processed and then treated for 10 minutes with proteinase K (Fermentas), and with acetic anhydride / triethanolamine (Sigma) for 10 minutes. Before hybridization, the samples were prepared in pre-hybridization buffer for 2 hours. Hybridization was carried out for 12–16 hours. Slides were then washed and incubated with Anti-Dioxigenin-AP fab fragments (Roche) antibody for 4 hours at RT. Color development was carried out with NBT/BCIP buffer (Sigma) for 4 hours at RT in dark.

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), pERK (1:2000, #4695, Cell Signaling), pERK (1:2000, #9101, Cell Signaling), and β-actin (1:5000; ab3280, Abcam). Membranes were visualized with ECL+ after incubation with anti-mouse or rabbit secondary antibodies (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 (Applied Biosystems). Experiments were done in triplicate on 7500 Real Time PCR System (Applied Biosystems). SPRY2 mRNA levels were normalized to GAPD and relative mRNA differences was calculated with the $2^{\Delta Ct}$ Method.

shRNA knockdown of SPRY2

Three separate pGIPZ lentiviral shRNA constructs targeting SPRY2 transcripts were purchased from Open Biosystems (RHS4430-101098640 (KD3), RHS4430-101103852 (KD2), RMM1766-96881511 (KD1)). A non-silencing construct (RHS4346) was used as a control. All constructs contained both a puromycin selection marker and green fluorescent protein (GFP). 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 8 µg/µl polybrene. Stable, $D492^{SPRY2\ KD}$ cells were established by puromycin selection (3 µg/µl) followed by flow-sorting, selecting GFP expressing cells.

Migration and proliferation assay

For migration experiments, a total of 2.5×10^4 starved cells were seeded in DMEM/F12 basic medium on collagen coated upper compartment of a transwell Boyden chamber (Corning) with an 8 µm 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 filter. 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 10% acetic acid and measured at 570 nm in a plate reader.

Statistical analysis

All migration and 3D culture experiments were performed in triplicate. 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.

Ethics Statement

The breast tissue samples were provided by written informed consent from women undergoing reduction mammoplasty. This procedure has been approved by the National Bioethics Committee of Iceland, Reference number VSNa2001050056. The Committee on the use of Experimental Animals (Tilraunadýranefnd) approves all protocols for experiments on animals performed in Iceland. The committee does not require special approval for collecting tissues after euthanasia, as was done in this report. Details of animal welfare; Mice were housed in Micro Isolator cages (Lab Products Inc.) according to the guidelines set out in the recommendation of the EU commission (2007/526/EC - June 18, 2007) for accommodation and care of animals used for experimental and other scientific purposes, and according to Icelandic law (number 15/1994) and regulations (number 279/2002). Mice were euthanized by inhalation of high concentrations of CO2. This method is classified as "acceptable" in the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Results

SPRY2 is predominantly expressed in luminal epithelial cells in the human breast gland

To explore the expression of SPRY2 in the human breast gland we performed immunostaining and *in situ* hybridization against SPRY2 in tissues representing the adult non-pregnant human mammary gland from reduction mammoplasties. SPRY2 expression was seen in epithelial cells, both in large ducts and in the terminal duct lobular units (TDLU) (Fig. 1A). Dual labeling with antibodies against SPRY2 and the linage restricted markers cytokeratin (CK) 18 (luminal epithelial cells) or CK14 (myoepithelial cell), demonstrated that SPRY2 was predominantly expressed within the luminal epithelial compartment (Fig. 1A). SPRY2 was also detected in discreet areas in the stroma, most likely endothelial cells (Fig. 1A arrows). This was supported by analyzing the expression of SPRY2 in purified myoepithelial and luminal epithelial cells isolated from three different breast tissue samples using quantitative real-time PCR. Luminal epithelial cells

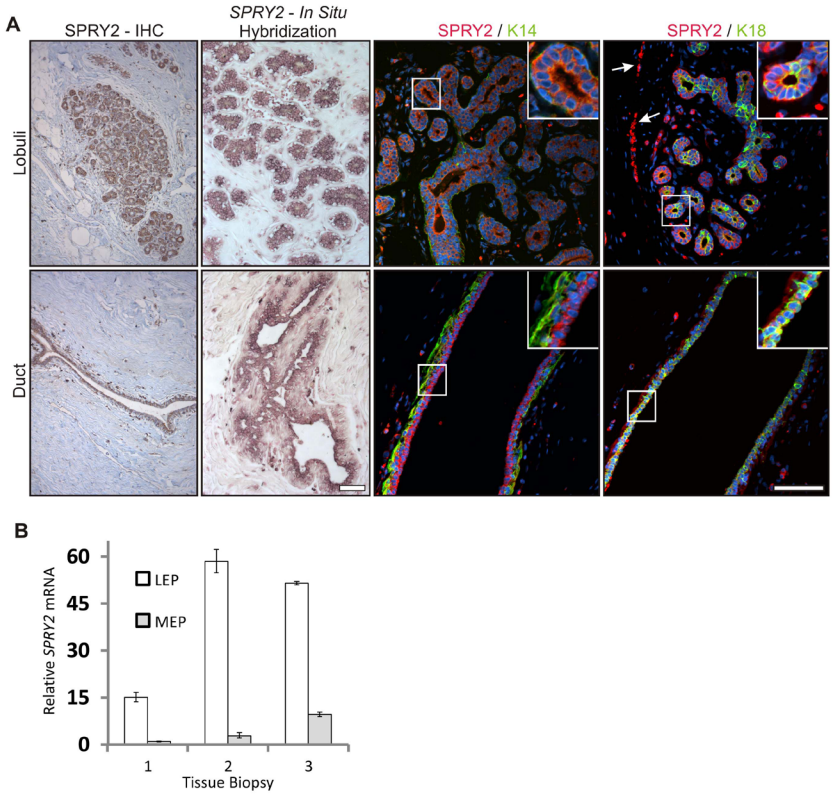

Figure 1. Expression of SPRY2 in lobules and ducts in the normal human breast gland. Expression of SPRY2 was evaluated in normal human breast tissue derived from reduction mammoplasty biopsies. *A) Expression of SPRY2 is most prominent in the luminal epithelial cells.* SPRY2 expression was predominantly found within the epithelial compartment of duct and lobuli as evidenced by immunohistochemistry and in situ hybridization. SPRY2 was predominantly expressed in luminal epithelial cells both in ducts and lobuli. SPRY2 was co-stained for K14 (myoepithelial cells) and K18 (luminal epithelial cells). Note the co-expression of SPRY2 and K18 in luminal epithelial cells. SPRY2 expression was also presence in the stroma, most likely in endothelial cells (arrows). Sections were counterstained with TOPRO-3. Bar = 100 μm. *B) Expression differences of SPRY2 in luminal- and myoepithelial cells.* Real time PCR was used to quantify expression difference of *SPRY2* between luminal- and myoepithelial cells. *SPRY2* expression was generally low in myoepithelial cells compared to luminal epithelial cells that expressed up to 58 fold more *SPRY2*. Measurement was done in paired luminal and myoepithelial cells from three different biopsies.
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showed 15–58 fold higher expression of SPRY2 compared to myoepithelial cells (Fig. 1B) in cell purifications from three different breast tissue samples.

SPRY2 expression is associated with activated EGFR signaling in the pregnant and lactating mouse mammary gland

A disadvantage of studying sprouty expression in tissue from reduction mammoplasty is that we are unable to analyze the temporal expression changes during different stages of branching morphogenesis. Therefore, we analyzed the expression of SPRY2 in mouse mammary gland at different stages of development. We isolated mammary glands from virgin, pregnant and lactating mice and analyzed total SPRY2 expression and pSPRY2 and correlated this with pEGFR level. Low level of SPRY2 was seen in the virgin gland. Expression was more prominent in the pregnant gland where the expression was co-localized with myoepithelial cells as evidenced by co-staining for SPRY2 and CK14 (Fig. 2Aa and b). SPRY2 expression reached its highest levels during lactation. Total EGFR showed similar expression pattern as SPRY2. pEGFR was low or absent in the virgin mammary gland but increased focally at branching end buds in the pregnant gland. Furthermore, a dramatic increase in pEGFR was seen in end buds during lactation (Fig. 2Ad). (Fig. 2A). The high EGFR phosphor-

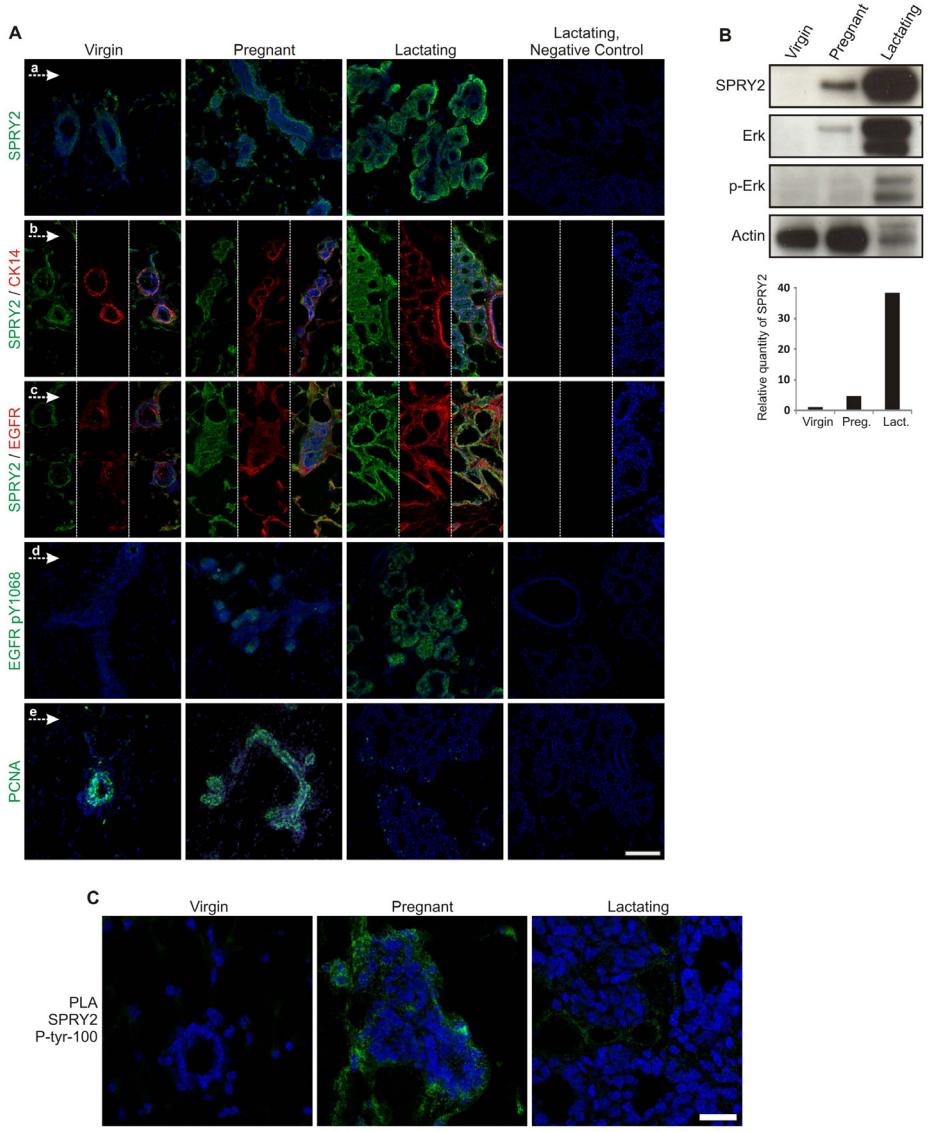

Figure 2. Expression of SPRY2 in virgin, pregnant and lactating mouse mammary gland. *A) Expression of SPRY2 and pEGFR is inversely correlated with cell proliferation.* Low expression of SPRY2 is found within the virgin gland with few positive stromal cells (a). Note, increased stromal expression of SPRY2 in pregnant gland accompanied with expression in myoepithelial cell as evidenced by double staining of SPRY2 and the myoepithelial marker CK14 (b). Dramatic increase in SPRY2 expression is seen during lactation (a and b). SPRY2 and EGFR show similar expression pattern at all stages (c) with pEGFR expression seen at terminal buds in pregnant gland. Dramatic increase in pEGFR expression is seen in the lactating

gland. Similar expression is found for SPRY2 and pEGFR in lactating gland. Proliferation is increased from virgin to pregnant gland but is reduced during lactation, with only few PCNA positive cells left. Cells counterstained with TOPRO-3, Bar = 100 μm. *B) SPRY2 expression is highest during lactation accompanied by activation of Erk/MAPK pathway.* Western blot demonstrated the expression differences of SPRY2 in virgin, pregnant and lactating glands. There is over 38 fold increase in SPRY2 expression during lactation compared to virgin state. Total ERK and pERK is also significantly increased during lactation. Actin was used as a loading control. *C) SPRY2 activity is peaking during pregnancy in the mouse mammary gland.* Using proximity ligation assay it was shown that phosphorylated SPRY2 was significantly more expressed during pregnancy compared to virgin and lactating gland. Bar = 25 μm.
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ylation and SPRY2 expression in the lactating state was not associated with cell proliferation marker PCNA (Fig. 2Ae). In order to get quantitative information on SPRY2 expression, western blot analysis was performed on mouse mammary gland tissues. SPRY2 was low or not detected in the virgin mammary gland whereas it was expressed at low levels in pregnant and high levels in lactating mammary glands (Fig. 2B and Figure S1). ERK1/2 mediates signaling through the RAS/MAPK cascade downstream of EGFR. Total ERK expression was correlated with total SPRY2 expression with highest levels seen during lactation. In contrast, phosphorylated ERK1/2 was increased substantially in lactating state only (Fig. 2B). There is no commercially available antibody against phosphorylated SPRY2. To analyze the tyrosine phosphorylation status of SPRY2 in the virgin, pregnant and lactating gland we carried out proximity ligation assay (PLA) using antibodies against SPRY2 and phosphorylated tyrosine residues (see material and methods for details). The PLA assay demonstrated strong SPRY2 phosphorylation in mammary glands from pregnant mice compared to virgin or lactating mice (Fig. 2C). These data suggest that loss of pSPRY2 during lactation is accompanied by increased ERK activity.

