Crosstalk between cells with different EMT profiles and endothelial cells in breast cancer progression

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Thesis for the degree of Philosophiae Doctor

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Samskipti milli frumulína með ólíkan EMT prófíl og æðaþelsfruma í framþróun brjóstakrabbameins

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Ágrip

Bandvefsumbreyting þekjufrumna (e. epithelial to mesenchymal transition (EMT)) er þroskunarfræðilegur ferill sem kemur fyrir í sjúkdómum eins og trefjun (e. fibrosis) og krabbameini. Í krabbameini hefur EMT verið tengt við ífarandi æxlisvöxt og meinvarpamyndun og er ferillinn almennt tengdur slæmum batahorfum. Í þessari rannsókn bar ég saman mun á svipgerð (e. phenotypic) og virkni (e. functional) tveggja frumulína með sama genetíska bakgrunn (e. isogenic) sem sýna mismunandi EMT prófíl, D492M og D492HER2. Þessar frumulínur eru komnar frá D492, brjóstþekjufrumulínu með stofnfrumueiginleika sem getur myndað bæði kirtilþekju- og vöðvaþekjufrumur, og í þrívíðri rækt myndar hún greinótta formgerð.

Í samrækt með æðaþeli, undirgekkst D492 frumulínan EMT og við það myndaðist D492M. D492HER2 var hins vegar mynduð með yfirtjáningu á HER2 krabbameinsgeninu. Svipgerð D492M frumulínunnar með tilliti til EMT er lengra komin (e. fixed EMT phenotype) miðað við D492HER2 (e. intermediate EMT phenotype). Ennfremur eru D492M og D492HER2 mismunandi hvað varðar eiginleika til að mynda æxli í músum, það er að segja D492M myndar ekki æxli á meðan D492HER2 myndar æxli. Markmið þessarar rannsóknar var að greina mun í tjáningu gena á milli þessara frumulína og skoða mismun í samskiptum þeirra við æðaþel og stoðvef. Þessi rannsókn hefur leitt af sér greiningu mögulegra krabbameinsgena (e. potential oncogenes) og mat á áhrifum þeirra á frumusvipgerð og æðamyndun í æðaþelsfrumum.

Við gerðum samanburðarkortlagningu á gena- (e. transcriptome) og próteinmengi (e. secretome) frumulínanna D492, D492M og D492HER2 og staðfestum tjáningu valinna gena. Í kjölfarið voru ákveðin gen (e. candidate genes) valin (sjá hér að neðan) til að slá niður eða yfirtjá með því að nota CRISPR og siRNA aðferðir. Greiningar á svipgerð og virkni voru gerðar í tvívíðri og þrívíðri rækt.

Við sýndum fram á að YKL-40 (einnig þekkt sem CHI3L1) er töluvert hærra tjáð í D492HER2 samanborið við D492 og D492M. Þó YKL-40 hafi verið tengt við langvarandi bólgusjúkdóma og krabbamein er hlutverk þess ekki þekkt að fullu. Þegar YKL-40 er slegið niður í D492HER2, dregur úr frumuskriði og ífarandi hegðun frumanna en einnig breytist hæfni frumanna til að örva æðasprotamyndun í frumurækt. Athyglisvert er að HER2 tjáning féll í

D492HER2 frumulínunni eftir að YKL-40 var slegið niður. Athugun á áhrifum lækkaðrar YKL-40 tjáningar á svipgerðarbreytingu D492HER2 í tvívíðri rækt leiddu ekki í ljós neinn mun, en í þrívíðri rækt jókst myndun spólulaga formgerðar (e. spindle-like structures), svipað og í D492M, á meðan myndun berjaklasaformgerðar (e. grape-like structures) lækkaði. Berjaklasaformgerð var viðhaldið með því að bæta raðbrigðnu (e. recombinant) YKL-40 við D492HER2 með niðurslátt (e. knockdown) af YKL-40 eða með því að yfirtjá YKL-40 í D492M.

Annar áhugaverður kandidat úr samanburðarkortlagningu á próteinmengjum var ECM1, vegna mikillar tjáningar þess í æxlismyndandi frumulínunni D492HER2, í samanburði við frumulínurnar, sem ekki mynda æxli í músum, D492 og D492M. Eins og með YKL-40 var sýnt fram á að ECM1 hafði áhrif á æðasprotamyndun, frumuskrið og ífarandi hegðun. Ennfremur var sýnt fram á að æðaþelsfrumur sem ræktaðar voru í æti frá D492HER2 með mismunandi magni af ECM1 sýndu mismunandi afturvirknisáhrif (e. feedback) á æxlisvaldandi frumulínuna, D492HER2.

Niðurstaða rannsóknarinnar er sú að samanburður á frumulínum með sama genetíska bakgrunn (e. isogenic) sem annars vegar geta ekki myndað æxli í músum (D492M og D492) og hins vegar mynda æxli í músum (D492HER2) hefur leitt til greiningar á YKL-40 og ECM1 sem mögulegum krabbameinsgenum (e. oncogenes) sem eru mikilvæg fyrir hreyfanleika frumna og eru hugsanlegir hvatar til æðasprotamyndunar. YKL-40 og ECM1 geta veitt D492HER2 aukna árásargirni sem styður framrás krabbameins.

Lykilorð:

Brjóstakrabbamein, EMT, æðaþel, YKL-40, ECM1, D492 frumulínur

Abstract

Epithelial to mesenchymal transition (EMT) is a developmental trait that is hijacked in some disease conditions such as fibrosis and cancer. In cancer, EMT has been linked to increased invasion and metastasis, and is generally associated with a poor prognosis. In this study, I have compared phenotypic and functional differences between two isogenic cell lines exhibiting an EMT profile, D492M and D492HER2, but to a distinct extent. These cell lines are derived from D492, a breast epithelial cell line with stem cell properties that can generate both luminal and myoepithelial cells, and in 3D culture, it forms branching terminal ductal lobular units (TDLU)-like structures.

When co-cultured with breast endothelial cells, D492 undergoes EMT, which is the origin of the D492M subline. Another subline of D492 was generated by overexpressing the HER2 oncogene. D492M has a more fixed EMT phenotype while D492HER2 expresses a more intermediate EMT phenotype. Moreover, D492M and D492HER2 differ in their tumorigenicity, that is, they are non-tumorigenic and tumorigenic, respectively. This study aimed to analyze the expression profile of these cell lines and study how they interact with endothelial cells. This study has prompted the identification of potential oncogenes and the evaluation of their effects on the cellular phenotype and angiogenic potential in endothelial cells.

We performed transcriptome and secretome analyses of D492, D492M and D492HER2 cell lines and verified the expression of selected genes at both RNA and protein levels. Subsequently, the candidate genes were selected (see below) for knock-down/in experiments using CRISPR and siRNA methods. Phenotypic and functional analyses were conducted in 2D and 3D culture.

Here we demonstrate that YKL-40 (also known as CHI3L1) is significantly higher expressed in D492HER2 compared to D492 and D492M. Though YKL-40 has been linked to chronic inflammatory diseases and cancer, its function is not fully understood. When YKL-40 is knocked-down in D492HER2, migration and invasion are reduced, and its potential to induce angiogenesis *in vitro* is also affected. Interestingly, a lower expression of HER2 was observed in the D492HER2 cell line after the YKL-40 knock-down. Phenotypic characterization of diminished YKL-40 expression in D492HER2 in monolayer did not reveal any differences, however, in 3D culture, there was an increase

in spindle-like structures, similar to D492M, while the number of grape-like structures was reduced. The grape-like phenotype was rescued by adding recombinant YKL-40 to D492HER2 with KD of YKL-40 or overexpressing YKL-40 in D492M.

Another interesting candidate from the secretome analysis was ECM1, due to its high expression in the tumorigenic isogenic cell line, D492HER2, compared to the non-tumorigenic cell lines, D492 and D492M. Similar to YKL-40, ECM1 was also shown to affect angiogenesis, migration and invasion. Furthermore, conditioned media from cells with different levels of ECM1 used to culture endothelial cells revealed different kinds of feedback from endothelial cells toward the tumorigenic D492HER2 cell line.

In conclusion, the comparison of isogenic the non-tumorigenic (D492M and D492) and tumorigenic (D492HER2) cell lines has identified YKL-40 and ECM1 as potential oncogenes that are important for the mobility of cells, and they are potential inducers of angiogenesis. YKL-40 and ECM1 may provide D492HER2 with increased aggressiveness that supports cancer progression.

Keywords:

Breast cancer, EMT, endothelial niche, YKL-40, ECM1, D492 cell lines

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Contents

List of abbreviations

2D	Two-dimension(al)
3D	Three-dimension(al)
ALDH	Aldehyde Dehydrogenase
BC	Breast Cancer
BM	Basement Membrane
BRENC	Breast Endothelial Cell
BLBC	Basal-like breast cancer
CAF	Cancer-Associated Fibroblast
CDH2	Neural (N) Cadherin. Also known as N-cad
CHI3L1	Chitinase 3-Like Protein 1. Also known as YKL-40
CK	Cytokeratin
CM	Conditioned Medium/ Media
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
CSC	Cancer Stem Cell
EC	Endothelial Cell
E-cad	Epithelial (E) Cadherin. Also known as CDH1
ECM	Extracellular Matrix
ECM1	Extracellular Matrix Protein 1
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMT	Epithelial to Mesenchymal Transition

EpCAM	Epithelial Cell Adhesion Molecule
ER	Estrogen Receptor
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
GFP	Green Fluorescent Protein
gRNA	guide RNA used in CRISPR-Cas9 editing
HER2	Epithelial Growth Factor Receptor 2 (EGFR2/ErbB2)
HGF	Hepatocyte Growth Factor
HIF-1α	Hypoxia Inducible Factor 1 alpha
HUVEC	Human Umbilical Vein Endothelial Cell
IF	Immunofluorescence
IGF	Insulin-like Growth Factor
IL	Interleukin
KD	Knock-down
KO	Knock-out
LEP	Luminal Epithelial Cell
lncRNA	Long non-coding RNA
MEP	Myoepithelial Cell
MET	Mesenchymal to Epithelial Transition
miR/ miRNA	MicroRNA
MMP	Matrix-Metalloprotease
mA^{YKL-40}	Monoclonal Antibody anti YKL-40
N-cad	Neural (N) Cadherin. Also known as CDH2
NF-κB	Nuclear Factor κB
OE	Overexpression
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
p-EMT	Partial-EMT