Spatial and temporal expression of SPRY2 and pEGFR during branching morphogenesis of breast epithelial cells in 3D culture

To directly study the functional role of SPRY2 in branching morphogenesis of the human breast epithelium we used the D492 cell line cultured in 3D reconstituted basement membrane (rBM). D492 has stem cell properties, i.e. it can differentiate into luminal- and myoepithelial cells and forms TDLU-like colonies through branching morphogenesis when cultured within a 3D rBM [33,34]. We first analyzed temporal expression of SPRY2 during TDLU formation in 3D rBM. D492 cells undergo most extensive branching during days 10–16 in 3D rBM culture (Fig. 3A). Initially, D492 forms solid round colonies that start to branch on days 10–12 (initial branching). After the first branching event ductal structures elongate and secondary branching occurs with bifurcation at the lobular-like ends (Fig. 3A). To analyze SPRY2 expression we isolated mRNA from culture days, 8, 10, 12, 14 and 16. Pre-branching (day 8), round colonies show high expression of SPRY2. Interestingly, during the initial branching period (days 10–12) the expression of SPRY2 decreases. At day 16 elaborate TDLU-like structures have formed and the expression of SPRY2 increases to more than 4-fold levels compared to day 10 (Fig. 3B). Expression was also confirmed with an immunoblot on D13, D16 and D19, showing the increase in SPRY2 expression from D13 to D16. pEGFR expression pattern was similar with increasing levels from D13 to D16 while decreasing on D19 when branching has stopped (Fig. 3B). This expression pattern suggests that SPRY2 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, immunofluorescent staining of branching colonies at day 16 shows that SPRY2 expression is mainly concentrated at the branching, lobular-like tips but is lowered at sites of cleft formation (Fig. 3C). The location of SPRY2 at day 16 is similar to that of pEGFR at branching tips while staining for total EGFR has a more general distribution in the branching colonies (Fig. 3C). Co-staining of EGFR and SPRY2 demonstrate co-localization at the edge of the branching structures. Phosphorylated SPRY2 followed the same pattern as pEGFR and total SPRY2 as seen using the PLA (3C). Staining for ß4-integrin and F-actin expression show the general outlines of the branching structures and its connection to the surrounding basement membrane (Fig. 3C).

SPRY2 knockdown in D492 stimulates branching morphogenesis

To further explore the functional role of SPRY2 in the regulation of branching morphogenesis we knocked down SPRY2 in D492 cells and explored their proliferative, migratory and morphogenic potential. We used a lentiviral based shRNA knockdown where D492 were transduced with a GFP-containing non-silencing (NS) control and 3 different knockdown (KD) shRNA constructs (SPRY2-KD1, SPRY2-KD2 and SPRY2-KD3) targeting SPRY2. The SPRY2-KD3 construct was most effective, decreasing SPRY2 expression levels 4 fold (Fig. 4A). Thus, we used this knockdown cell line and a single cell subclone referred to as SPRY2-KD3A. No morphological difference was seen between NS cells and KD3A cells when visualized in a monolayer (Fig. 4B) but $D492^{SPRY2\text{-}KD3}$ and $D492^{SPRY2\text{-}KD3A}$ showed increased migration compared to $D492^{NS}$ cells (Fig. 4C). There was no significant difference in the proliferation of $D492^{SPRY2\text{-}KD3}$ and $D492^{SPRY2\text{-}NS}$ cells (Fig. 4D). However, increased expression of pERK is seen in $D492^{SPRY2\text{-}KD3A}$ (Figure S2) which could explain the migration ability of these cells. To analyze the effects of SPRY2 knockdown on branching morphogenesis we compared $D492^{SPRY2\text{-}NS}$, $D492^{SPRY2\text{-}KD3}$ and $D492^{SPRY2\text{-}KD3A}$ 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 SPRY2 knockdown was quantified by counting colonies of simple/early branching, complex/late branching and other (solid round colonies) morphology (Fig. 5B). In a setup with 1×10^4 cells both $D492^{SPRY2\text{-}KD3}$ and $D492^{SPRY2\text{-}KD3A}$ formed more branching colonies in total and substantially more colonies that showed complex branching phenotype compared to $D492^{SPRY2\text{-}NS}$ cells (Fig. 5C). Large complex colonies (>250 μm) were twice as common in both $D492^{SPRY2KD3}$ and $D492^{SPRY2\text{-}KD3A}$ compared to $D492^{SPRY2\text{-}NS}$ cells (Fig. 5D). All cell lines were cultured in three different cell concentrations (1.3×10^4, 1×10^4 and 7×10^3) 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 cultures 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 SPRY2 at D16 in $D492^{SPRY2\text{-}NS}$ and $D492^{SPRY2\text{-}KD3}$ cells we saw that the $D492^{SPRY2\text{-}NS}$ cells showed normal expression of SPRY2 at the lobular tips whereas the $D492^{SPRY2\text{-}KD3}$ cells showed markedly suppressed expression (Fig. 5F).

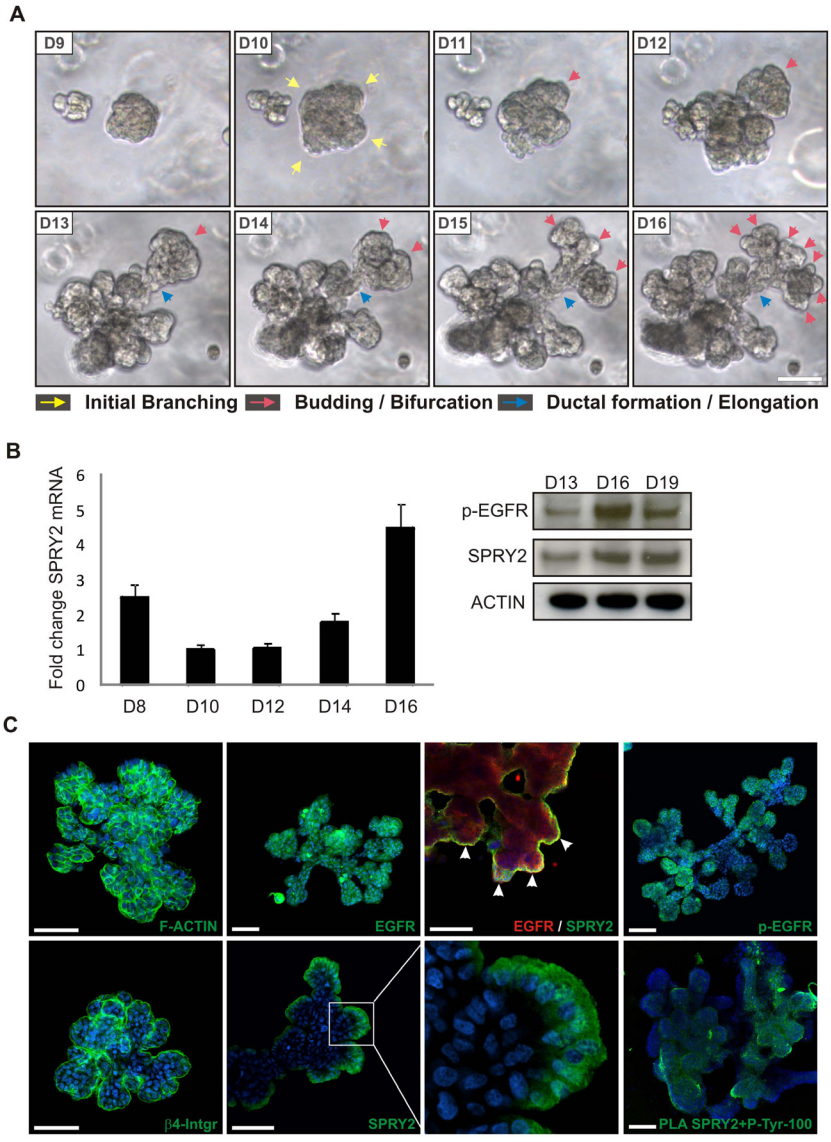

Figure 3. SPRY2 expression is correlated with critical points in branching morphogenesis of D492 breast stem cell line. *A) D492 cells generate branching structures when cultured in rBM.* When seeded in rBM D492 cells generate TDLU-like structures. By generating in vitro TDLU-like structures it is possible 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) SPRY2 expression shows a dramatic shift during TDLU formation in 3D culture.* Colonies were isolated from 3D cultures at different time points as indicated.

Initially at day 8 there is relative high expression of SPRY2 mRNA 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 pEGFR is slightly decreasing for day 16 to day 19. Actin was used as a loading control. *C) pEGFR and SPRY2 are expressed at the growing tips of TDLU-like structures.* D492-derived TDLU-like structures generated in 3D culture were stained with antibodies against SPRY2, EGFR, pEGFR, β4-integrin and F-actin. pEGFR was predominantly expressed at the branching tips while total EGFR had a more general distribution. SPRY2 was also expressed at branching tips but not in clefts. Co-staining of SPRY2 and EGFR show strong expression at the branching tips (arrows). F-actin staining gives a general outlook of a branching colony while β4-integrin outlines their connection to the surrounding rBM matrix. Phosphorylated SPRY2 (right) was analyzed using proximity ligation assay as described above. pSPRY2 was predominantly expressed at the branching tips showing similar pattern as total SPRY2. Cells were counterstained with TOPRO-3 nuclear stain. Bar = 100 μm.
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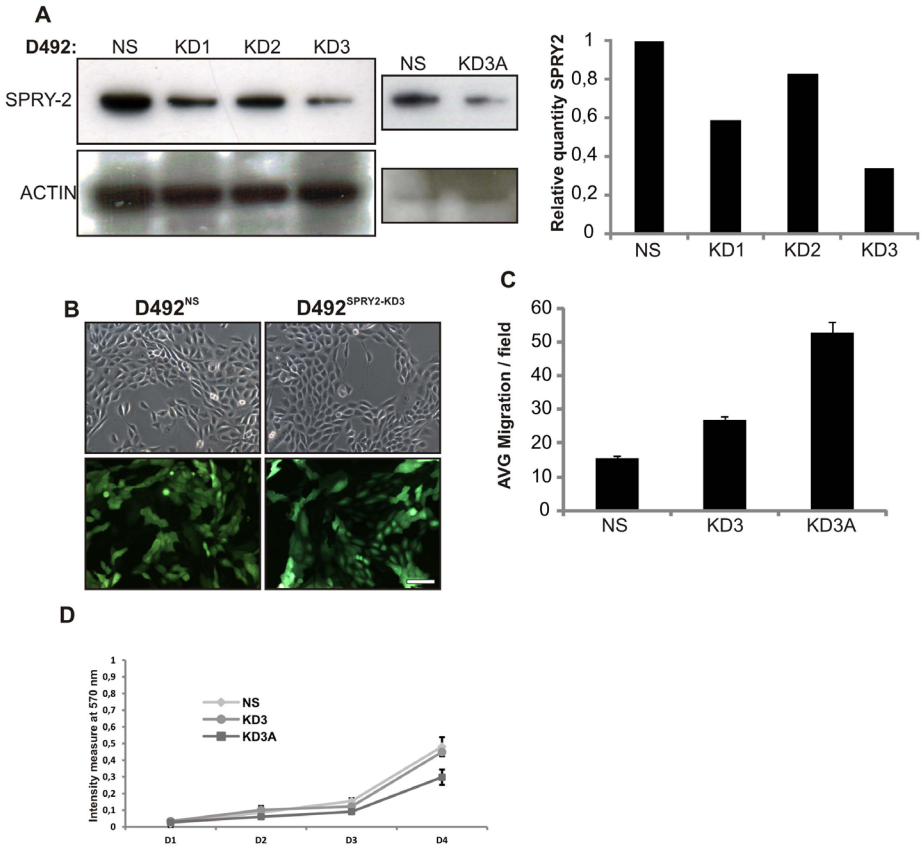