PR	Progesterone Receptor
qRT-PCR/ qPCR	Quantitative Real Time Polymerase Chain Reaction
rBM	Reconstituted Basement Membrane
rECM1	Recombinant ECM1 protein
RFP	Red Fluorescent Protein
siRNA	Small Interfering RNA
TAM	Tumor Associated Macrophage
TCA	TriCarboxylic Acid cycle/Krebs cycle
TDLU	Terminal Duct Lobular Unit
TEB	Terminal End Bud
TEC	Tumor-associated Endothelial Cell
TGF-β	Transforming Growth Factor beta
TIC	Tumor Initiating Cell
TNBC	Triple Negative Breast Cancer
TNF-α	Tumor Necrosis Factor alpha
TF	Transcription Factor
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WB	Western Blot
YKL-40	Chitinase 3-Like Protein 1 (CHI3L1)
YKL-40^{r}	Recombinant YKL-40 (or CHI3L1) protein

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List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-III):

I. **YKL-40/CHI3L1 facilitates migration and invasion in HER2 overexpressing breast epithelial progenitor cells and generates a niche for capillary-like network formation.** Erika Morera, Sarah Sophie Steinhäuser, Zuzana Budkova, Saevar Ingthorsson, Jennifer Kricker, Aileen Krueger, Gunnhildur Asta Traustadottir, Thorarinn Gudjonsson. *In Vitro Cell Dev Biol Anim* (2019) doi:10.1007/s11626-019-00403-x

II. **ECM1 secreted by HER2-overexpressing breast cancer cells promotes the formation of a vascular niche accelerating cancer cell migration and invasion.** Sophie Sarah Steinhäuser, Erika Morera, Zuzana Budkova, Qiong Wang, Ottar Rolfsson, Angela Riedel, Aileen Krueger, Bylgja Hilmarsdóttir, Gunhild Mari Maelandsmo, Bjarni Agnar Agnarsson, Jon Gunnlaugur Jonasson, Saevar Ingthorsson, Gunnhildur Asta Traustadottir, Thordur Oskarsson, Thorarinn Gudjonsson *Lab Invest* (2020) doi:10.1038/s41374-020-0415-6

In addition, some **unpublished data** are presented:

Crosstalk between endothelial cells and D492, D492M and D492HER2

The relation between YKL-40 and the tumorigenic properties provided by HER2 in breast cancer

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Declaration of contribution

Paper I. YKL-40/ CHI3L1 facilitates migration and invasion in HER2 overexpressing breast epithelial progenitor cells and generates a niche for capillary-like network formation (*published in 2019 in In Vitro Cellular & Developmental Biology – Animal*).

This article is the main article of my thesis and represents the principal outcome of my Ph.D. project. In this paper, I compared the phenotypic and functional differences between D492M and D492HER2, and I subsequently found that YKL-40, which was highly expressed and secreted in D492HER2 cells, was important for migration and invasion of D49HER2 cells and formation of the capillary-like network in endothelial cells. Here, I am the first author, I have designed and analyzed the experiments and optimized the protocols together with my supervisors. I have performed the majority of the experiments. The co-authors helped me performing some of the experiments. I wrote the article in collaboration with my supervisors.

Paper II. ECM1 secreted by HER2-overexpressing breast cancer cells promotes the formation of a vascular niche accelerating cancer cell migration and invasion (*published in 2020 Laboratory Investigation*).

In this article, similar to article I, cell lines were compared, but focusing on D492 and D492HER2. ECM1 was selected as the candidate to explain the differences between D492 and D492HER2. ECM1 is highly expressed and secreted in D492HER2 compared to D492 and induces the formation of the capillary-like network in endothelial cells and feedback mechanisms toward D492HER2. Here, I am the second author, I have worked closely with Sophie Steinhauser and this was the main manuscript for her Ph.D. project. I helped in the development of the study, optimization of the protocols and analyzing results. I have also helped in writing, discussing and adding critical comments.

Unpublished data.

Heterotypic interactions between endothelial cells and the isogenic D492 cell lines revealed differences between the cell lines regarding the effects of ECs. Moreover, the relation between YKL-40 and HER2 in the tumorigenic properties provided by HER2 was studied. I have designed and analyzed the experiments together with my supervisors. I have performed most of the experiments.

1 Introduction

1.1 The human mammary gland

The mammary gland is the organ responsible for the production of milk in female mammals to feed their offspring and this is indeed one of the main characteristics that define the family of mammals. It is believed that the mammary gland is a modified apocrine sweat gland that has evolved from the epidermis.

The female human breast gland is under continuous remodeling from the onset of puberty until the female reaches menopause which is caused by hormonal influences that happen during each menstruation cycle and, more drastically, during pregnancy and lactation. Epithelial cells in the breast gland are organized in ductal branching “trees” to produce and secrete milk. This ductal system starts at the nipple and ends in the Terminal Ductal Lobular Units (TDLUs) (Fig. 1). It is at the end of pregnancy that the TDLUs completely reach maturity and their function of milk production takes place during lactation. TDLUs are surrounded by stroma and the interactions between cells of both compartments are necessary for the maintenance and differentiation of the cells of the breast (Sternlicht, 2006; Sternlicht et al., 2005).

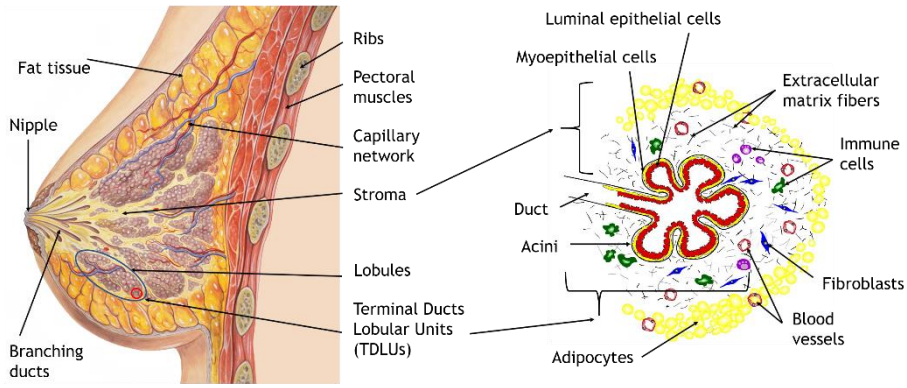

Figure 1. Schematic structure of the human breast gland and the Terminal Ductal Lobular Units (TDLUs). Left: Overview of the components of the human breast gland. It includes the branching ducts that end in the TDLUs. Right: Components of the TDLUs and the surrounding stroma. Image on the left adapted from Patrick J. Lynch, Creative Commons Attribution 3.0 Unported (*CC BY 3.0*).

1.1.1 The breast epithelium and its stem cell origin

The branching ducts in the breast gland are composed of epithelial cells surrounded by a highly vascularized stroma. Epithelial cells are classified into luminal epithelial (LEP) and myoepithelial (MEP) cells and are organized into two layers. LEPs, lining the inner part of the ducts and TDLUs, are responsible for the milk production that is secreted to the lumen. MEPs, on the other hand, are located in the outer layer in direct contact with the basement membrane (BM) and their contractile morphology helps LEPs with the secretion of milk during lactation. LEPs and MEPs are not only situated in different locations in the ducts and acini, but they also express different proteins that can be used to distinguish between these two cell types. LEPs express specific cytokeratins (CKs) such as CK8, CK18 and CK19 and other proteins like sialomucin (MUC1) and epithelial-specific antigen, Epithelial Cell Adhesion Molecule (EpCAM) (Gudjonsson et al., 2002b; Pechoux C, 1999). MEPs express other epithelial markers including basal CKs (CK5/6, CK14, and CK17), P-cadherin, P63 and α-smooth muscle actin (ASMA or α-SMA) (Gudjonsson et al., 2005).

Although LEPs are the cells that produce and secrete the milk, they need the presence of MEPs not only to secrete the milk but also to have correct polarization and differentiation. For instance, MEPs secrete laminin-1 that is essential for the proper formation of the BM and, consequently, for the apical polarization of LEPs (Gudjonsson et al., 2002a). Furthermore, the expression of P63 contributes to LEPs differentiation and has a key role in lactation in the last stage of maturation of LEPs (Forster et al., 2014). ASMA is the ultimate differentiation marker for MEPs and is the main protein that controls the contractile role of MEPs in the breast gland (Jolicoeur, 2005). It has been suggested that MEPs could even have a protective role against cancer by maintaining tissue integrity and polarity (Gudjonsson et al., 2005; Gudjonsson et al., 2002a; Runswick et al., 2001).

LEPs and MEPs are believed to have a common cell-of-origin, usually considered to be a stem cell that is defined as a progenitor with bipotent properties to differentiate into luminal and myoepithelial cells (Fridriksdottir et al., 2011; Gudjonsson et al., 2002b; Pechoux C, 1999; Visvader & Stingl, 2014). Breast epithelial stem cells remain quiescent and undifferentiated until there is a need for self-renewal, or when they receive the required signals to differentiate toward specialized cell types by an asymmetric cell division that will lead to the hierarchic organization of epithelial cell types (Fig. 2) through ducts and TDLUs (Knoblich, 2008). Bipotent progenitor cells together with cells that present different undifferentiated states drive morphogenesis and

homeostasis of the breast gland, including during the adult stage (Inman et al., 2015; Rios et al., 2014; Visvader & Stingl, 2014), through repeated cycles of differentiation, proliferation and apoptosis (Fridriksdottir et al., 2011; Inman et al., 2015). Breast stem cells are also believed to be strongly linked to the initiation and progression of cancer (Colacino et al., 2018; Visvader, 2011; Visvader & Lindeman, 2008; Ye et al., 2015).