Figure 4. SPRY2 Knockdown in D492 breast epithelial stem cell line. *A) D492 cells show significant knockdown of SPRY2.* D492 were transfected with non-silencing (NS) shRNA and different version of knockdown (KD) shRNA against SPRY2. KD3 showed most efficient knockdown (70%) measured by western blot. KD3A is a single cell cloned subline from KD3. *B) D492SPRY2-KD3 retains an epithelial phenotype in monolayer culture.* No phenotypic differences were observed in monolayer of $D492^{NS}$ and $D492^{SPRY2\text{-}KD3}$ (upper row). Transfection efficacy was evaluated by GFP (lower row). *C) $D492^{SPRY2\text{-}KD}$ cells have acquired increased migration potential.* When plated on porous transwell filter $D492^{SPRY2\text{-}KD3}$ showed increased migration compared to $D492^{NS}$. Single cell derived clone KD3A from KD3 had the highest migration potential. *D) SPRY2 knockdown has no effect on cell proliferation*. Monolayer proliferation of $D492^{NS}$, $D492^{SPRY2\text{-}KD3}$ and $D492^{SPR2\text{-}KD3A}$ was evaluated at different time points, as indicated. There was no remarkable difference in the proliferation rate of the NS and KD cells, although at day 4 $D492^{SPRY2\text{-}KD3A}$ seemed to proliferate slightly less.
doi:10.1371/journal.pone.0060798.g004

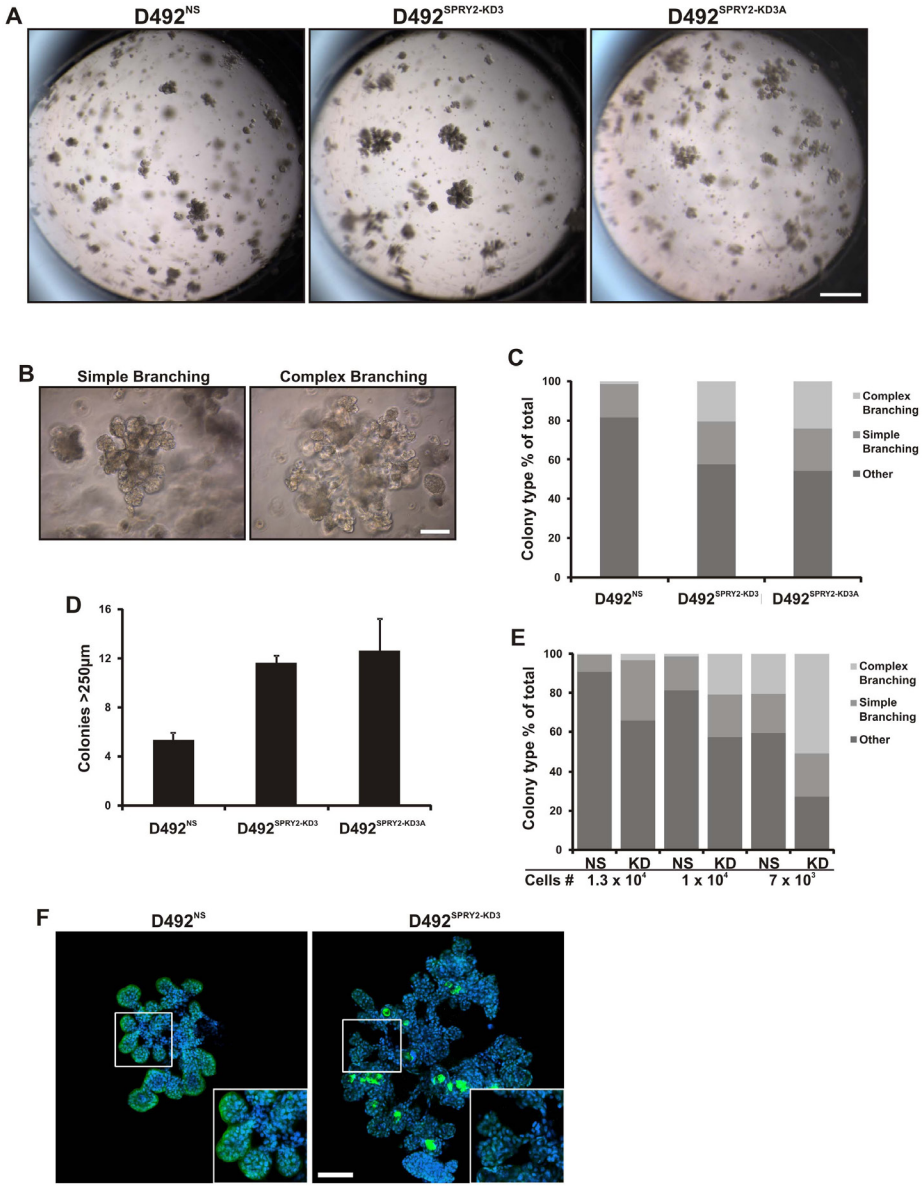
A
D492NS
D492SPRY2-KD3
D492SPRY2-KD3A
B
Simple Branching
Complex Branching
C
Colony type % of total
100
80
60
40
20
0
Complex Branching
Simple Branching
Other
D492NS
D492SPRY2-KD3
D492SPRY2-KD3A
D
Colonies >250µm
16
12
8
4
0
D492NS
D492SPRY2-KD3
D492SPRY2-KD3A
E
Colony type % of total
100
80
60
40
20
0
Complex Branching
Simple Branching
Other
NS KD NS KD NS KD
Cells # 1.3 x 10^4 1 x 10^4 7 x 10^3
F
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Figure 5. SPRY2 Knockdown in D492 promotes increased branching morphogenesis. *A) SPRY2-KD resulted in increased branching colonies in 3D culture.* Stereoscopic images of representative 3D rBM gels for $D492^{NS}$, $D492^{SPRY2-KD3}$ and $D492^{SPRY2-KD3A}$ showing increased branching upon SPR2 knockdown. Bar = 2mm. *B) Types of morphogenesis in 3D culture.* Epithelial colonies were divided into three morphotypes: simple branching, complex branching and other (mostly solid round). Representative images of simple- and complex branching are shown. *C) 3D morphogenesis of $D492^{NS}$, $D492^{SPRY2-KD3}$ and $D492^{SPRY2-KD3A}$ cells.* In a setup with 10^4 cells both $D492^{SPRY2-KD3}$ and $D492^{SPRY2-KD3A}$ cells showed an increase in simple- and complex branching pattern. Bar = 100 μm. *D) Large complex colonies in 3D rBM culture.* Complex branching colonies over 250 μm were counted. This showed a 2 fold increase in size of the SPRY2 KD cells. *E) 3D morphogenesis of $D492^{NS}$, $D492^{SPRY2-KD3}$ and $D492^{SPRY2-KD3A}$ cells with variable amount of cells.* In a setup using 1.3×10^4, 10^4 and 7×10^3 cells, the SPR2-KD cells showed superior branching abilities compared to NS cells. *F) SPRY2 expression in branching colonies from $D492^{NS}$ and $D492^{SPRY2-KD3}$.* As seen before SPRY2 was located at branching tips in NS cells. SPRY2 KD cells showed reduced expression of SPRY2 with some areas of diffuse staining. Bar = 100 μm.
doi:10.1371/journal.pone.0060798.g005

Endothelial cells stimulate EMT in $D492^{SPRY2-KD}$ cells

We have recently shown that endothelial cells support morphogenesis and improve clonal efficiency in both lung and breast epithelial cells [35,36]. Furthermore, we have shown that breast endothelial cells (BRENCs) induce EMT in D492 cells [37]. Interestingly, when D492 cells are co-cultured with BRENCs we also see marked stimulation in branching morphogenesis at clonal dilution (Fig. 6A). Branching TDLU-like structures in co-culture were generated from as few as 100 D492 cells in 300 μl rBM compared to the usual 10,000 cells used in monocultures 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. 6B). Dual immunostaining against cytokeratin 14 and CD31 demonstrates the perilobular location of endothelial cells surrounding the TDLU structures (Fig. 6B). Thus, TDLU-like colonies generated in co-cultures with BRENCs mimic TDLUs *in situ* with a bi-layered epithelium consisting of an inner layer of luminal epithelial cells, an outer layer of myoepithelial cells and an extralobular location of endothelial cells.

To see if SPRY2 KD cells responded differently to BRENCs we set up co-cultures with $D492^{SPRY2-NS}$, $D492^{SPRY2-KD3}$ and $D492^{SPRY2-KD3A}$ cells. $D492^{SPRY2-NS}$ formed 50% spindle-like (EMT) colonies and 40% branching colonies while $D492^{SPRY2-KD3}$ cells formed over 65% spindle-like colonies (Fig 6C and D). The $D492^{SPRY2-KD3A}$ clone which produced larger and a higher number of branching colonies in the monoculture was also used in the co-culture and interestingly they exclusively produced spindle-like colonies. The spindle-like colonies were similar to previously reported endothelial-induced EMT colonies [37] and this was confirmed by an E- to N-cadherin switch (Fig. 6C). The proliferation was similar in both branching and spindle colonies as evidenced by Ki67 expression. 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 possibly through inhibition of RAS/ERK pathway. SPRY2 knockdown cells might thus be prone to both increased branching and more susceptible to full EMT with formation of spindle-like EMT colonies under co-culture conditions.

Discussion

The sprouty protein family is increasingly recognized as a key regulator of receptor tyrosine kinase signaling in different species and organs where SPRY2 has captured most attention [43–45]. Furthermore, SPRY2 has been shown to be downregulated in a number of cancers, including breast cancer [29,30,46]. In this study, we have analyzed the expression of SPRY2 in the mouse and human mammary gland.

In the human breast gland SPRY2 was equally expressed in ducts and TDLU, and its expression was most prominent within luminal epithelial cells. We also demonstrate that the expression of total SPRY2 is low in virgin mouse mammary glands but is focally increased at branching tips during pregnancy and reaches maximum expression during lactation. The presence of phosphorylated SPRY2 was most profound during pregnancy where it seems to dampen signaling through RAS/MAPK pathway. In support of this the pERK1/2 levels increase during lactation when a reduction is seen in pSPRY2. Previously, Lo et al. [29] demonstrated by *in situ* hybridization that SPRY2 was highly expressed in pregnant mouse mammary glands but decreased during the lactating stage. In our study total SPRY2 expression was high at lactating stage but pSPRY2 level was low.

The functional role of sprouty in branching morphogenesis during trachea development in Drosophila was first demonstrated in spry-/- mutants which showed excessive branching [47]. Similarly, Tefft et al. [48] demonstrated that inhibition of SPRY2 expression in mouse embryos at E11.5 produced a significant increase in lung branching. Development of the uretic bud is another example of controlled branching morphogenesis that is regulated by sprouty proteins [43]. In the nephric duct, cells with high Ret tyrosine kinase receptor expression preferentially move to the dorsal nephric duct adjacent to the metanephric mesenchyme where they form the first uretic bud. Interestingly, SPRY1-/- mutants show elevated expression of RET and increased branching [49]. These data collectively demonstrate the regulatory role of sprouty proteins during branching morphogenesis in various epithelial organs.

Mouse studies have shown that the mammary organoid branches and migrates by bifurcation and collective migration [50]. Furthermore, end bud and TDLU formation requires growth factor induced cell proliferation and studies show that this cell proliferation is mediated through ERK1/2 [7,50]. These results are consistent with our data. D492 cells in 3D culture capture, by collective migration, the morphogenic process in the mammary gland, including the formation of TDLU like structures. SPRY2 expression was most prominent at the peripheral branching buds whereas it was significantly reduced in clefts/furrows both in *in vitro* 3D cultures of human breast epithelium and in the growing mammary gland in pregnant mice. The same expression pattern was also seen for p-EGFR. This indicates that the D492 cell line activates both EGFR and SPRY2 pathways during branching morphogenesis.