Several investigators have worked on the identification of a profile signature of stem cells in the breast gland (Eirew et al., 2012; Gudjonsson et al., 2002b; Shehata et al., 2012; Stingl et al., 2001; Stingl et al., 2006; Stingl et al., 1998; Villadsen et al., 2007). It is considered that stem cells from the breast express markers, particularly CD49f (also known as integrin α6 or ITGA6) and EpCAM (Eirew et al., 2012; Shehata et al., 2012; Stingl et al., 2001). Mammary stem cells can express EpCAM at medium levels and CD49f^{high}, while luminal progenitors are EpCAM^{high} and CD49f^{high}, and myoepithelial cells are EpCAM^{low} CD49f^{high} (Eirew et al., 2012; Shehata et al., 2012).

In humans, EpCAM^{high}CD49f^{high} luminal progenitors that are ALDH^{+} have been identified as progenitors with an alveolar signature and this fraction expresses low levels of luminal cell differentiation (Eirew et al., 2012; Shehata et al., 2012; Stingl et al., 2001). In mice, the luminal progenitors that are ER- are analogous to the EpCAM^{high} CD49f^{high} ALDH+ population since they express genes involved in the alveolar differentiation (Shehata et al., 2012). The common luminal epithelial marker, EpCAM, has also been associated with stem cell properties. In that regard, Gudjonsson *et al.* showed that a breast stem cell population expressed high levels of EpCAM. The EpCAM^{high} cells were MUC1 negative, expressed high levels of CK19 and were able to form TDLU-like structures. Therefore, these progenitor cells are suggested to be early LEP progenitor TDLU precursors (Gudjonsson et al., 2002b). In the research by Villadsen *et al*., the progenitor cells in the human breast gland were observed to express basal markers (CK14) and luminal markers (CK19) (Villadsen et al., 2007).

Other cell surface markers are used to identify mammary stem epithelial cells such as CD24 (Eirew et al., 2012; Shackleton et al., 2006; Stingl et al., 2006) and CD29 (Asselin-Labat et al., 2007; Shackleton et al., 2006) in both, human and mouse. Additional markers that can be used to detect progenitor luminal populations are GATA3 (Asselin-Labat et al., 2007; Shehata et al., 2012), ErbB3 (Shehata et al., 2012) in humans and Elf5 (Oakes et al., 2008; Zhou et al., 2005) in mice. While markers for the basal progenitor populations are Lgr5 (Van Keymeulen et al., 2011) and Bcl11b (Cai et al., 2017).

Breast epithelial progenitor cells are considered to be located in a basal or suprabasal location in the breast gland (Fridriksdottir et al., 2017; Gudjonsson et al., 2002b; Pechoux C, 1999; Rios et al., 2014), more likely in the breast ducts of the gland rather than in the TDLUs (Gudjonsson et al., 2002b; Villadsen et al., 2007). On the other hand, recent publications have suggested that myoepithelial cells could act as stem cells conferring different profiles and functions in the ducts and the TDLUs of the breast gland (Fridriksdottir et al., 2017; Prater et al., 2014).

Studies trying to elucidate the location and biological function of stem cells in the mammary gland include *in vitro* assays and transplantation in animal models (Ginestier et al., 2007; Inman et al., 2015; Mani et al., 2008; Stingl et al., 2006). The most rudimentary transplantation assays were performed with a pool of cells from the mammary gland of mice (Inman et al., 2015; Stingl et al., 2006). Later, single progenitor cells were transplanted into cleared mammary fat pads, resulting in epithelial branching structures and repopulation of the mammary gland (Stingl et al., 2006). The use of 3D cell cultures *in vitro* has clarified the potential of human breast multipotent progenitor cells to self-renew and form colonies respecting the hierarchy of breast epithelial cells (Dontu et al., 2003).

1.1.2 The composition of breast microenvironment and the interactions with the epithelium

Breast epithelial cells are embedded in a rich vascular stroma where interactions and exchange of signals take place. The composition of the human breast stroma that surrounds the epithelium includes both cellular and non-cellular components (Fig. 1). The cellular components include fibroblasts, immune cells, endothelial cells and fat cells (Clevers, 2011; Russo & Russo, 2004).

Remodeling of the breast gland depends on epithelial cells (Fridriksdottir et al., 2011; Inman et al., 2015), but also requires signals from the stromal cells (Wiseman & Werb, 2002). The cells surrounding the breast epithelium control the production of cytokines and growth factors that are secreted to the extracellular matrix (ECM). These factors are implicated in the interactions between stromal and epithelial cells in the development and the correct morphogenesis of the breast gland (Hynes, 2009; Lu et al., 2012).

1.1.2.1 Cellular components

The microenvironment of the breast gland is rich in fibroblasts. They release soluble factors to the ECM that supply signals for normal morphogenesis and

homeostasis. They are responsible for the structural integrity of the tissue, producing fibers of the extracellular matrix such as collagen, elastin, laminin and fibronectin (Kass et al., 2007). Fibroblasts maintain the composition of the ECM under normal conditions but also during development and in wound healing after an injury (Barrientos et al., 2008). Furthermore, fibroblasts secrete soluble factors that provide growth and morphogenic signals to the epithelium (Kass et al., 2007). They secrete FGF and EGF, which are the principal signals that facilitate and initiate branching morphogenesis in the breast gland (Sternlicht, 2006; Sternlicht et al., 2005).

There are other molecules secreted by fibroblasts that induce the response of immune cells and endothelial cells under inflammatory or malignant processes (Carmeliet, 2005; Coussens & Werb, 2002; Kalluri & Neilson, 2003; Kalluri & Zeisberg, 2006). Some of the most prominent pro-inflammatory cytokines that fibroblasts produce and secrete to induce the action of immune cells are Transforming Growth Factor β (TGF-β), interleukin 1 (IL-1), as well as Platelet-Derived Growth Factor (PDGF), IL-33, CXC, and CC chemokines (Jordana et al., 1994; Kendall & Feghali-Bostwick, 2014; Wynn, 2008). Moreover, pro-angiogenic factors are secreted by fibroblasts. Signaling through the Vascular Endothelial Growth Factor (VEGF) and TGF-β have important roles in the creation of new blood vessels. In fibroblasts, TGF-β induces the expression of VEGF which is secreted to the ECM and stimulates the receptors in endothelial cells for angiogenesis (Carmeliet & Jain, 2000; Relf et al., 1997).

Immune cells reside in the microenvironment of the breast gland and activate the immune response when needed. Monocytes arrive from the bone marrow to the breast gland and differentiate into macrophages. Macrophages are cells from the immune system whose main function is to phagocytose dead cells, debris, foreign substances, microorganisms and even cancer cells. They have important roles as sensors of damages in the local organ and maintaining the homeostasis as well as repairing the damaged tissue and inducing wound healing (Gordon & Martinez, 2010; Martinez & Gordon, 2014). Macrophages are involved in the innate immune defense and also they contribute to activating the adaptive immune response by the recruitment of lymphocytes (Gordon & Martinez, 2010; Gordon et al., 2017; Martinez & Gordon, 2014). Resident macrophages are able to secrete pro- or anti-inflammatory molecules depending on the requirements of the organ (Martinez & Gordon, 2014). They can secrete factors such as IL-6, IL-12 and Tumor Necrosis Factor Alpha (TNFα) as well as IL-10, VEGF-A and TGF-β (Balkwill et al., 2005) that can induce angiogenesis of the blood vessels (Balkwill et al., 2005; Martinez &

Gordon, 2014).

In the breast gland, the vascular niche plays an important role. It transports oxygen and nutrients to the organ but also releases signals for its correct development (Carmeliet & Jain, 2000; Relf et al., 1997). Endothelial cells cover the inner part of the blood vessels and are responsible for sustaining the barrier of the vascular niche. Endothelial cells establish tight junctions between each other and other cells which require molecules of adhesion such as Vascular Endothelial Cadherin (VE-Cadherin), Platelet Endothelial Cell Adhesion Molecule (PECAM-1), Junctional Adhesion Molecule (JAM) family, and claudins (Reglero-Real et al., 2016). Also, other molecules support the inflammatory responses, mainly Vascular Cell Adhesion Molecule 1 (VCAM1), Intercellular Adhesion Molecules (ICAM) and E-selectin (van Buul et al., 2010). Additionally, endothelial cells have been shown to induce growth and morphogenesis of breast epithelium (Briem et al., 2019b; Inman et al., 2015). It has been well described how endothelial cells increase branching in the breast epithelial cells in 3D cell cultures (Ingthorsson et al., 2010; Shekhar et al., 2000; Sigurdsson et al., 2011) and they can even induce EMT (Briem et al., 2019b; Sigurdsson et al., 2011).

1.1.2.2 Non-cellular components

The basement membrane (BM) of the breast gland encloses the epithelial cells and separates them from their microenvironment. The BM is composed of collagen (mainly collagen IV), laminin, fibronectin, and linker proteins (e.g. nidogen and entactin) (Lu et al., 2012). Cells of the stroma are embedded in the interstitial matrix that is rich in fibrillar collagen but also has proteoglycans, glycoproteins (mainly tenascin C) and fibronectin (Egeblad et al., 2010; Lu et al., 2012). BM and the interstitial matrix compartment form the extracellular matrix (ECM), whose most abundant component is fibrillar collagen in normal conditions (Lu et al., 2012). However, the ECM composition and stiffness are altered in pathogenic and malignant environments increasing the deposition of fibers and increasing their thickness (Acerbi et al., 2015; Lu et al., 2012; Provenzano et al., 2006).

The main function of the ECM is to support the morphology of the organ but is also essential for the interactions between cells that take place in development (Hynes, 2009; Lu et al., 2012) and in cancer (Belgodere et al., 2018; Kaushik et al., 2016; Troester et al., 2009). For instance, the ECM is essential for the proper polarity of the epithelial cells and the laminin produced by myoepithelial cells is important for this process (Gudjonsson et al., 2002a). The ECM is dynamic, its unique composition arranges the tissue architecture

and provides versatility and integrity (Lu et al., 2012). Epithelial and stromal cells are important mediators of the production, degradation and remodeling of the ECM (Lu et al., 2012). Reciprocal interactions exist between cells and ECM which provide regulatory mechanisms to the cells and the ECM to adapt to their microenvironment (Egeblad et al., 2010; Lu et al., 2012).

Cells release molecules to the ECM that can interact directly or indirectly with other cells to induce transduction cascades providing the signals for cell survival, proliferation and differentiation (Hynes, 2009), and also supporting in initiating events for malignant processes and metastasis (Hynes, 2009; Lu et al., 2012; Oskarsson, 2013).

The signals generated by growth factors and hormones can regulate the morphogenesis of the breast gland (Macias & Hinck, 2012; Sternlicht, 2006). Growth factor signals derived from the Wnt pathway and tyrosine kinase receptors, such as EGFR and FGFR families, play essential roles for branching morphogenesis of the breast through cell-cell interactions (Hu & Li, 2010; Hynes, 2009). For instance, amphiregulin (AREG), a ligand of the EGF receptor family, transmits signals from the fibroblasts in the microenvironment to induce correct polarization and branching of the breast epithelial cells (Macias & Hinck, 2012; Sternlicht, 2006; Sternlicht et al., 2005). Moreover, TGF-β can act as an auto-negative proliferation regulator, when it is secreted by epithelial cells (Hansen & Bissell, 2000; Van Obberghen-Schilling et al., 1988; Yingling et al., 2004).

Lactogenic hormones are involved in the differentiation of mammary epithelial cells by releasing signals that regulate the process. Particularly, prolactin can induce signal transducer and activator of transcription 5 (STAT5) pathway which induces maturation and differentiation of mammary epithelial cells when signals from laminin are in the ECM, leading to the production of milk in the mammary gland (Xu et al., 2009b). If there is a deficiency in ß1-integrin, the main binding receptor of laminin in the mammary epithelial cells, the differentiation and production of milk are not possible (Xu et al., 2009b; Zhu et al., 2014). The differentiation of the epithelial cells can also be inhibited when the fibrilar collagen, proteoglycans, hyaluron and some glycoproteins are in high concentrations in the ECM (Zhu et al., 2014). This evidence shows the relevance of the ECM composition in the differentiation and maturation of epithelial cells in the breast gland.

The ECM can also induce changes in the stroma cells. Regarding this, an ECM with an increased concentration of fibrillar collagen and proteolysis can

be a chemoattractant for macrophages leading to tissue remodeling and even cancer (O'Brien et al., 2010).

1.2 Breast cancer and Cancer Stem Cells

Breast cancer (BC) is the most prevalent cancer type and the leading cause of cancer-related deaths among women. It is estimated that 5,03% of women worldwide were diagnosed with BC in 2018 and the mortality rate was one-third of all the diagnosed cases (GLOBOCAN 2018 database version 1.0, https://gco.iarc.fr/). In Iceland, the incidence rate between 2014 and 2018 was 85 of 100.000 women and the mortality rate was 15 of 100.000 (https://www.krabb.is/krabbameinsskra). Therefore, even though treatments and therapies have improved in the last decades, still many lives are lost to the disease. The prognosis depends on age, genetic factors and the stage when cancer is discovered, therefore there is an urgent need to improve in preventative treatments.

Classification of breast cancers is organized in subtypes depending on the expression of estrogen receptor (ER), progesterone receptor (PR) and the amplification of HER2 (Sørlie et al., 2001). The ERs are transcription factors required for estrogen-stimulated growth and can control the gene expression of PR which are also transcription factors. These receptors are involved in development and sexual behavior. The overexpression of ER, PR or both is present in breast cancer is classified as luminal cancer (Jacobsen & Horwitz, 2012; Osborne, 1998). Luminal cancers are usually tumors of a good prognosis and are subtyped as luminal A and luminal B. Luminal B tumors progress faster and have a worse prognosis than Luminal A (Eroles et al., 2012).

HER2 (also known as ERBB2, EGFR2 or CD340) is a tyrosine kinase that intracellularly activates signaling pathways that promote cell proliferation and survival (Hsu & Hung, 2016; Mitri et al., 2012). HER2 belongs to the epidermal growth factor receptor (EGFR) family that is involved in the normal development of the breast gland and is composed of EGFR1, HER2, HER3 and HER4. HER2 has no identified ligands and remains in a constitutively active conformation being the preferred dimerization partner of the EGFR family (Hsu & Hung, 2016; Mitri et al., 2012). The overexpression of HER2 has been linked to aggressive cancers with poor prognoses and it is present in approximately 30% of breast cancer patients (Mitri et al., 2012).

Those tumors that do not express hormone receptors nor HER2 are defined as triple-negative breast cancer (TNBC) and are the BC with the worst

prognosis (Dent et al., 2007; Foulkes et al., 2010). The lack of specific therapeutic targets makes TNBCs especially difficult to treat. TNBC is a heterogeneous group containing subgroups such as normal-like, basal-like (BLBC) and claudin-low breast cancers. Epithelial to Mesenchymal Transition (EMT) frequently occurs in TNBCs and is linked to stem cell signature, increased migration, metastasis and apoptotic resistance (Dent et al., 2007; Foulkes et al., 2010; Hennessy et al., 2009; Prat et al., 2010).

Expression profiling of breast cancer subtypes enhances effective treatments against specific targets. Luminal breast cancers are regulated by hormonal signals and can be treated with drugs like tamoxifen (Goldhirsch et al., 2011). HER2-overexpressing tumors usually have a poor prognosis because they have a high potential to metastasize and are treated with trastuzumab that acts specifically against the tyrosine kinase receptor HER2 (Goldhirsch et al., 2011; Slamon et al., 2001). TNBCs have no specific treatments and are commonly treated with chemotherapy and drugs that are used in other subtypes of breast cancers (Foulkes et al., 2010; Prat et al., 2010). It is, therefore, of high demand to study TNBCs to develop therapies with specific treatments. Targeting EMT is one possible avenue that is being pursued by many research groups (Fedele et al., 2017; Shibue & Weinberg, 2017; Singh & Settleman, 2010).

During carcinogenesis, a cell is transformed into a malignant phenotype after an accumulation of oncogenic mutations (Hanahan & Weinberg, 2000) but the cell-of-origin of cancer has not been clarified yet. It is believed that Cancer Stem Cells (CSCs) have an important role in carcinogenesis, but it does not need to be related to the origin (Reya et al., 2001; Skibinski & Kuperwasser, 2015). Tumors may be originated by the accumulation of mutations in normal stem cells, progenitor cells, or differentiated cells that escape the regulation of homeostasis and become Tumor-Initiating Cells (TICs). Therefore, TICs can be CSCs, but CSCs are not always TICs. However, in literature, it is frequent to find CSC and TIC terms used indistinctly.

In this context, CSCs would be cancer cells with stem cell properties that maintain their self-renewal and the heterogeneity of the tumor but not necessarily are responsible for the origin of the tumor and can emerge in later stages of tumor progression (Aponte & Caicedo, 2017; Reya et al., 2001; Skibinski & Kuperwasser, 2015). CSCs have mechanisms to protect themselves from extrinsic damages, which may lead to resistance to treatments (Al-Hajj et al., 2003; Stingl et al., 2006). CSCs secrete factors, such as cytokines that can stimulate the generation and organization of the tumor

microenvironment, increasing the aggressiveness and heterogeneity (Junttila & de Sauvage, 2013; Scadden, 2014).

The group of Al-Hajj in 2003 discovered that cells isolated from breast cancer patients with the expression profiling of $CD44^{hi}/CD24^{low/neg}$ were able to generate tumors, even when a small number of cells were injected in serial transplantations into immunodeficient mice. Other factors related to stemness have been linked to cancer, such as Oct4 (Ponti et al., 2005) and Nanog (Kong et al., 2010). In addition, ALDH1, an enzyme that oxidizes intracellular aldehydes and is important for stem cell differentiation, has been related to populations of cancer cells with stem or progenitor properties, capable of self-renew and generate tumors reflecting the heterogeneity of the primary tumor. Cells that are positive for ALDH1 in a cancer microenvironment originate and facilitate the progression of the tumor in animal models (Colacino et al., 2018; Ginestier et al., 2007). Nonetheless, some CSCs with tumor-initiating properties have been described to not express the $CD44^{hi}/CD24^{low/neg}$ signature and ALDH1 at the same time, suggesting the existence of different signatures in the cells that initiate tumors (Liu et al., 2014).

Some CSCs have been suggested to be involved in the metastasis of cancer cells (Shibue & Weinberg, 2017; Singh & Settleman, 2010). In the work of Al-Hajj and co-workers, cells with the $CD44^{hi}/CD24^{low/neg}$ signature were also detected in metastatic pleural effusions in mice with breast cancer (Al-Hajj et al., 2003). Moreover, this CSC signature was found in the bone marrow in a higher percentage than in the primary tumors of breast cancer patients (Balic et al., 2006). These findings suggest a possible relation between CSCs and metastasis in breast cancer.

The percentage of cells expressing $CD44^{hi}/24^{low/neg}$ has been related to the stemness/ differentiation state of breast cancer. BLBC cells are usually the most undifferentiated BC cells and show a higher percentage of $CD44^{hi}/24^{low/neg}$ cells, while luminal BCs express less percentage of this cancer stem cell profile (Fig. 2) (Fillmore & Kuperwasser, 2008). BLBCs have been shown to have not only CSCs properties but also EMT phenotype (Mani et al., 2008; Morel et al., 2008; Sarrio et al., 2008). The differences in differentiation stages in BC cells are also related to prognosis, being the luminal the subtype BC with most differentiated cells and it is considered the BC with better prognosis. Conversely, HER2-positive BC and TNBC have cells more undifferentiated and these BCs have a worse prognosis (Fig. 2) (Fillmore & Kuperwasser, 2008).