It is becoming clear that the stromal microenvironment plays a critical role in tissue morphogenesis of many organs, including the breast [51]. It is also widely acknowledged that cancer progression is dependent on signals from the surrounding microenvironment [52]. Fibroblasts and extracellular matrix molecules, such as laminin, fibronectin and extracellular matrix-entrapped growth factors have received much attention [28]. Our recent results demonstrate that endothelial cells stimulate growth and morphogenesis of breast epithelial cells [35] and induce EMT [37]. We have also shown that endothelial cells can induce bronchial epithelial cells with stem cell properties to generate bronchioal-

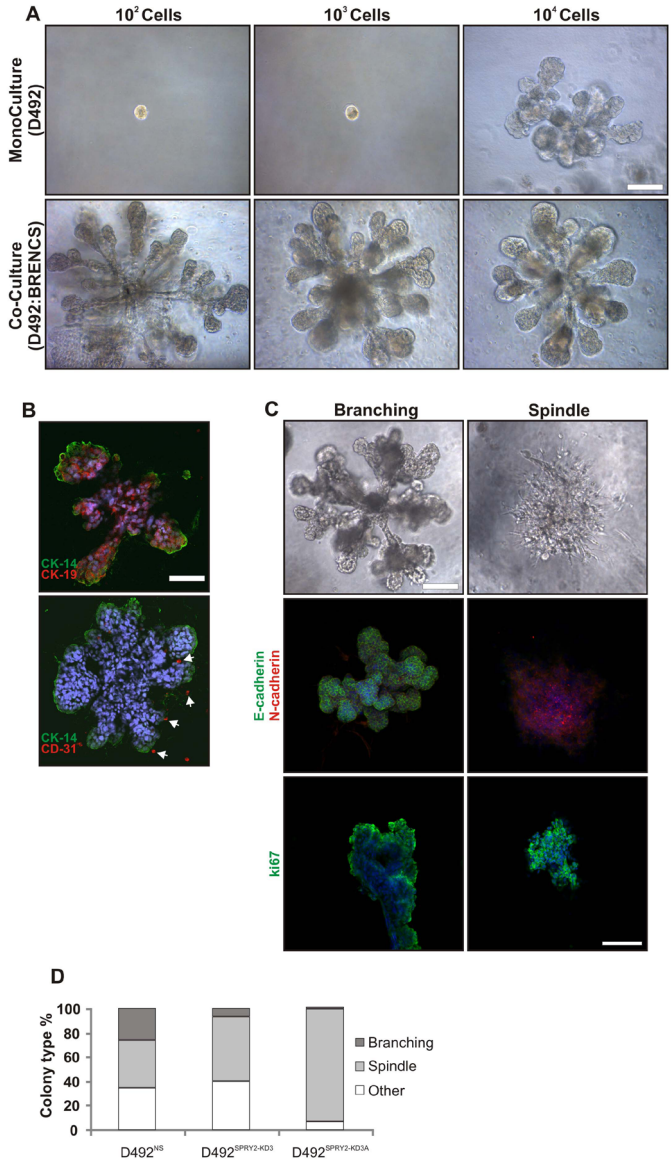

Figure 6. Epithelial integrity is disturbed in SPRY2 KD cells when co-cultured with endothelial cells. *A) Endothelial cells stimulate growth of D492 cells.* When plated in 3D rBM culture with breast endothelial cells (BRENCs), D492 cells can form complex branching colonies from as little as 100–1000 cells compared to 7×10^3 –10^4 in 3D monoculture. *B) D492-derived branching structures form bi-layered epithelium with BRENCs positioned extralobular.* The branching colonies are bi-layered and polarized structures as evidenced by the expression of the myoepithelial marker CK14 on the

outer side and the luminal epithelial marker CK19 on the inner side (upper figure). In co-cultures endothelial cells stay as single cells positioned outside the branching structures as seen with CD31 staining (lower figure, arrows). Bar = 100 μm. Sections counterstained with TOPRO-3 nuclear stain. *C) Phenotypes of D492 in co-culture with BRENCs.* In co-culture with endothelial cells D492 cells form both branching- and spindle-like colonies. The branching colonies show strong expression of E-cadherin, while spindle like colonies have undergone EMT as evidenced by cadherin switch from E- to N-cadherin. Staining for the proliferation marker ki67 shows that both the branching and spindle like colonies are viable and growing. Bar = 100 μm. Sections counterstained with TOPRO-3 nuclear stain. *D) Spry2-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 $D492^{Spry2\text{-}KD3}$ cells up to 65%. The $D492^{Spry2\text{-}KD3A}$ form almost exclusively spindle-like colonies in co-culture with endothelial cells.
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veolar branching structures in 3D culture [36]. Interestingly, in co-culture of endothelial cells and D492 we see a dramatic increase in TDLU formation. This further demonstrates the proliferative and morphogenetic induction potential of endothelial cells.

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 morphogenetic signals and under regulation of the microenvironment [53]. 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 SPRY2 expression in D492 we see hyperplasia-like effects and increased branching morphogenesis. Furthermore, when the SPRY2 knockdown cells are co-cultured with endothelial cells they showed increased EMT susceptibility. We have previously shown that endothelial-induced EMT in D492 is partially mediated by hepatocyte growth factor (HGF) [37]. The increase in EMT after SPRY2 knockdown suggests that SPRY2 might be critical in temporally regulating EMT during branching morphogenesis through modulation of RTK signaling.

Conclusion

Our data suggest that breast epithelial branching morphogenesis is regulated by SPRY2. Furthermore, our data indicate that SPRY2 is an important regulator of epithelial integrity as SPRY2 knockdown cells are prone to endothelial induced EMT.

Supporting Information

Figure S1 Expression of SPRY2 in virgin, pregnant and lactating gland. SPRY2 is present at all developmental stages in the adult mammary gland with highest expression seen during lactation. Actin used as a loading control.
(TIF)

Figure S2 SPRY2 knock down result in increased pERK activity. Knock down of SPRY2 result in approximately 20% increase in pERK activity. Actin used as a loading control.
(TIF)

Author Contributions

Conceived and designed the experiments: MKM TG VS SI. Performed the experiments: VS SI SMG BH AJA SRF. Analyzed the data: MKM TG VS SI. Contributed reagents/materials/analysis tools: ES. Wrote the paper: TG MKM VS SI.

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Paper IV

ErbB2 induced EMT and tumorigenicity is inhibited by EGFR in mammary epithelial cells with stem cell properties,

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Abstract

The members of the EGFR kinase family are important players in breast morphogenesis and cancer. EGFR and ErbB2 expression has been shown to have prognostic value in certain subtypes of breast cancer such as, basal-like, HER-2 amplified and luminal type B. These subtypes are highly metastatic and enriched with cancer stem cells. D492 is a breast epithelial cell line with stem cell properties that generates luminal- and myoepithelial cells and forms elaborate branching structures in 3D culture. Here, we show that overexpression of ErbB2 in D492 ($D492^{ErbB2}$) results in epithelial to mesenchymal transition (EMT) as evidenced by reduced expression of E-cadherin and keratins and gain of the mesenchymal markers such as N-cadherin and AXL. In contrast, overexpression of EGFR in D492 ($D492^{EGFR}$) drives differentiation towards myoepithelial phenotype when cultured in monolayer. The EMT phenotype of $D492^{ErbB2}$ is further exaggerated in 3D culture where cells form grape or spindle-like structures. When EGFR is overexpressed in $D492^{ErbB2}$ ($D492^{EGFR/ErbB2}$), we do not see any changes in phenotype in monolayer culture and cells retain their EMT phenotype. In contrast, in 3D culture, $D492^{EGFR/ErbB2}$ cells are reverted towards epithelial differentiation. When cells were injected into nude mice, $D492^{ErbB2}$ cells formed large tumors. In contrast $D492^{EGFR/ErbB2}$ cells formed smaller tumors with phenotype similar to cells in 3D culture. Our data indicate that in ErbB2 overexpressing D492 cells, EGFR can behave as a tumor suppressor, by pushing the cells towards epithelial differentiation. This is in concordance with tumor data showing that EGFR expression in ErbB2 expressing luminal B tumors is linked to increased survival.

Introduction

In the breast, tissue remodeling is a normal recurrent event. Active stem- and progenitor cells in the epithelium are controlling and maintaining the epithelial integrity during pregnancy, lactation, involution, as well as in each menstruation cycle (1-6). Breast epithelial stem- and progenitor cells has in recent years been in the spotlight as candidate targets for breast cancer initiation (7-9). Carcinomas of the breast can be classified by their intrinsic gene expression pattern, and the phenotype of the various tumor subgroups has been postulated to mimic the cell of origin, to an extent (10, 11). Researchers hypothesize that luminal cancers arise from the more differentiated luminal compartment, while the HER2 and triple negative sub groups of breast cancer might originate from the more undifferentiated luminal and basal-progenitor cells, respectively (12, 13).

Whether breast cancer originates in stem cells or differentiated cells that adopt stem cell like behavior is still under debate (14). The reason for this is partly the lack of *in vitro* culture systems modeling both normal breast stem cell biology, and neoplastic transformation. The D492 cell line is a breast epithelial cell line with stem cell properties. It was established by isolating and immortalizing a $MUC1^-$, $EpCAM^+$ suprabasal cell population from normal primary tissue (15). D492 cells form branching structures resembling the terminal duct lobular units (TDLUs) when grown in three dimensional (3D) organotypic cultures, i.e. they differentiate into both luminal- and myoepithelial cells. Furthermore, these cells respond to microenvironmental signals and undergo EMT (16). EMT is a pivotal step during cancer progression where cells gain increased motility, lose epithelial characteristics such as cytokeratins as well as E-cadherin, and increase expression of mesenchymal markers, such as vimentin, fibronectin and N-cadherin (reviewed by (14)). Changes

to microRNAs (miR) expression, such as the miR-200-141 cluster have also been implicated with EMT in breast cancer (17, 18). Given the stem cell nature of this cell line, it has a unique potential to model the effect of critical signaling molecules on cellular morphogenesis, differentiation, EMT and neoplastic transformation.

Dysregulation of the EGFR receptor family activity are oncogenic in epithelial cells, and amplification of the ErbB2 gene is especially injurious in breast epithelium where upregulation of ErbB2 can increase cell motility and promote metastasis (19). These receptors are also key components of correct polarization and development of the breast gland (reviewed by (20)).

The clinical effects of ErbB2 and EGFR dysregulation is well known, and a range of options for treatment of their adverse effects exist (21). New breast cancer treatment regimes have, however, revealed new clinical challenges. Development of resistance against EGFR family targeted therapy is common, and in the HER2 breast cancer subgroup, systemic disease is now well controlled, while metastatic disease in the CNS seems to escape both targeted and conventional therapy (22). Increased knowledge on the cellular effects of unbalanced HER2 and EGFR expression in breast epithelial cells is obviously needed. The overall objective of this study was therefore to elucidate the functional consequences of HER2 and EGFR overexpression in multipotent breast epithelial cells. 3D *in vitro* models and *in vivo* tumorigenicity assay were employed to measure changes in phenotype, stemness and tumorigenicity. ErbB2 overexpressing D492 cells acquired *in vivo* tumorigenic capacity accompanied by loss of epithelial morphogenic and molecular properties including branching morphogenesis and expression of epithelial genes. overexpression of EGFR in ErbB2 expressing cells did partially reverse the Her2 induced mesenchymal transition allowing the cells to retain epithelial properties.

Moreover, EGFR overexpressing D492 cells, on the other hand, showed myoepithelial differentiation, retained the branching potential of the parental D492 cells and generated smaller tumors in NOD/SCID mice than D492^{ErbB2}. This study emphasizes the importance of understanding the context driven signaling effects mediated by tyrosine kinase receptors acting individually or in concert on cellular differentiation in both normal and cancerous breast.

Materials and Methods

Cell culture

D492 cells were maintained in H14 media as described in (1) Cells were maintained of tissue culture treated, collagen I (Advanced Biomatrix) coated T25 Falcon flasks (BD Biosciences). The cells were subcultured weekly in a 1:20 ratio on new flasks. Cells were fed three times per week.

Immunohistochemistry

Cryosections from fresh-frozen tissue samples from reduction mammoplasties (Icelandic National Bioethics Committee No. VSN-13-057) or three dimensional experiments were fixed in 3.5% formaldehyde for 15 minutes and permeabilized with 0.1% Triton-X for 10 minutes. Samples were blocked using 5% Normal Goat serum (Life Technologies) and incubated with primary antibody overnight at 4°C. Samples were then stained with isotype specific secondary fluorescent Alexa Fluor goat antibodies (Life Technologies) and counterstained with TO-PRO-3 (Life-Technologies). Samples were visualized using a Zeiss 510 LSM Pascal confocal microscope (Carl Zeiss).

Sections from paraffin embedded mouse tissue was processed in xylene and ethanol to remove paraffin, and then rehydrated in dH_2O. After rehydration, samples were stained as described above.

Antibodies used for Immunostaining/western blotting

The following antibodies were used in this study: CK14 (Leica Microsystems, LL-002 and abcam, ab 15461), CK19 (abcam, ab7754), Actin (abcam ab8229), GAPDH (abcam, ab9484) p63 (abcam, ab3239), E-cadherin (BD, BD610682), N-cadherin, (BD, BD610921) P-cadherin (Cell Signaling, #2130), EGFR (CS#4267), EGFR pY1068 (CS#3777), EGFR pY1173 (CS#4407), ErbB2 (CS#2165) ErbB2

pY1221/1222 (CS#2243), Axl (CS#8661), Phalloidin (Life Technologies, A22283, A12379).

Viral transduction

All vectors used for viral production or cloning were acquired from Addgene (Cambridge, MA). pBABE-EGFR and empty backbone (#11011, #1764, respectively) were used as it was. ErbB2 coding sequence (#16254), was cloned into the lentiviral vector pWPI (#11254). Retroviral (EGFR) virus production was performed in Phoenix HEK293 cells, using Arrest-In transfection (Thermo-Scientific). D492 cells were infected overnight with viral supernatant containing 8μg/ml Polybrene (Sigma). EGFR transduced cells were selected using 2μg/ml Puromycin (Sigma-Aldrich). Lentiviral plasmids were transfected into HEK293T cells using ArrestIn, and D492 cells were infected with viral supernatant containing 8μg/ml polybrene. ErbB2 over-expressing cells were selected based on eGFP expression in FACSaria (BD-Biosciences). Control cells were transduced with both empty vector backbones for EGFR and ErbB2.

Primary cultures.

Primary normal human mammary tissue was processed as described in (23). Luminal epithelial cells were sorted by positive selection for MUC-1 (biogenesis, 1510-5025) and EpCAM (Leica, NCL-ESA) using magnetic cell sorting (Miltenyi biotech), whereas myoepithelial cells were selected for using Thy-1 (Dianova, DIA100) and beta 4 integrin (Millipore, MAP1964) selection.

3D assays

For 3D assays, cells were trypsinized and seeded into Growth Factor Reduced Matrigel matrix (BD Biosciences). 10^4 cells were seeded into 300μl Matrigel and maintained over 15 days. Culture media (H14) was changed thrice per week. After

the culture period, cultures were either frozen in chilled N-hexane for cryosectioning, or processed for protein extraction or immunostaining of isolated colonies.

Proliferation assay

Cells were seeded in triplicate into collagen coated 24 well trays at a density of 10.000 cells per well in H14 media. Daily, cells were fixed in 3.5% formaldehyde and stained with 0.1% Crystal violet. After the culture period (10 days) wells were de-stained using 10% acetic acid and crystal violet absorbance measured at 570nm.

Western blotting

Protein lysates were acquired using RIPA lysis buffer supplemented with both phosphatase and protease inhibitor cocktails (Thermo Scientific). For western blots, 5µg protein was used per lane, unless otherwise stated. Samples were denatured using 10% mercaptoethanol and run on NuPage 10% Bis-Tris gels (Invitrogen) in MES running buffer. Samples were then transferred on to Immobilon FL PVDF membranes (Millipore). Membranes were blocked in Li-cor blocking buffer and primary antibodies were incubated overnight at 4°C. Near-IR fluorescence visualization was measured using Odyssey CLx scanner (Li-Cor).

Low attachment assay

For Low attachment assays, 6 well trays were coated with 1.2 mg/ml Poly-HEMA (Sigma) overnight at 37°C. Cells were seeded at a density of 10^3 per well, and cultured for two weeks. After the culture period, colonies were imaged and counted.

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 EGFR (Hs00540086_m1, AB), ErbB2 (Hs01076092_m1, AB), ZEB1

(Hs00232783_m1) and GAPDH (Hs99999905_m1). Experiments were done in triplicate on 7500 Real Time PCR System (Applied Biosystems). EGFR, ErbB2 and ZEB1 mRNA levels were normalized to GAPDH and relative mRNA differences was calculated with the $2^{\Delta Ct}$ Method.

Quantitative RT-PCR analysis of miRNAs was performed using miRCURY LNA™ microRNA PCR System (Exiqon). Gene expression levels were quantified using primers for hsa-miR-141 (#204504) and hsa-miR-200c (#2044852)(Exiqon). Normalization was done with U6 RNA(#203907)(Exiqon).

NOD/SCID Xenografts

The *in vivo* tumorigenicity assays were performed using NOD/SCID interleukin-2 receptor gamma chain null (Il2rg-/-) (NSG) mice. The animals were randomized into four groups according to size, and $5x10^5$ cells were injected bi-laterally in 100µl PBS/matrigel Mice were kept under pathogen-free conditions, at constant temperature (21.5±0.5 ºC) and humidity (55±5%), 20 air changes/hr and a 12 hr light/dark cycle. Distilled tap water was given *ad libitum*, supplemented with 17-β-estradiol at a concentration of 4 mg/l. All mice used in the experiment were locally bred at the animal facility at Oslo University Hospital, Oslo, Norway. All procedures and experiments involving animals were approved by the National Animal Research Authority (http://www.fdu.no/), and were conducted according to the regulations of the Federation of European Laboratory Animal Science Association (FELASA), and all efforts were made to minimize suffering.Tumors were measured three times weekly, and after the experimental period, tumors were formalin fixed and embedded in paraffin.

Results

EGFR and HER2 show distinct expression pattern in the human breast epithelium

Although, EGFR and HER2 have been intensively studied in terms of development and cancer there are few studies that that show their co-expression pattern in the normal breast. To pattern the expression of these critical tyrosine kinases in the normal human breast epithelium, we initially stained normal breast tissue with antibodies against CK19 and CK14 to identify luminal epithelial- and myoepithelial cells, respectively (Fig. 1A). Co-staining of EGFR and ErbB2 with either CK19 or CK14 revealed distinct expression pattern with EGFR expression associated with the basal/myoepithelial compartment and HER2 expression with the luminal epithelial cells (Fig. 1B). In rare instances, basally located HER2 positive cells, and luminally located EGFR cells could be seen (Fig. 1B, arrows). We also isolated luminal epithelial- and myoepithelial cells from primary cultures and immunoblotting for EGFR and ErbB2 demonstrated higher expression of ErbB2 in luminal epithelial cells compared to higher expression of EGFR in myoepithelial cells (Fig. 1C).

Overexpression of ErbB2 in D492 leads to reduction of endogenous EGFR expression and EGF independent activation of EGFR and ErbB2

To analyse the oncogenic and transforming effect of ErbB2 on breast epithelium, we introduced ErbB2 into D492. Corresponding to the basal-like properties of D492 cells, they express very low levels of ErbB2 (Figure 2A and B). ErbB2 transduction ($D492^{ErbB2}$) gave a strong increase in ErbB2 expression at both protein (Fig.2A) and transcriptional level (Fig. 2B). Interestingly, endogenous EGFR expression was greatly reduced in the $D492^{ErbB2}$ cells (Figure 2A and B). Transduction of EGFR into

$D492^{ErbB2}$ ($D492^{EGFR/ErbB2}$) partially restored EGFR levels as seen in the immunofluorescence images (Fig. 2A). These results were further confirmed by WB (Supplementary Fig. S1A). qRT-PCR was performed to confirm that the reduced EGFR levels was caused by transcriptional repression of EGFR mRNA. EGFR transcription could be rescued in $D492^{ErbB2}$ cells by retroviral transduction of EGFR. Transduction of EGFR alone ($D492^{EGFR}$) showed a modest increase in both protein and RNA levels (Fig. 2A and B). ErbB2 is known to convey signaling through formation of heterodimers with EGFR. It was therefore of interest to investigate whether overexpression of ErbB2 led to changes the activation state of the two receptors. Cells were starved, and then treated with EGF for up to three hours. As expected, in the absence of EGF, $D492^{ctrl}$ and $D492^{EGFR}$ show no phosphorylation of either tyrosine (Y)1173 or 1068 on EGFR. In contrast, while strong EGFR downregulation can be seen in $D492^{ErbB2}$, the cells show detectable $EGFR^{Y1068}$ and $ErbB2^{Y1221/1222}$ phosphorylation, demonstrating EGF independent activation of both receptors (Fig. 2C). Upon addition of EGF, strong phosphorylation at Y1173 and Y1068 was detected after 3 and 30 minutes in $D492^{ctrl}$ and $D492^{EGFR}$ cells, respectively (supplementary Fig. S1B). This signal is maintained after three hours (Fig. 2C). Interestingly, in $D492^{ErbB2}$ cells, the response to EGF treatment is much less pronounced, presumably in part due to the reduced EGFR levels. Phosphorylation of Y1068 shows minimal change, and Y1173 phosphorylation returns to baseline levels within three hours. Phosphorylation of ErbB2 can be detected in ErbB2 expressing cells only, and does not respond to EGF treatment. These data demonstrate that ErbB2 overexpression in D492 makes cells EGF independent and nonresponsive to EGF, and that the cell line model mimics both the ligand dependency of EGFR signaling, and the ligand independent Her2 signaling.

ErbB2 induces epithelial to mesenchymal transition in D492 cells

The effect of EGFR and ErbB2 overexpression on cellular differentiation was evaluated by analyzing the expression pattern of various epithelial markers in the three transduced cell lines, and compared to control D492 cells. $D492^{ErbB2}$ cells show near complete disappearance of CK14 and CK19, E-cadherin and P-cadherin. In $D492^{EGFR/ErbB2}$ partial recovery of the four markers was seen. $D492^{ctrl}$ and $D492^{EGFR}$ show a heterogeneous expression pattern of CK19 and CK14 with most cells being either CK19 positive or CK14 positive while a smaller subpopulation was double positive (Fig. 3A). N-cadherin staining was not observed. In $D492^{EGFR}$ no change in E-cadherin expression was seen, but expression of P-cadherin was increased, suggesting increased myoepithelial differentiation. To further look at the differentiation we compared luminal-, myoepithelial and mesenchymal markers on western blotting. The loss of epithelial markers was clearly visible on western blotting in both $D492^{ErbB2}$ and $D492^{EGFR/ErbB2}$ cell lines as evidenced by reduced expression of P63, CK14 and CK19 as well as E- and P-cadherins. In contrast, these cells showed increased mesenchymal traits as shown by N-cadherin and Axl expression, markers that both are linked to EMT (Fig. 3B). $D492^{EGFR}$ show an enhanced basal/myoepithelial differentiation, as evidenced by stronger CK14, P63 and P-cadherin staining and weaker CK19 staining (Fig. 3B). Based on these data, we analysed the transcription levels of the miR-200-141 cluster in the D492 sublines. Compared with $D492^{ctrl}$, both miR-200c and miR141 were greatly downregulated in $D492^{ErbB2}$ cells (Fig. 3C), accompanied with upregulation of ZEB1, a mesenchymal transcription factor. Constitutive expression of EGFR, with or without ErbB2 resulted

in increased miR-200-141 expression levels, and reduced ZEB1 (Fig. 3C). Collectively, we have shown that the tyrosine kinases EGFR or ErbB2 have distinct effects on the differentiation pathways in the breast epithelial progenitor cell line, D492. ErbB2 overexpression causes a loss of EGFR, cytokeratin and E-cadherin expression and increase in mesenchymal markers reminiscent of EMT, EGFR expression leads to a myoepithelial differentiation and partial rescue of the EMT induced by ErbB2.

Expression of ErbB2 leads to non-adherent growth

To study the effects of ErbB2 and EGFR overexpression in D492 on proliferation we carried out a proliferation assay on $D492^{ctrl}$, $D492^{EGFR}$, $D492^{ErbB2}$ and $D492^{EGFR/ErbB2}$. When $D492^{ctrl}$ cells reach confluency they show contact inhibition. The same pattern holds for $D492^{EGFR}$ (Fig. 4A). In contrast, $D492^{ErbB2}$ and $D492^{EGFR/ErbB2}$ cells continue to proliferate after reaching confluence, forming ridges and piling up in culture (Fig. 4A). Furthermore, colonies of viable cells could be seen floating in the culture medium, indicating a loss of adhesion and resistance to anoikis (not shown). We therefore seeded cells into low-attachment plates and quantified survival based on colony formation (Fig. 4B). In this low attachment environment, a subset of D492 cells is able to survive and proliferate, forming small spheroid colonies in suspension (Fig. 4B, GFP images). $D492^{EGFR}$ does not have significantly higher number of spheroids formed compared to D492, but colony size is significantly increased. $D492^{ErbB2}$ and $D492^{EGFR/ErbB2}$ show greatly increased colony formation; in addition, colonies are not spherical with robust cell-cell adhesion as seen in control/EGFR cells, instead colonies appear to be grape-like aggregates. (Fig. 4D, bottom GFP

images). These data indicate unresponsiveness to growth inhibition signals, suggesting increased malignant potential.