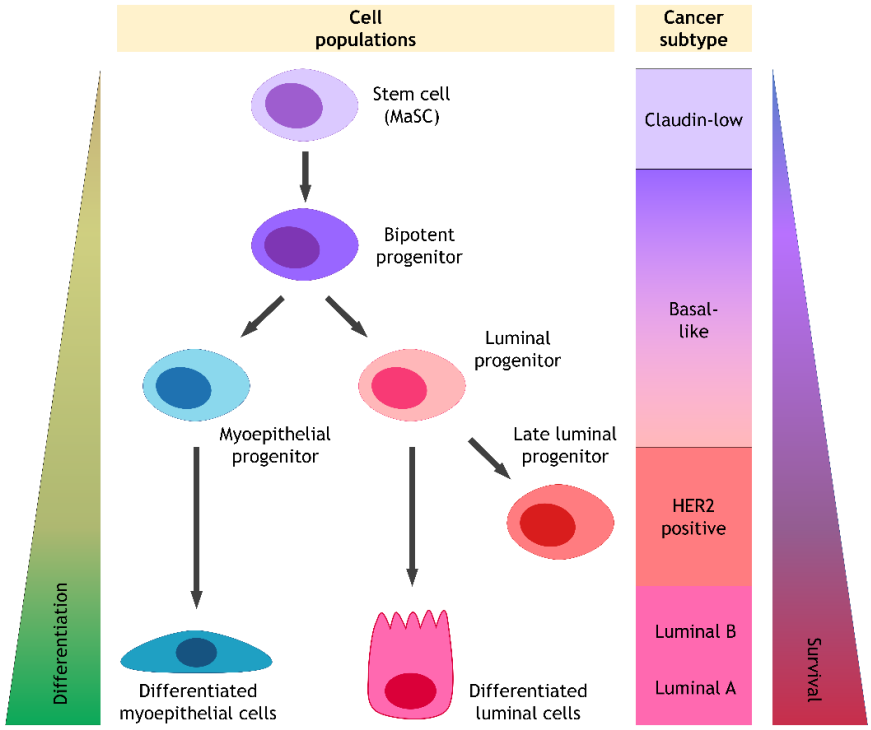

Figure 2. Cellular differentiation hierarchy of the breast epithelial cells and their possible association to breast cancer subtypes. Differentiation hierarchy of breast epithelial cells starting from the stem cell, followed by different progenitor states and ending in matured differentiated epithelial cells. Corresponding cell types can lead to different breast cancer subtypes. Cell types in different stages in differentiation can be linked to different grades of malignancy and, therefore, survival. Adapted from (Fillmore & Kuperwasser, 2008; Oakes et al., 2014; Prat et al., 2010; Prat & Perou, 2011; Skibinski & Kuperwasser, 2015; Visvader & Stingl, 2014).

The identification of cancer cells with progenitor properties could help to find the cell-of-origin of many breast cancers. In that regard, some *in vitro* assays have been used, such as low attachment assays, in which, similar to normal stem cells, colonies are generated and are referred to as tumorspheres (Mani et al., 2008; Morel et al., 2008). This technique has been useful in the study of resistance to paclitaxel and 5-fluorouracil (Fillmore & Kuperwasser, 2008). Also, *in vivo* studies inserting TICs in mouse models have improved the identification of these cells with tumor-initiating properties (Al-Hajj et al., 2003; Stingl et al., 2006). Therefore, it is very important to elucidate the cell-of-origin of cancer to improve treatment options and develop new cancer therapies.

1.2.1 Composition of the breast cancer microenvironment and breast cancer cells-stroma interactions

Epithelial-stromal interactions are important for normal breast morphogenesis, but it is also becoming more evident that this crosstalk contributes to cancer progression. A proper hierarchy of the cell types in the branching ducts and the stroma, as well as a correct conformation of the fibers and proteins of the ECM, are needed in the breast. Thus, an alteration or dysregulation in the homeostasis in the breast gland can lead to cancer (Fig. 3) (Hynes, 2009; Kalluri & Zeisberg, 2006; Kaushik et al., 2016; Lu et al., 2012).

Heterotypic interactions between cancer cells and stromal cells are believed to generate an environment that contributes to migration and invasion of cancer cells (Hansen & Bissell, 2000; Kalluri & Zeisberg, 2006; Provenzano et al., 2006; Wiseman & Werb, 2002). It has been found that cancer cells can alter their niche by secreting growth factors that induce angiogenesis, modifications in the ECM, proliferation of fibroblasts, and increase recruitment of macrophages (Bhowmick et al., 2004; Ronnov-Jessen et al., 1996).

1.2.1.1 Cellular components in breast cancer

In the breast cancer microenvironment, fibroblasts secrete growth factors that stimulate the progression and growth of tumors (Bhowmick et al., 2004; Kalluri & Zeisberg, 2006). Fibroblasts are able to modulate the synthesis, deposition and remodeling of the ECM in tumors and facilitate metastasis of the primary tumor cells through infiltration to the vascular systemic circulation (Bhowmick et al., 2004; Kalluri & Zeisberg, 2006). These fibroblasts that surround tumors and secrete the ECM of the tumor microenvironment are called myofibroblasts and are also referred to as cancer-associated fibroblasts (CAFs) (Fig. 3) (Kalluri & Zeisberg, 2006).

Some growth factors secreted by fibroblasts have been found in high levels in patients with fibrosis and cancer (Carmeliet, 2005; Goustin et al., 1986; Kalluri & Neilson, 2003), such as FGF and EGF (Sternlicht, 2006; Sternlicht et al., 2005). Additionally, overexpression of inflammatory cytokines produced by fibroblasts can lead to chronic inflammation, fibrosis or cancer (Carmeliet, 2005; Coussens & Werb, 2002; Kalluri & Neilson, 2003; Kalluri & Zeisberg, 2006). The most common pro-inflammatory cytokines are TGF-β, some interleukins, and chemokines (Jordana et al., 1994; Kendall & Feghali-Bostwick, 2014; Wynn, 2008).

CAFs are important regulators of the immune response. They contribute to the innate and adaptive immune action, facilitating the differentiation and

activation of the immune cells (Harper & Sainson, 2014). Myofibroblasts can increase the migration of monocytes to the tumor microenvironment and activate the differentiation of M2-macrophages (Cohen et al., 2017; Zhang et al., 2017). Furthermore, CAFs secrete TGF-β for the recruitment of macrophages and participate in the macrophage polarization and functions (Gong et al., 2012; Mills et al., 2000).

In addition, CAFs can induce angiogenesis to support the increased energetic requirements of the tumor. When TGF-β is released in the tumor microenvironment by cancer cells, fibroblasts are activated in a paracrine manner and they are induced to secrete TGF-β which acts as an autocrine signal as well. Consequently, the expression of VEGF is induced in fibroblasts and VEGF stimulates the receptors in endothelial cells for angiogenesis and proliferation (Carmeliet & Jain, 2000; Relf et al., 1997). VEGF (subtype A) can promote the migration of cancer cells through microenvironmental modulation in a paracrine manner (Wu et al., 2018b). Besides, TGF-β has paracrine activity and promotes the migration of epithelial cells by inducing EMT, thus increasing the invasiveness of cancer cells (Xu et al., 2009a). TGF-β and VEGF have positive feedback towards cancer cells in their growth, survival and migration.

The immune system can have harmful effects when it is activated in aberrant conditions or inappropriate contexts promoting chronic inflammatory diseases or cancer (Balkwill et al., 2005; Carmeliet, 2005; Condeelis & Pollard, 2006; Mantovani et al., 2002). Macrophages that are implicated in cancer and tumor growth are called tumor-associated macrophages (TAMs) (Fig. 3) (Condeelis & Pollard, 2006; Gordon & Martinez, 2010; Mantovani et al., 2002). TAMs secrete growth factors such as EGF, FGF, PDGF and TGF-β to modulate the ECM, which can originate an aberrant microenvironment that promotes tumor progression and supports metastasis (Condeelis & Pollard, 2006; Peinado et al., 2017). Moreover, TAMs secrete pro-inflammatory factors like TNFα which stimulates the nuclear factor-kappa B (NF-κB) and subsequently allows the transcription and translation of anti-apoptotic proteins that increase the survival and proliferation in cancer cells (Hagemann et al., 2008). TAMs are also able to produce pro-angiogenic factors and cytokines such as TGF-β, VEGF-A and TNFα, as well as IL-1, IL-6 and macrophage colony-stimulating factor (M-CSF/CSF1) (Lin et al., 2006). Additionally, TAMs are associated with breast cancer resistance to chemotherapeutic agents, such as paclitaxel, doxorubicin and etoposide (Ruffell & Coussens, 2015) and with metastasis through EMT signals (Condeelis & Pollard, 2006; Qian et al., 2015).

Angiogenesis is an important process in cancer progression that can lead toward metastasis and, therefore, often results in poor prognosis (Bhowmick et al., 2004; De Palma et al., 2017; Lee et al., 2015a). When cells advance in the formation of the tumor, there is an increase in nutrient and oxygen supplies in order to respond to increased proliferation of the malignant cells (Fig. 3) (Carmeliet & Jain, 2000; Hanahan & Weinberg, 2011). Endothelial cells form the inner layer of the blood vessels, having an important role in angiogenesis. Moreover, in cancer, tumor-associated endothelial cells (TECs) play roles in immune suppression and metastasis as they regulate the infiltration of other cells (Buckanovich et al., 2008). It has been described how endothelial cells are able to block the extravasation of T cells by the deregulation of adhesion molecules in cancer niches, which interferes with the defense system, making recovery for the patients more difficult after cancer treatments (Buckanovich et al., 2008). Furthermore, while tumors are growing, cells can leave the primary organ and disseminate to distant organs transported by blood vessels (Pein & Oskarsson, 2015; Potente et al., 2011).

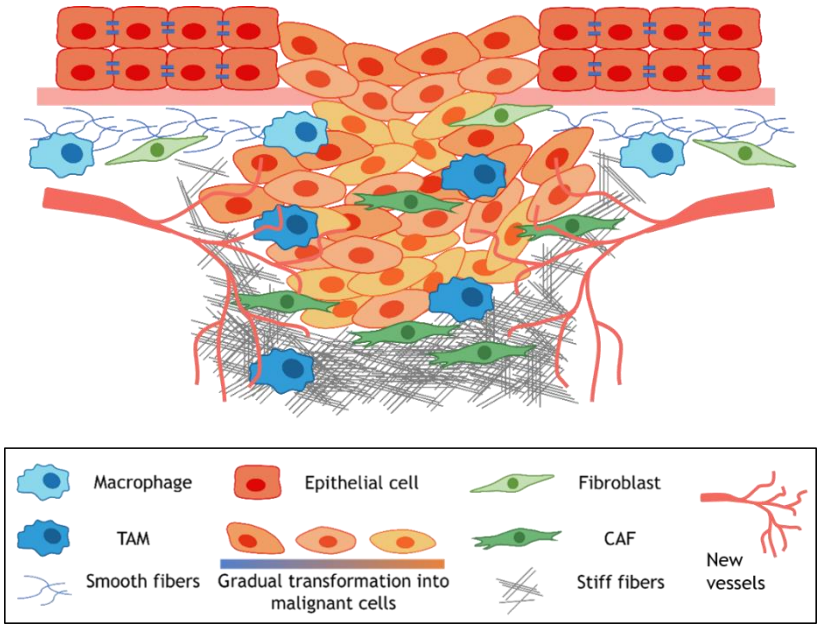

Figure 3. Heterotypic interactions in cancer progression. During cancer progression, epithelial cells are transformed into malignant cells and establish heterotypic interactions with stromal cells. Stromal cells in cancer niches differentiate into cancer-associated stromal cells and the stiffness of extracellular matrix (ECM) fibers is induced. Angiogenesis is induced and new vessels are formed. Adapted from (Carmeliet & Jain, 2000; Condeelis & Pollard, 2006; Hanahan & Weinberg, 2011; Kalluri & Zeisberg, 2006; Lu et al., 2012; Nieto et al., 2016).

1.2.1.2 Non-cellular components in breast cancer

In the breast gland, ECM regulates the behavior of cells in terms of cell communication, survival, proliferation, differentiation, and immune response. Modifications in the biochemical, physical or molecular ECM composition can change its dynamic morphology and stiffness which supports cancer progression and metastasis (Kaushik et al., 2016; Lu et al., 2012; Pickup et al., 2014).

Due to the increased proliferation of cancer cells, the interstitial pressure within the tumor rises, as well as the cell density. In addition, stromal cells such as myofibroblasts also increase their proliferation which, subsequently, triggers the increase of collagen secretion (Kaushik et al., 2016). Moreover, there is an intensification of collagen deposition, linearization and thickening of the fibers; therefore, the stiffness of ECM rises (Fig. 3) (Lu et al., 2012; Pickup et al., 2014). This process is referred to as desmoplasia (Ayala et al., 2003). In breast cancer, desmoplasia is mainly caused by secretions from CAFs but also immune cells and cancer cells contribute (Lu et al., 2012), and causes an increase in deposition of collagen I, II, III, V, and IX (Kaushik et al., 2016). In the diagnosis of breast cancer, measurements of density and stiffness are often used as clinical signs (Lopez et al., 2011). The increment in ECM stiffness is usually linked to aggressiveness in breast cancer. It has been seen that TNBC and HER2-positive breast cancers, which are the subtypes with worse prognoses, have stiffer ECMs (Kaushik et al., 2016).

Increased ECM stiffness may facilitate cancer progression and metastasis. In the tumor vasculature, the BM becomes more permeable and porous, enabling the infiltration and extravasation of cancer cells, allowing the exit of the cancer cells through blood vessels or lymph vessels to distant organs (Egeblad et al., 2010; Lu et al., 2012). Furthermore, secreted molecules in the ECM may contribute to tumor remodeling and pre-metastatic niches (Kaushik et al., 2016). Before tumor cells are disseminated to distant organs, pre-metastatic niches require some preparation (Kaplan et al., 2006). In this context, the collagen crosslinking protein lysyl oxidase (LOX) promotes invasion of cancer cells and when LOX is repressed, metastasis is reduced (Erler & Weaver, 2009). Additionally, LOX is secreted by breast cancer cells in hypoxic conditions and has been seen to be accumulated in pre-metastatic niches together with fibronectin in the BM of the lungs (Erler et al., 2009).

In addition, matrix metalloproteinases (MMPs) are secreted by cancer cells, fibroblasts and macrophages in the tumor microenvironment. When MMPs are activated, they degrade the ECM facilitating tumor growth and migration of

tumor cells (Egeblad et al., 2010; Lu et al., 2012). Also, other secreted molecules in the ECM have been linked to metastasis. For instance, tenascin C that is typically an ECM protein of stem cell niches has been found in increased levels in breast cancer patients and supporting metastasis to lungs (Oskarsson et al., 2011).

1.3 Epithelial to mesenchymal transition (EMT) in breast cancer

Epithelial to mesenchymal transition (EMT) is a developmental process that describes the plasticity of epithelial cells to change phenotype from compact adherent epithelial cells to mesenchymal-like cells that have lost adherence properties. This is accompanied by increased migratory abilities and a spindle-shaped morphology (Kalluri & Weinberg, 2009; Thiery et al., 2009).

The concept was first referred to as epithelial-to-mesenchymal transformation in 1967 by Betty Hay (Trelstad et al., 1967) and was developed as a crucial feature of embryogenesis in the 1980s (Greenburg & Hay, 1982). This is an important event in normal development during gastrulation in the formation of the mesoderm and the formation of the neural crest cells (Kalluri & Weinberg, 2009; Thiery et al., 2009). EMT also takes place in wound healing and some diseases, mainly fibrosis and cancer (Nieto, 2013; Nieto et al., 2016; Thiery, 2002).

During EMT, there is a loss or decrease of epithelial markers, such as E-cadherin and cytokeratins, and the expression of mesenchymal markers is at increased levels, especially N-cadherin and others, for instance, vimentin, α-smooth muscle actin, fibronectin and Axl (Fig. 4) (Gjerdrum et al., 2010; Peinado et al., 2007).

Epithelial cells that undergo EMT gain increased ability to migrate and invade, and acquire increased resistance to apoptosis (Polyak & Weinberg, 2009; Singh & Settleman, 2010; Thiery et al., 2009). These properties have linked EMT to cancer aggressiveness and EMT has been proposed to be necessary for the initial steps of metastasis (Nieto et al., 2016; Polyak & Weinberg, 2009; Singh & Settleman, 2010; Tan et al., 2014; Thiery et al., 2009; Thiery, 2002). Thereby, EMT-phenotype is related to a worse prognosis whose mechanisms are not completely understood yet, thus it is imperative to investigate the subject further.

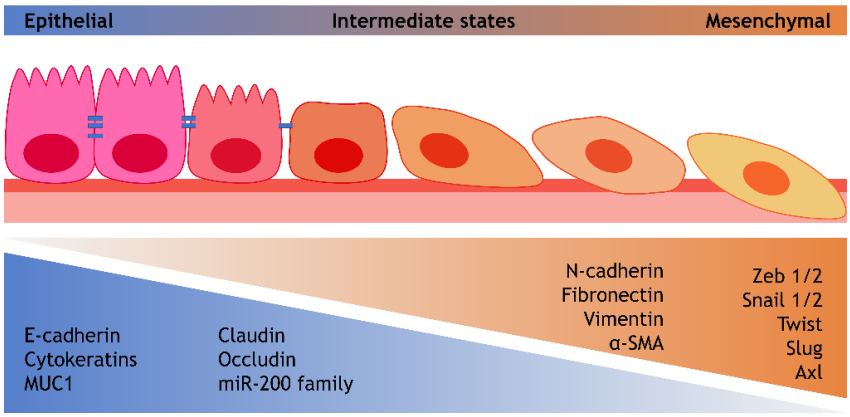

Figure 4. Epithelial to Mesenchymal Transition (EMT). Transition of epithelial cells to the mesenchymal phenotype. Partial-EMT takes place, giving rise to a range of intermediate EMT phenotypes.Epithelial markers decrease their expression, while mesenchymal markers arise. Polarization and contact between cells are lost, while migration and invasion are increased. Adapted from (Gjerdrum et al., 2010; Kalluri & Weinberg, 2009; Korpal et al., 2008; Peinado et al., 2007; Thiery et al., 2009).

1.3.1 Partial-EMT and plasticity

Cellular plasticity is an important property in embryonic development where cells switch between epithelial and mesenchymal phenotypes to adapt to different conditions. EMT and the reverse process, Mesenchymal to Epithelial Transition (MET), are dynamic processes in which cells swap from an apico-basal polarization and cell-cell adhesive state to more migratory and invasive states (Brabletz, 2012; Thiery et al., 2009).

There are similarities between embryonic development and cancer progression as both processes involve cells increasing their migratory abilities in order to disseminate to other organs. It is believed that in both cases, this high mobility is raised by signals from the stroma that induce EMT and once in the distant organ, cells restore their phenotype by undergoing MET and proliferating (Brabletz, 2012; Kalluri & Weinberg, 2009; Thiery et al., 2009).

It is important to note that these states are not always either completely epithelial or mesenchymal, there are intermediate states that provide plasticity and advantages to cells to adapt to the microenvironment. This program is referred to as partial-EMT (p-EMT) (Nieto et al., 2016; Tam & Weinberg, 2013). This partial-EMT has been described in several developmental, wound healing, fibrosis, and cancer processes (Huang et al., 2013; Nieto et al., 2016; Yu et al., 2013). Indeed, most scientific articles about EMT published today are describing partial-EMT or cellular plasticity rather than complete EMT (Nieto, 2013; Nieto et al., 2016).