ErbB2 disrupts colony integrity and induces EMT in 3D culture

The HER2 induced phenotypes seemed to render the D492 cells less sensitive to inhibition signals from the microenvironment, and this was further demonstrated in 3D culture. When cultured alone, both $D492^{ctrl}$ and $D492^{EGFR}$ form predominantly colonies with a solid epithelial phenotype with robust cell-cell adherence. Colonies are either solid round or branching (Fig. 5A). These colonies demonstrate robust expression of epithelial markers such as keratins 14 and 19, and E-Cadherin (Fig. 5B). In the same assay $D492^{ErbB2}$ cells form colonies with a diffuse phenotype containing both grape-like and spindle colonies These colonies do not express keratins 14 and 19, or E-cadherin. Branching- and solid colonies were virtually absent in $D492^{ErbB2}$ cultures (Fig. 5A). Next, to see whether ErbB2 receptor signaling was crucial for formation of the EMT colonies in 3D, cultures were treated with the ErbB2 inhibitor CP-724,714 (CP). Spindle colony formation was completely inhibited in all cells when CP-treated, while grape-like colonies were still seen. Furthermore, CP-treated, $D492^{ErbB2}$ cells now also formed solid round colonies not seen in without ErbB2 inhibition. Re-introduction of EGFR expression in $D492^{EGFR/ErbB2}$ cells on led to the formation of a large number of solid colonies in addition to grape-like and spindle colonies. These colonies demonstrated the re-appearance of keratin and E-cadherin expression as evidenced by western blotting (Fig. 5C), opposite of what was seen in monolayer lysates (Fig. 3B). Additionally, N-cadherin was detected in $D492^{ctrl}$ and $D492^{EGFR}$, while none was detected in monolayer, further emphasizing the differences between 2D and 3D environment. These data indicate that EGFR partially

inhibits this EMT phenotype with re-appearance of keratins and E-cadherin, in addition to solid epithelial morphology in colonies.

D492^{ErbB2} tumorigenicity in NOD/SCID mice is partially suppressed in D492$^{EGFR/ErbB2}$

To test whether EGFR, ErbB2 or double overexpression of these two receptors in D492 affects cell growth and behavior in vivo, we injected cells into fat pads of NOD/SCID mice. One week post injection 3/6 and 2/5 injections of D492^{ctrl} and D492^{EGFR} cells had palpable tumors, respectively (Fig. 6A); six weeks after injection tumors were detected at 5/6 and 5/5 injections, respectively. D492^{ErbB2} and D492$^{EGFR/ErbB2}$ led to palpable tumors in all injections from week one. Tumor size varied greatly among the subtypes of D492. Both D492^{ctrl} and D492^{EGFR} had limited tumor growth throughout the observation period (Fig. 6B) D492^{ErbB2} alone led to large tumor volumes over 250mm^3 after 6 weeks. Interestingly, D492$^{EGFR/ErbB2}$ formed smaller tumors than D492^{ErbB2} indicating that EGFR might inhibit ErbB2 tumor growth in these mice (Fig. 6B). During the first month post injection, limited growth was seen in D492$^{EGFR/ErbB2}$ tumors; afterwards, proliferation increased. Supplementary Fig. S2A shows the growth of individual tumors.

Tumor samples were then prepared for immunohistochemistry. Figure 7A shows whole mount mosaic images of representative D492^{ErbB2} and D492$^{EGFR/ErbB2}$ tumors, black boxes denote the area used for Immunofluorescent imaging. Immunostainings of tumors show that the phenotype correlates well to the 3D *in vitro* phenotype (Fig. 7B). D492^{ErbB2} cells express ErbB2 but have low EGFR expression. Furthermore, they are CK14 negative demonstrating lack of myoepithelial differentiation and are E-cadherin and ck19 negative (Supplementary Fig. S2B). In contrast, D492$^{EGFR/ErbB2}$ form tumors that are heterogenous in terms of marker expression. These tumors are

positive for both ErbB2 and EGFR with ErbB2 expressed in all tumor cells. In contrast CK14 expression is restricted to EGFR expressing cells, thus indicating retention of epithelial characteristics within these tumors (Fig. 7B) Additionally, E-cadherin and CK19 staining were seen in EGFR expressing cells (Supplementary Fig. S2B). Collectively, these data show that co-expression of EGFR and ErbB2 result in slower tumor growth than in ErbB2 alone and suggest that EGFR might suppress the ErbB2 tumor phenotype, potentially through maintaining the epithelial phenotype in these cells.

Discussion

In this paper we show that EGFR and ErbB2 are expressed in luminal breast epithelial- and myoepithelial cells, respectively. Overexpressing these two oncogenes in D492 breast epithelial stem cell line resulted in changed phenotype. $D492^{ErbB2}$ undergoes EMT as evidenced by loss of epithelial behavior and expression pattern and gain of mesenchymal marker expression accompanied by formation of spindle-like colonies in 3D culture. Furthermore, $D492^{ErbB2}$ proliferate beyond confluence, and showed increased low attachment survival. When injected into mice, $D492^{ErbB2}$ demonstrated greatly increased tumor volume compared to $D492^{ctrl}$ and $D492^{EGFR}$. Overexpression of EGFR in $D492^{ErbB2}$ ($D492^{EGFR/ErbB2}$) had limited effects in monolayer but resulted in a partial reversion of the EMT phenotype in three-dimensional culture. Furthermore, $D492^{EGFR/ErbB2}$ generated smaller tumor volumes than $D492^{ErbB2}$ and showed a mixed luminal/myoepithelial phenotype along with the EMT phenotype. $D492^{EGFR}$ adopts increased myoepithelial differentiation while maintaining the branching potential in 3D culture. These data indicate that EGFR may act as a tumor suppressor when co-expressed with ErbB2 by retaining or inducing epithelial differentiation.

Understanding the lineage relationship between luminal- and myoepithelial cells in the human breast gland is important to be able to understand how the normal gland is maintained and different subgroups of breast cancer occur and how different clinical outcomes are related to the particular phenotype. In the ErbB2/HER2 tumor group, ErbB2 is amplified. however, expression (although not amplified) of ErbB2 can also been found in other subtypes such as luminal B and even TNBC (24).

Luminal A and B cancers are both ER/PR positive but in contrast to Luminal A, a large number of Luminal B tumors express ErbB2. Luminal B tumors, although

positive for ER, do not respond to tamoxifen treatment and have worse prognosis than Luminal A tumors (25). Balk-Møller and colleagues have recently demonstrated that HER2 rich tumors and luminal tumors likely share a common origin (26). This correlates well with the fact that ErbB2 is predominately expressed in the luminal epithelial compartment (Fig. 1).

There are a number of studies that show that myoepithelial cells can act as a tumor suppressor in the breast gland (reviewed by (27)). Myoepithelial cells have been shown to maintain the correct polarity of luminal epithelial cells by providing basement membrane proteins such as laminins (15). In cancer, the transition from ductal carcinoma in situ to invasive carcinoma is among other things characterized by the progressive loss of myoepithelial cells (28). Forster et al. have recently shown that P63 positive myoepithelial cells are important regulators of epithelial integrity in the breast gland. Loss of P63 resulted in failure of luminal epithelial cells to lactate. They demonstrated that this was due to failure of myoepithelial cells to express the EGF family member neuregulin (29).

The heterogeneous expression of luminal and myoepithelial markers within different subtypes of breast cancer indicates that stem cells or progenitor cells may be critically involved in these tumors. Stem cells are of particular importance in the adult cycling breast gland where they are responsible for renewal of luminal and myoepithelial cells after each menstruation cycle and for the massive branching morphogenesis observed during pregnancy (6). Branching morphogenesis is a partially invasive phenomenon where epithelial tissue use collective migration to invade the surrounding mesenchyme (30). The branching process requires that the invading epithelium acquire a partial EMT phenotype (31). The EMT process has been captured by many invasive cancer types. This process is seen as escape way

for cancer cells into the adjacent tissue and subsequently into the lymphatic or blood system (31, 32). It is, however, important to discriminate between partial EMT where cells retain large part of their epithelial properties and complete/full EMT where epithelial cells lose their properties and adopts a complete mesenchymal differentiation. The latter is one of the hallmarks of cancer (8). EGFR is one of the key markers used to identify Basal-like breast cancer within TNBC, along with CK5/6 (33). EGFR has several known ligands that share EGF-like binding site. This includes EGF, Amphiregulin (AREG) and TGF-a. Interestingly, Pasic et al (3) have shown that EGF and AREG have distinct effects on breast epithelial organoids in 3D matrigel culture. EGF induces myoepithelial expansion while AREG support duct elongation. This is in line with our data showing that EGFR facilitates myoepithelial differentiation. We have also previously shown that EGFR amplification results in more pronounced basal/myoepithelial phenotype (34). In contrast to EGFR, ErbB2 has no known ligands. It rather heterodimerizes with other EGF receptors, preferably EGFR and becomes phosphorylated and activated.

ErbB2 overexpression has been shown to favor luminal differentiation in mouse models (35). The changes in EGFR expression in our model following ErbB2 overexpression are not due to increased receptor breakdown, but rather due to reduced transcription, as evidenced by qRT-PCR (Fig. 3). D492 cells are dependent on EGF in the culture media. Omission of EGF results in greatly reduced cell proliferation. Y1068 of EGFR has been shown to be linked with increased MAPK signaling (36). Y1173, on the other hand has been shown to be associated with attenuation of EGFR downstream signaling by binding SHP-1 (37). Phosphorylation of EGFR is virtually absent in D492 cells after starvation, but reaches high levels upon EGF stimulation, and is maintained for long periods. Both Y1068 and Y1173

are phosphorylated in $D492^{ctrl}$ and $D492^{EGFR}$. Overexpression of ErbB2 caused an increase in Y1068 phosphorylation, independent of EGF stimulation and reduced Y1173 phosphorylation, indicating a sustained activation of EGFR. Taking into account the reduced total level of EGFR, these findings become even more pronounced.

The loss of keratin and epithelial cadherins following ErbB2 overexpression has been described before (38-40). In monolayer experiments, ErbB2 was dominant over EGFR as evidenced by EMT phenotype. Interestingly, in three-dimensional assays, EGFR partially reversed the EMT phenotype caused by ErbB2. Phenotypic reversion of $D492^{EGFR/ErbB2}$ was only seen in 3D culture demonstrating the importance of the microenvironment.

In our model, overexpression of ErbB2 results in reduced expression of EGFR. One can speculate if the loss of EGFR, which we know to be a driver of myoepithelial differentiation results in loss of myoepithelial differentiation in our cell line, therefore allowing the more invasive ErbB2 expressing cells to proliferate unhindered. When we injected our cells into nude mice, all cell types formed palpable lesions, including the control $D492^{ctrl}$ cells. On the other hand, the time until palpable tumors were found was much shorter in $D492^{ErbB2}$ and $D492^{EGFR/ErbB2}$. Tumor growth was greatly increased in $D492^{ErbB2}$ cells compared $D492^{ctrl}$ and $D492^{EGFR}$. $D492^{EGFR/ErbB2}$ resulted in smaller tumors compared to $D492^{ErbB2}$. In addition, keratin expression coincided completely with EGFR expression within the tumor. The reasons for areas of low EGFR expression within $D492^{EGFR/ErbB2}$ tumors are unclear, but the delay in tumor growth for the first month might indicate some selection within the tumors, perhaps selecting for loss of EGFR, and subsequent loss of keratins and cadherins.

In this paper, we have introduced a novel and important role for EGFR in breast morphogenesis and cancer. We have shown that EGFR epithelial characteristics and if expressed in defined context with ErbB2 may act as tumor suppressor by maintaining epithelial integrity. These data indicate that in certain tumors, where EGFR expression is present, inhibiting the receptor might lead to changes in marker expression and loss of epithelial characteristics; emphasizing the importance of context when devising treatment strategies.

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Figure 1. Expression of EGFR and ErbB2 in normal human breast gland

A) *Luminal and myoepithelial cells are identified by expression of CK19 and CK14, respectively.* CK19 is predominately expressed by luminal epithelial cells (red) while CK14 is expressed in myoepithelial cells. Bar=50μm.