The most common partial-EMT phenotype described in the literature involves a hybrid state where there is co-expression of epithelial and mesenchymal markers in the cell (Huang et al., 2013; Yu et al., 2013). However, there is a wide range of phenotypes representing the heterogeneity of the cells involved in the program. Chu, Halbleib and co-workers published in 2006 intermediate EMT states where the loss of E-cadherin did not imply the gain of N-cadherin, but it reflected differences in adhesion (Chu et al., 2006; Halbleib & Nelson, 2006).

In cancer, resistance to drugs may follow the anti-apoptotic nature of EMT-phenotypes but also the heterogeneity of cells in the tumor that is representing a wide range of EMT intermediate states (Huang et al., 2013; Nieto et al., 2016; Yu et al., 2013). Tumors that are originated in mesoderm or neural crest-derived organs are less heterogeneous compared to solid tumors with an epithelial origin. There is a high heterogenicity of partial-EMT states in carcinomas in breast, ovaries and lung (Nieto et al., 2016), which may obstruct the identification of effective treatments against these cancers. Moreover, it has been shown that cancer cells that express partial-EMT are more efficient in metastasis possibly because of the need for the reverse process, MET (Jolly et al., 2018).

The switch of E-cadherin to N-cadherin in the cytoplasmic membrane, besides the loss of other epithelial proteins and the increase of mesenchymal markers, are characteristic of some TNBCs, but EMT does not only happen in this BC type. There are also EMT-derived phenotypes in luminal and HER2 breast cancers although at a lower percentage (Tan et al., 2014; Yu et al., 2013), which suggests that EMT is a local event rather than a global event (Nieto et al., 2016).

1.3.2 Regulation of epithelial to mesenchymal transition

EMT can be induced in response to external conditions, such as hypoxia or inflammation. Consequently, molecules are secreted by stromal cells (mainly macrophages and activated fibroblasts, but also endothelial cells) and they provide the intercellular signals to induce EMT in epithelial cells. These EMT-inducing signals include TGF-β, PDGF, EGF, HGF and FGF (Kalluri & Weinberg, 2009). In addition, cells that have undergone EMT can secrete those molecules to maintain their phenotype in an autocrine manner (Nieto, 2011; Scheel et al., 2011)

Particularly, TGF-β is an important EMT-inducer that is able to increase migration and invasion of epithelial cells in development, but also in malignant

processes. In cancer, TGF-β is secreted by myofibroblasts and other cells of the tumor niche and increases the mobility and aggressiveness of cancer cells (Kalluri & Weinberg, 2009; Kasai et al., 2005; Moustakas & Heldin, 2012; Willis & Borok, 2007; Xu et al., 2009a).

In breast cancer, the reduction in estrogen receptors and hypoxic conditions can induce the activation of TGF-β which activates the NOTCH signaling pathway and, subsequently, EMT and metastasis (Nieto, 2011). Furthermore, NOTCH signaling can also be activated in EMT by the induction of the ligand Jagged that is activated by the transcription factors (TFs) Snail and Slug (Wang et al., 2010). The activation of NOTCH can contribute to the development of the EMT program but also the stemness in CSCs together with the NF-kB signaling pathway through the STAT3 and PI3K-AKT signaling pathways (Hsu et al., 2016).

The cellular modifications that occur during EMT require gene reprogramming, repressing epithelial genes and upregulating mesenchymal genes. These changes are driven and regulated by transcription factors, which are crucial for the progress of EMT (EMT-TFs). Increased expression of transcription factors related to development involves typically Zeb1/2, Snail, Slug and Twist (Bolós et al., 2016; Cano et al., 2000; Peinado et al., 2007; Puisieux et al., 2014). These EMT-TFs have also been linked to cancer progression and stemness. In the work of Mani and colleagues, Snail and Twist have been described to be overexpressed in breast cancer in animal models and tissue from patients (Mani et al., 2008). However, it is believed that EMT can be controlled in breast stem cells and tumor-initiating cells by different programs, as it has recently been shown that Slug and Snail had different roles in those kinds of cells in the mammary gland of a mouse model (Ye et al., 2015). Snail has been linked to aggressiveness since when Snail was knocked-down in mammary tumor-initiating cells, the developed tumors were less aggressive, but not by Slug knock-down. Conversely, the normal mammary stem cells were affected in their organoid-forming properties when Slug was knocked-down, but not by Snail knock-down (Ye et al., 2015).

Other important EMT-TFs that are associated with stemness are members of the Wnt/β-catenin signaling pathway (Pattabiraman & Weinberg, 2016). When EMT occurs, β-catenin translocates to the nucleus where it induces the transcription of target genes in collaboration with the lymphoid enhancer factor/T-cell factor (LEC/TCF) (Clevers et al., 2014). These target genes are frequently involved in stemness and cancer (Lee et al., 2009; Yuan et al., 2015). In addition, it has been described that the repression of the Wnt/β-

catenin signaling can inhibit EMT in breast cancer and, even, metastasis to the lungs in BLBC (DiMeo et al., 2009).

The transcription factors that regulate EMT may work together with microRNAs (miRNAs or miRs) and epigenetic regulators in embryonic development, but also in wound healing, fibrosis and cancer (Nieto et al., 2016; Tam & Weinberg, 2013). In the last decade, the regulatory functions in EMT executed by non-coding RNAs have attracted the attention of scientists. Downregulation of miRNAs, especially the miR-200 family, has been linked to EMT (Briem et al., 2019a; Dvinge et al., 2013; Hilmarsdottir et al., 2014; Hilmarsdottir et al., 2015; Korpal et al., 2008). In epithelial cells, the expression of miR-200 is high, but this expression is often reduced or lost in EMT. Re-introduction of miR-200 family members into cells with EMT phenotype can revert the EMT phenotype through MET (Briem et al., 2019b; Burk et al., 2008; Hilmarsdottir et al., 2014; Korpal et al., 2008). Moreover, epigenetic modulation of the miR-200 family is associated with methylation of the stem cell-like state (Lim et al., 2013).

1.4 2D and 3D *in vitro* cell culture models to study breast morphogenesis and cancer

Animal models are valuable tools in life science and the use of mice has contributed to a better understanding of what is happening in the mammary gland through the different stages. Mouse strains with induced or spontaneous mutations and, more recently, genome-edited by CRISPR have been useful for studying gene function and regulation (Wang et al., 2013). The mouse and human mammary glands share some similarities (Dontu & Ince, 2015; Mani et al., 2008), although they differ in numerous aspects. In mice, the ducts of the mammary gland have terminal end buds (TEBs) that are more rudimentary and have less branching compared to human TDLUs, and the stromal composition and hormonal regulation are different (Dontu & Ince, 2015; Kuperwasser et al., 2004). Therefore, animal models should be used carefully and there is a need for proper human models that do not compromise ethical issues.

The establishment of *in vitro* models enables the study of breast morphology and cancer as an excellent alternative to animal models. The isolation of primary breast cells from reduction mammoplasties has become a good source of cells that can be cultured in laboratories. Primary cells from the breast are more representative of the phenotype and behavior of the cell-of-origin. However, primary cell cultures have disadvantages including short lifespan, limited access to tissue and technical difficulties, for instance, the

time-consuming process of cell isolation. Nonetheless, even if conditions have been improved and the lifespan has been extended by the culture of organoids (review by (McCauley & Wells, 2017)) or improving cell culture media (Sachs et al., 2018), many laboratories depend on established immortalized cell lines. Immortalization of cell lines is commonly generated by ectopic transfection in primary cells with agents that can inhibit or bypass replicative senescence, which permits us to perform long term studies (Kaur & Dufour, 2012).

Despite the development of *in vitro* techniques, there is still a need for representative cell cultures containing heterotypic interactions between cells and cell-substrate. For this effect, 3D cell cultures are useful tools using both primary cells or cell lines. The scaffolds are made of reconstituted basement membranes (rBM), which supply cells with collagens, laminins and other components of the BM. Cells are able to develop different 3D structures depending on the cell type. Moreover, there is the possibility of having more than one cell type in co-cultures. For instance, co-culturing of epithelial cells with stromal cells supports the epithelial cells to differentiate but also can lead to EMT (Briem et al., 2019b; Ingthorsson et al., 2010; Sigurdsson et al., 2011). The signals that stromal cells provide to epithelial cells can be driven by direct contact of the cells or indirectly where the rBM acts as the vehicle for growth factors that are delivered to epithelial cells (Aiello et al., 2018; Briem et al., 2019b; Calon et al., 2015; Chaudhuri et al., 2014; Hynes, 2009; Mina J. Bissell, 1982; Sigurdsson et al., 2011; Thiery et al., 2009).

1.4.1 *In vitro* cell culture models: immortalized cell lines

Immortalized cell lines are valuable tools to represent different cell types. There are representative breast normal cell lines that have been utilized to characterize breast differentiation and morphology. The most known breast normal cell lines are MCF-10a (Soule et al., 1990) and HMT-3522 (Petersen et al., 1992). Both cell lines were isolated from non-cancer individuals which had a breast proliferative or fibrocystic disease, respectively, and became immortalized spontaneously (Petersen et al., 1992; Soule et al., 1990).

Other breast normal cell lines, such as HMLE and D492, were isolated from mammoplasties from healthy donors and, afterward, were artificially immortalized. In HMLE, the immortalization took place by the insertion of the simian virus SV40 (Elenbaas et al., 2001) and, in D492, by the transduction of the E6 and E7 genes of the human papillomavirus (Gudjonsson et al., 2002b). These cell lines have been characterized and sublines have been generated in order to elucidate mechanisms in EMT and cancer progression (Briem et al., 2019b).

In addition, breast cancer cell lines are widely used in cancer research. For this purpose, there are many commercially available breast cancer cell lines and they try to provide representative sources of material for different BC subtypes. MCF-7, MDA-MB-231, SkBr3 and T-47D were isolated from pleural effusion of patients with invasive ductal carcinomas. MCF-7 (Soule et al., 1973) and T-47D (Horwitz et al., 1978) express estrogen and progesterone receptors, while SkBr3 overexpresses HER2 (Trempe & Fogh, 1973) and MDA-MB-231 shows a TNBC phenotype (Cailleau et al., 1974).