B) *EGFR and ErbB2 are expressed in myoepithelial- and luminal epithelial cells, respectively*. EGFR and CK14 are co-expressed in myoepithelial cells and ErbB2 is localized with CK19 in luminal epithelial cells. Occasional staining of EGFR is seen in Luminal cells (arrow, top right). Rare ErbB2 expression can also be found within basal/myoepithelial cells (arrows, bottom), Bar=50μm Left. Western blot showing expression of EGFR and ErbB2 in purified myoepithelial and luminal epithelial cells. GAPDH = loading control.

Figure 2 - ErbB2 overexpression causes a drop in EGFR expression

A) *Expression of endogenous EGFR in D492^{ErbB2} is reduced compared to D492^{ctrl}.* All cell lines (D492^{ctrl}, D492^{EGFR}, D492^{ErbB2} and D492$^{EGFR/ErbB2}$) were analyzed by immunofluorescence staining for EGFR and ErbB2 expression. ErbB2 expression suppressed both endo- and ectopic levels of EGFR. Bar=50μm.

B) *ErbB2 overexpression leads to reduced EGFR transcription levels.* ErbB2 partially suppresses EGFR transcription. Where ErbB2 is overexpressed, transcription of native EGFR is reduced, which is reversible by transducing EGFR into D492^{ErbB2} cells (D492$^{EGFR/ErbB2}$) Actin = loading control.

C) *ErbB2 overexpression leads to ligand independent EGFR and ErbB2 phosphorylation.* Cells were starved for 24 hours in media without EGF. Cells were then given EGF containing culture media. Protein lysates were collected

at +180 minutes and blotted for total and phosphorylated EGFR and ErbB2. Limited EGFR phosphorylation can be seen in $D492^{ctrl}$ and $D492^{EGFR}$. Upon EGF stimulation strong EGFR pY1173 and pY1068 bands can be seen, and maintained beyond 180 minutes. ErbB2 expression in D492 leads to a decrease in ligand induced EGFR phosphorylation. Phosphorylation of ErbB2 seems to be ligand independent and nonresponsive. $D492^{EGFR/ErbB2}$ show strong ligand independent EGFR phosphorylation

Figure 3 – ErbB2 overexpression leads to gain of mesenchymal phenotype.

A) *$D492^{ErbB2}$ cells lose epithelial phenotype in monolayer culture.* Keratin and cadherin expression is reduced in $D492^{ErbB2}$ and $D492^{EGFR/ErbB2}$. $D492^{ErbB2}$ cells show reduced expression of both CK19 and CK14. $D492^{ErbB2}$ demonstrates a near-complete dysregulation of E- and P-cadherin staining pattern. $D492^{EGFR/ErbB2}$ partially reverses this, as cadherin signal can be seen at cell periphery, in addition to keratin positive cells. D492 largely stains positive for E-cadherin, with a mixed staining pattern of P-cadherin. $D492^{EGFR}$ show increased P-cadherin expression, compared with $D492^{ctrl}$. N-cadherin staining is limited in all cells. Bar = 50µm

B) *$D492^{ErbB2}$ and $D492^{EGFR/ErbB2}$ gain mesenchymal phenotype in monolayer culture.* ErbB2 overexpression causes loss of epithelial cadherins, keratins and p63. Mesenchymal markers Axl and N-cadherin are upregulated. EGFR overexpression leads to stronger basal/myoepithelial phenotype as shown by increased CK14, P63 and P-cadherin. Concomitantly there is reduction in expression of the luminal epithelial marker CK19. Actin = Loading control.

C) *In D492^{ErbB2}, miR-200c and miR141 are strongly downregulated*. Overexpression of ErbB2 led to a strong downregulation of the miR200-141 cluster, accompanied with an upregulation of ZEB1. This phenotype was reversed by dual expression of EGFR and ErbB2.

Figure 4. ErbB2 overexpression leads to loss of growth arrest in monolayer and increased survival in low-attachment

A) *D492^{ErbB2} cells have lost contact inhibition in monolayer culture.* D492 and D492^{EGFR} cells show contact inhibition when confluent in culture. In contrast, D492^{ErbB2} andD492$^{EGFR\text{-}ErbB2}$ cells pile up in culture and form multilayered ridges.

B) *ErbB2 leads to higher colony formation in low attachment* When cultured in poly-Hema coated plates, D492 cells form solid round colonies, reminiscent of mammospheres. EGFR overexpression does not affect colony number but leads to greatly larger colonies. ErbB2 expression increases both colony number and size, and in addition colonies have a irregular, grape-like phenotype. Bar=200µm.

Figure 5 – In 3D culture, EGFR partially reverses ErbB2 induced EMT .

A) *D492^{ErbB2} cells show disrupted branching morphogenesis and generate spindle- and grape like colonies in 3D culture.* When seeded at high density, and cultured in serum free media, D492^{ctrl} cells form a mixture of solid round and branching/budding colonies. D492^{EGFR} overexpression does not significantly change colony phenotype. D492^{ErbB2} show near complete loss of solid, highly cell-cell adherent colonies, with appearance of grape-like and

spindle like colonies. Inhibiting ErbB2 causes a loss of Spindle colonies, corresponding gain in grape-like colonies and re-appearance of solid colonies in ErbB2 overexpressing cells. Bar=100µm.

B) *Grape- and spindle structures show reduced expression of E-cadherin, CK14 and CK19.* Budding/Solid structures show strongly adherent, E-cadherin expressing staining. Cells are positive to both cytokeratin 14 and 19, with 19 towards the inside and 14 at the periphery. ErbB2 expression results in reduction of cell-cell adhesion as evidenced by e-cadherin staining. Additionally, complete loss of cytokeratin 14 can be seen. Bar=50µm.

C) *D492$^{EGFR/ErbB2}$ restores the epithelial phenotype in 3d culture.* As before EGFR expression leads to stronger myoepithelial/basal phenotype. ErbB2 leads to loss of keratin expression and E-Cadherin. Dual expression of EGFR in addition to ErbB2 rescues keratin expression and E-cadherin.

Figure 6, Overexpression of ErbB2 in D492 leads to increased tumor growth in nude mice which is partially reversed in D492$^{EGFR/ErbB2}$.

A) *D492^{ErbB2} and D492$^{EGFR/ErbB2}$ generate tumors efficiently in NOD/SCID mice.* When ErbB2 is overexpressed, palpable tumors appear after 7 days in all injection sites, whereas in control mice, tumors appear later.

B) *EGFR negatively affects ErbB2 induced tumor growth* overexpression of ErbB2 resulted in increased tumor growth. Dual expression of EGFR and ErbB2 reduced tumor volume, compared with ErbB2 alone.

Figure 7. EGFR expression reverses ErbB2 induced loss of keratin expression in xenografts tumors

A) *Whole mount H&E staining of D492^{ErbB2} and D492$^{EGFR/ErbB2}$ tumors.* Tumors were paraffin embedded, sliced and stained with H&E. black squares denote areas used for immunofluorescence in B). Bar=1mm

B) *EGFR reverses ErbB2 induced loss of keratin expression.* When ErbB2 was overexpressed alone, EGFR expression is reduced. No keratin expression was seen in ErbB2 tumors. In EGFR/ErbB2 tumors, Keratin expression was seen in EGFR expressing cells. Bar=100μm.

Supplementary Figure S1

A) *ErbB2 overexpression leads to reduced EGFR protein levels.* Compared with Control. ErbB2 overexpressing cells have reduced EGFR signal on Western blot.

B) *EGFR and ErbB2 signaling +3 and +30 minutes after EGF addition.* Phosphorylation of Y1173 is stron at +3 minutes, while Y1068 is stronger at +30 minutes. ErbB2 phosphorylation does not respond significantly to EGF addition.

Supplementary Figure S2

A) *Growth of individual tumors over time* Each tumor was measured three times per week, graph shows the growth of each individual tumor.

B) *CK14, CK19, E-cadherin staining of mouse tumors.* Images show CK14 and CK19, and E-cadherin staining pattern in tumors from all D492 sublines. In general, ck14 and 19 have similar staining patterns. E-cadherin staining is seen in cells that in figure 7 were shown to express EGFR.

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Figure 1. Expression of EGFR and ErbB2 in normal human breast gland.

EGFR and ErbB2 are expressed in myoepithelial- and luminal epithelial cells, respectively.

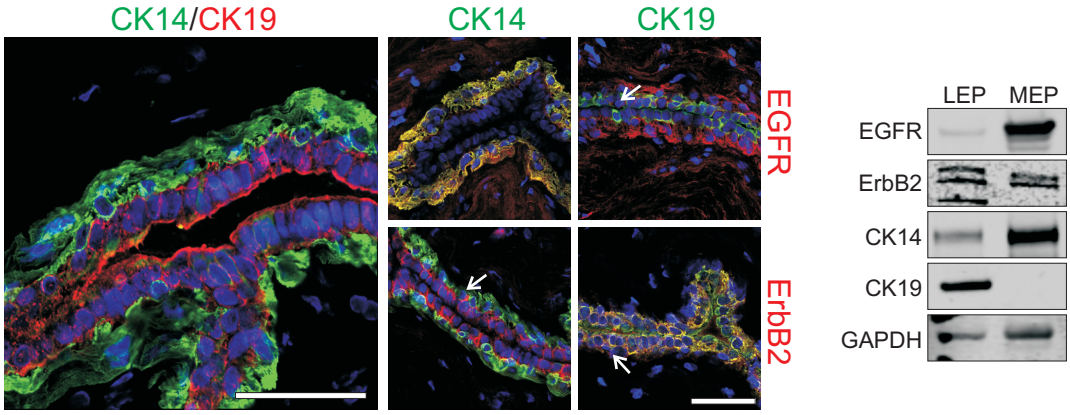
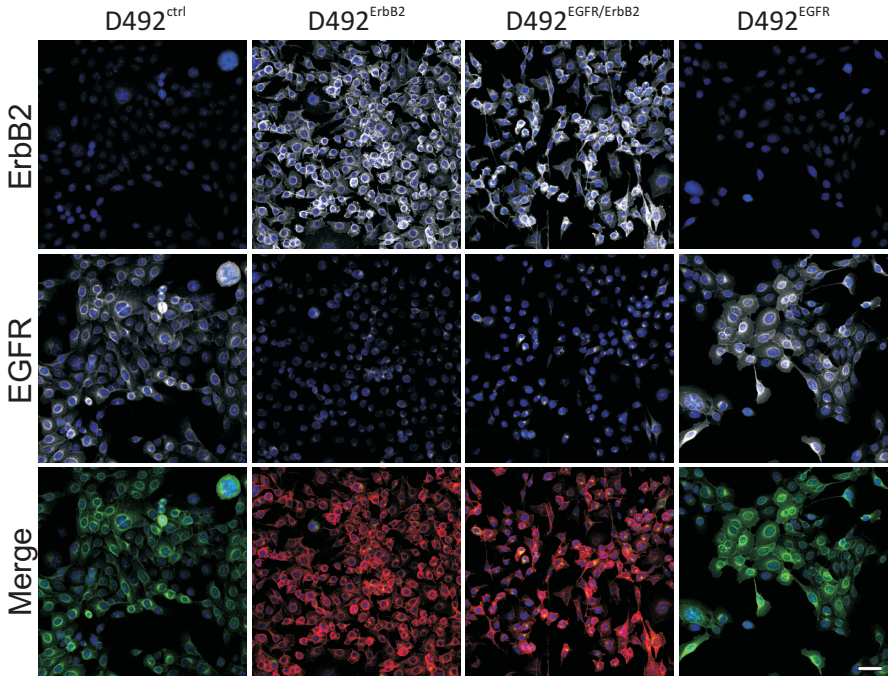
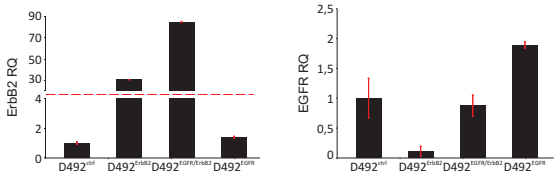


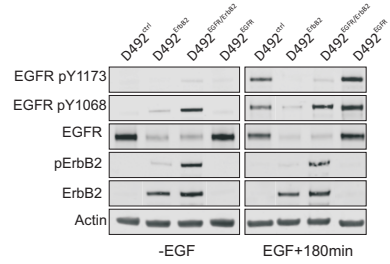
Figure 2. ErbB2 overexpression leads to loss of EGFR and gain of mesenchymal phenotype

A. Expression of endogenous EGFR in D492^{ErbB2} is reduced compared to D492^{ctrl}



C - Dual expression of EGFR and ErbB2 leads to EGF independent EGFR and ErbB2 phosphorylation

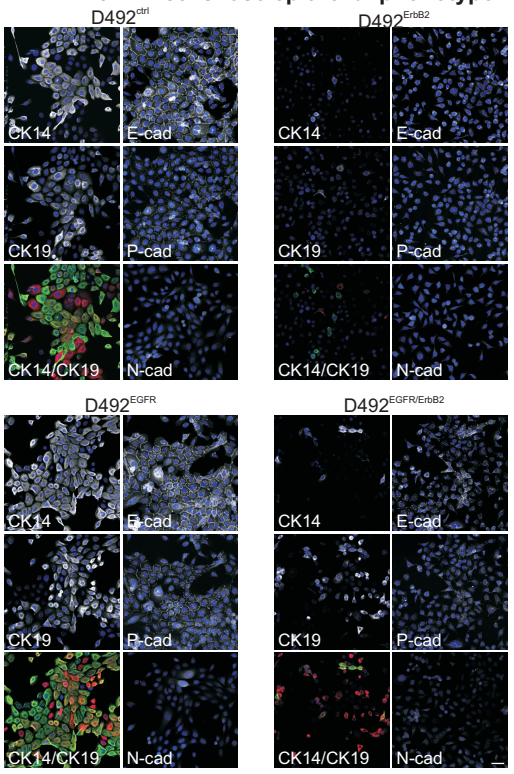




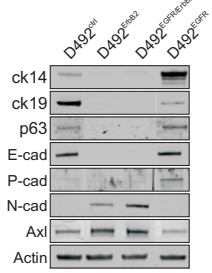
B. Upregulation of ErbB2 leads to downregulation of endogenous EGFR transcription

Figure 3. ErbB2 overexpression leads to gain of mesenchymal phenotype

A - D492^{ErbB2} cells lose epithelial phenotype in monolayer culture



B D492^{ErbB2} and D492$^{EGFR/ErbB2}$ gain mesenchymal phenotype in monolayer culture.



C miR-200c and 141 are downregulated in D492^{ErbB2}, followed by increased ZEB1

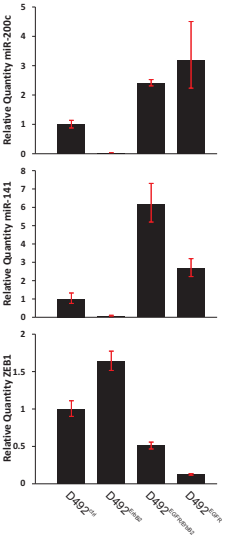
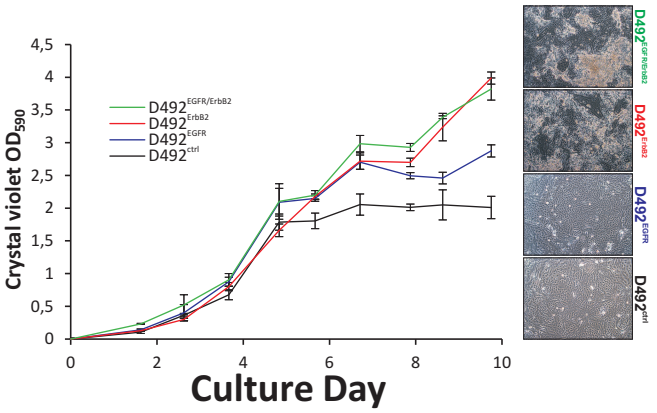


Figure 4. ErbB2 overexpression leads to loss of growth arrest in monolayer and increased survival in low attachment

A - D492^{ErbB2} and D492$^{EGFR/ErbB2}$ cells have lost contact inhibition in monolayer culture



B - ErbB2 leads to higher colony formation in low attachment

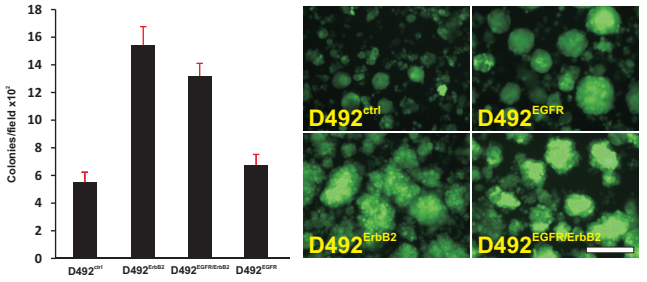
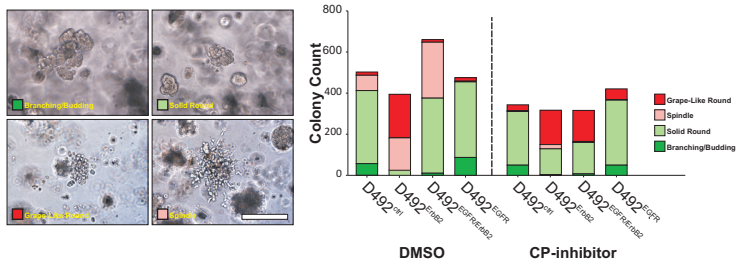
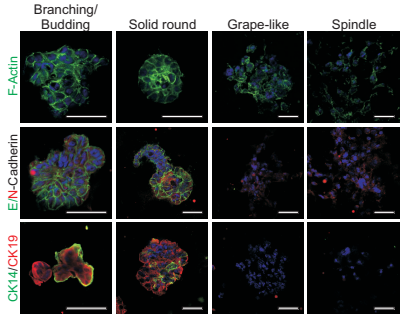


Figure 5 - In 3D culture, EGFR partially reverses ErbB2 induced EMT

A. D492ErbB2 cells show disrupted branching morphogenesis and generate spindle- and grape like colonies in 3D culture



B. Grape- and spindle structures show reduced expression of E-cadherin, CK14 and CK19



C. D492$^{EGFR/ErbB2}$ partially restores the epithelial phenotype in 3D culture.

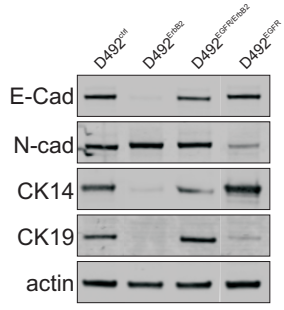


Figure 6. D492^{ErbB2} demonstrates increased tumor growth in NOD/SCID mice which is partially reversed in D492$^{EGFR/ErbB2}$

A. D492^{ErbB2} and D492$^{EGFR/ErbB2}$ generate tumors efficiently in NOD/SCID mice

	7	12	18	21	28	32	35	39	42	46
D492^{ctrl}	3/6	2/6	4/6	4/6	4/6	4/6	4/6	5/6	5/6	5/6
D492^{EGFR}	2/5	2/5	2/5	3/5	4/5	5/5	5/5	5/5	5/5	5/5
D492^{ErbB2}	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
D492$^{EGFR/ErbB2}$	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5

B. EGFR negatively affects ErbB2 induced tumor growth

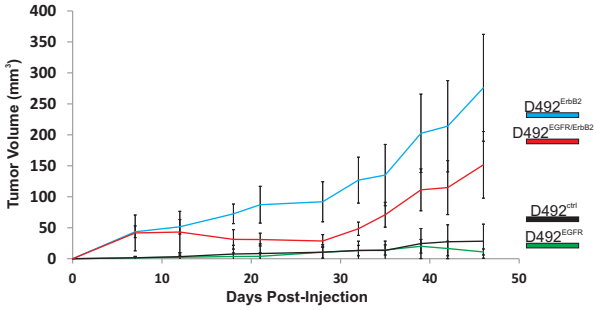
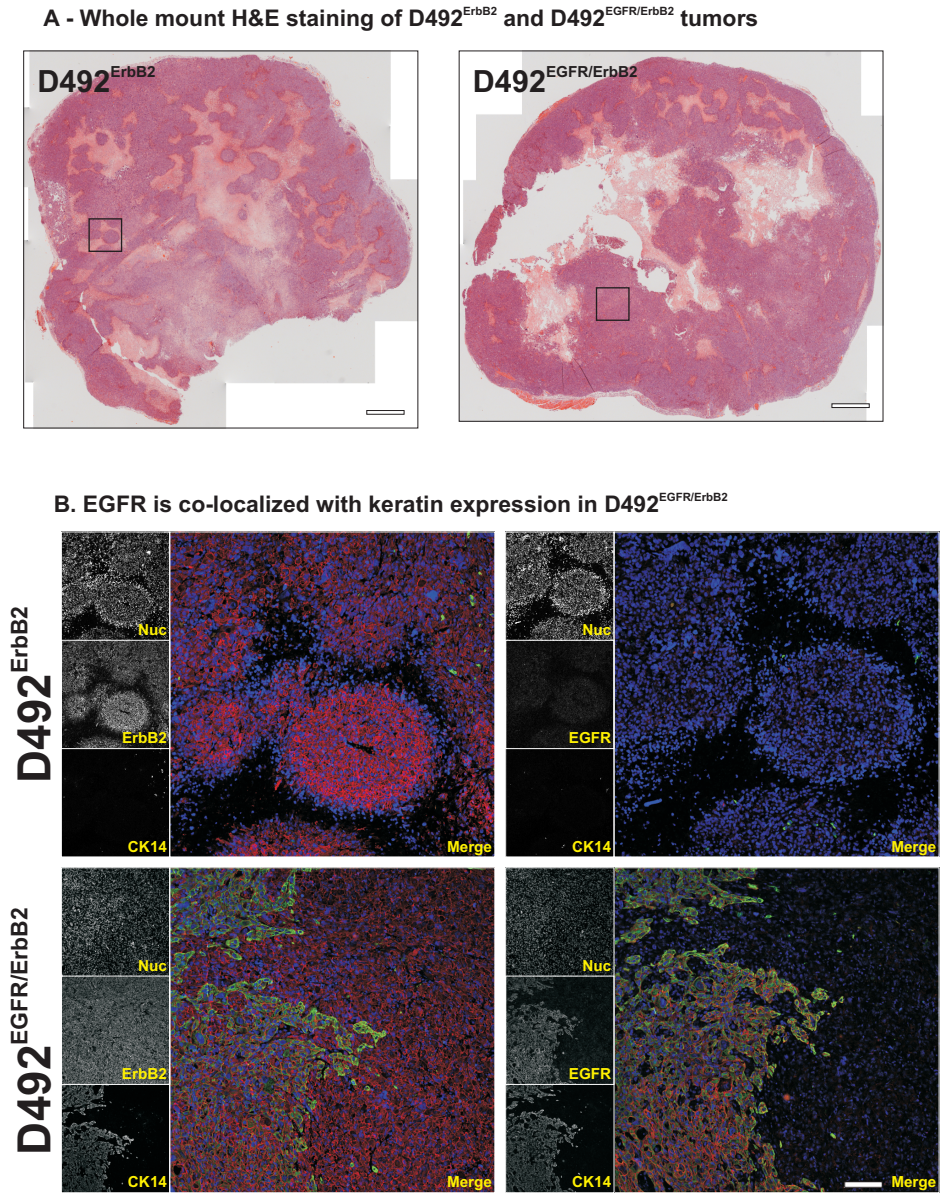
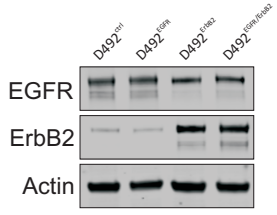


Figure 7. EGFR expression reverses ErbB2 induced loss of keratin expression in xenograft tumors

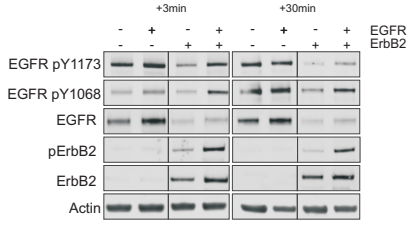


Supplementary Figure S1

A. ErbB2 overexpression leads to reduced EGFR protein levels

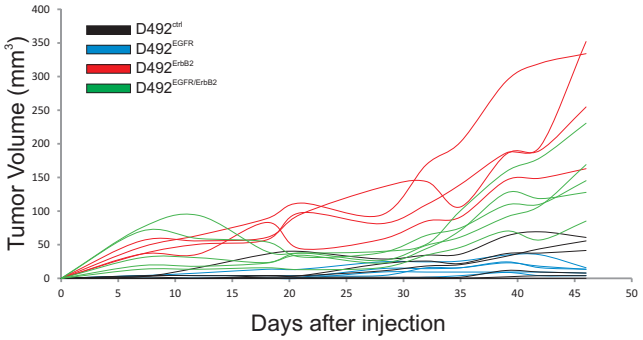


B. after 3 and 30 minutes, EGF induced phosphorylation can be seen in D492^{ctrl} and D492^{EGFR}, not in ErbB2 cells



Supplementary Figure S2

A- growth of individual tumors



B-EGFR rescues keratin expression
and E-cadherin in ErbB2 expressing cells