Despite being useful tools in cancer research, immortalized cell lines have their limitations. For instance, it has been difficult to obtain a suitable ER-positive normal cell line that could be used as a control in comparison with ER-positive breast cancer (included in the luminal BC subtype) cell lines in order to identify oncogenes and mechanisms that lead to tumorigenicity. Nevertheless, recently, Fridriksdottir and co-workers were able to isolate ER-positive breast primary cells from reduction mammoplasties and maintain its characteristics in culture by inhibiting the TGF-β receptor and using a specific culture medium (Fridriksdottir et al., 2015). Following the strategy, the same laboratory has successfully immortalized an ER-positive normal cell line (Hopkinson et al., 2017). Since over 70% of breast cancers are ER-positive (Fridriksdottir et al., 2015), a proper protocol for isolating and maintaining ER-positive normal cells in culture and their subsequent immortalization was a significant advance.

Nonetheless, cell lines have several drawbacks. First and foremost, the immortalization process itself is of concern, as immortalization involves disruption of conserved check-points in the cell cycle that can drive modifications in their genome that, consequently, can affect the cellular behavior (Garbe et al., 2014; Gudjonsson et al., 2002b; Gudjonsson et al., 2004; Hopkinson et al., 2017; Kaur & Dufour, 2012; Reddel, 2000). Furthermore, cell lines have commonly drifted significantly from their initial cell of origin, due to the high number of passages in culture and artificial culture environment (Kaur & Dufour, 2012). In addition, cell lines are not able to reflect the heterogeneity in an organ or a tumor. For these reasons, cell lines should be used carefully, and all their limitations must be taken into account.

1.4.2 D492 cell line and its cell sublines as *in vitro* model

The use of immortalized cell lines allows us to achieve long-term studies but also opens the opportunity to work with and compare isogenic cell lines that share a large part of their genome but differ in terms of gene expression, phenotype and other traits, such as tumorigenicity. This is the case of the cell

lines used in this thesis, D492 and its EMT-induced sublines D492M and D492HER2 (Fig. 5).

D492 is a breast epithelial cell line with stem cell properties that was isolated from a suprabasal subpopulation of normal breast tissue. It was initially established by isolating MUC1 negative, EpCAM positive cells from a reduction mammoplasty and subsequently immortalized using the E6 and E7 oncogenes from the human papillomavirus 16 (Gudjonsson et al., 2002b). Although D492 is non-tumorigenic, it is not completely normal because E6 and E7 bind and inactivate P53 and retinoblastoma, respectively. D492 expresses epithelial markers such as E-cadherin and cytokeratins (CK), including both luminal (CK8, CK18 and CK19) and myoepithelial (CK5/6, CK14). When D492 is cultured in 3D, it forms branching structures, similar to the TDLUs formed in the breast gland (Gudjonsson et al., 2002b; Sigurdsson et al., 2011). D492 has been characterized and used as a model to study morphogenesis in normal conditions, EMT (Sigurdsson et al., 2011) and tumorigenicity (Ingthorsson et al., 2016).

To study the heterotypic interactions between D492 and cells of the breast endothelium, breast endothelial cells (BRENCs) were co-cultured with D492 in 3D. The colony branching was increased in comparison to monocultures and interestingly, some colonies underwent EMT. This is how D492M was established. Further characterization revealed a switch of E-cadherin to N-cadherin and down-regulation of other epithelial markers (Sigurdsson et al., 2011).

Additionally, a tumorigenic cell line was generated by overexpressing the HER2 oncogene in D492. The exogenic expression was established by the lentiviral transduction of HER2. This effect triggered D492 to undergo EMT and as a result, it shows a similar phenotype to D492M in monolayer cultures. However, D492HER2 was able to form tumors when cells were injected into mice (Ingthorsson et al., 2016) while D492M is non-tumorigenic (Sigurdsson et al., 2011).

The isogenic cell lines D492, D492M and D492HER2 are cultured in H14 medium (Blaschke et al., 1994) that is supplemented with defined hormones and growth factors (detailed in Material and Methods chapter). This is a serum-free medium that has the advantage of being homogeneous with known components and amounts, as well as avoiding the additional risk of contaminants from the serum. This results in a more controlled environment for D492 and its sublines than for other breast cell lines that require the supplement of serum, such as MCF7 or MDA-MB-231.

In this thesis, I have mainly compared D492M (the endothelial-induced EMT cell line) and D492HER2 (the oncogene-induced EMT cell line), having D492 (the progenitor cell line) as control (Paper I) (Fig. 5) and then, D492 and D492HER2 (Paper II), in order to identify oncogenes that could support the tumorigenic abilities of D492HER2, evaluate their effects on cellular phenotype, and their crosstalk with the endothelial microenvironment.

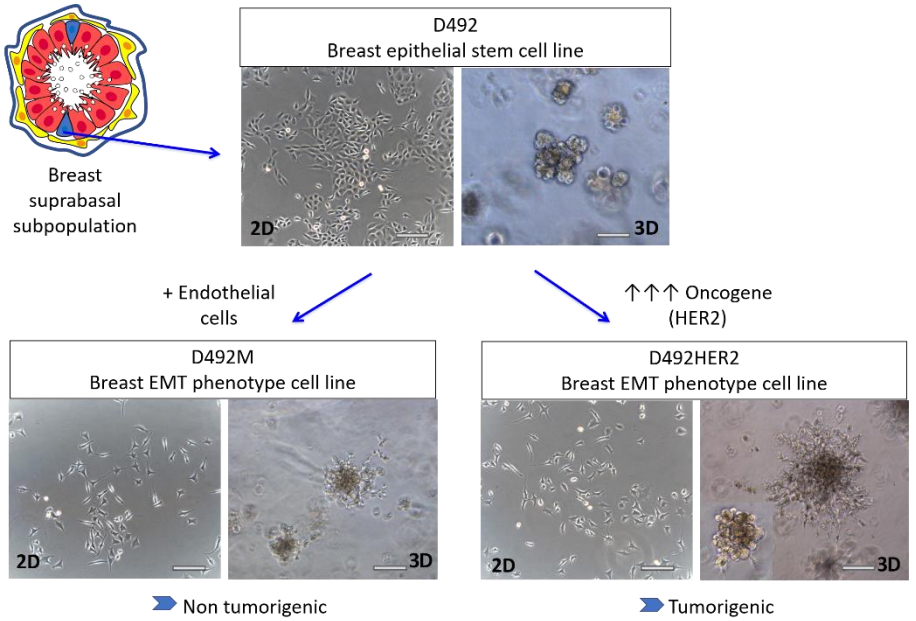

Figure 5. D492 isogenic cell lines: D492, D492M and D492HER2. At the top left corner, there is a representation of the organization of the epithelial cells in the breast gland. In the inner layer luminal epithelial cells (cells in red) produce the milk (drops in white). The outer layer is composed by myoepithelial cells (cells in yellow) and is surrounded by the basement membrane (line in blue). Stem cells (cells in blue) are in the suprabasal area. The D492 cell line originates from the isolation of a suprabasal subpopulation from healthy breast tissue. D492 has progenitor properties and forms TDLU-like structures (tree-like structures with polarized epithelial cells) in 3D cell cultures. The co-culture of D492 with endothelial cells gave rise to EMT in some colonies, establishing the D492M cell line, which forms spindle-like structures (elongated stellate-like structures) in 3D and is non-tumorigenic. When HER2 was overexpressed in D492, cells underwent EMT and generated the D492HER2 cell line. D492HER2 forms spindle-like (main picture) and grape-shaped structures (disorganized cells with poor cell-cell adhesion) (inserted picture at the same scale) in 3D and is tumorigenic. *Scale bar = 200μm.* Adapted from (Briem et al., 2019b; Morera et al., 2019).

2 Aims

Epithelial to mesenchymal transition (EMT) is a developmental process essential for organogenesis but also important in cancer where EMT is believed to be necessary for cell migration and metastasis. EMT can occur in multiple ways involving a wide range of molecules. Marker expression and cellular behavior define the state of the EMT phenotype. The objective of my Ph.D. project was to study two isogenic cell lines with EMT phenotype that differ in terms of marker expression and their ability to form tumors in mice. My objective was also to analyze how these isogenic cell lines interacted with the surrounding vascular stroma.

In my study, I mainly used three isogenic cell lines, thereof two with EMT profile. 1) D492, a breast epithelial cell line with progenitor properties that was isolated from a suprabasal population of healthy breast tissue (Gudjonsson et al., 2002b). 2) D492M, an EMT phenotype cell line that was isolated from a D492 colony that underwent EMT in a 3D co-culture with endothelial cells (Sigurdsson et al., 2011). 3) D492HER2, an EMT phenotype cell line that was oncogene-induced after overexpressing HER2 in D492 (Ingthorsson et al., 2016). Both D492M and D492HER2 show EMT phenotype, but D492M has a more fixed EMT phenotype and is non-tumorigenic while D492HER2 has a partial-EMT phenotype and is highly tumorigenic.

In this thesis, the objectives were focused on the comparison of these cell lines in order to:

1. Have a better understanding of the effects of the heterotypic interactions between the cell lines with different EMT profiles and endothelial cells.

2. Identify and select gene/ protein candidates that could explain the differences between the cell lines regarding EMT and tumorigenic properties.

3. Study in detail the selected candidates regarding differences in gene/ protein expression between the cell lines and their biological function.

To achieve these aims, functional and phenotypic analyses were performed in this Ph.D. project.

3 Materials and methods

In this chapter, I will describe the materials and methods I have used during my Ph.D. Technical considerations to some specific methods are detailed and discussed in the Aspects on methodology and experimental approach chapter.

3.1 Cell culture (2D and 3D)

The main cell lines used in this project are D492 (Gudjonsson et al., 2002), its endothelial-induced EMT cell line, D492M (Sigurdsson et al., 2011) and its oncogene-induced EMT cell line, D492HER2 (Ingthorsson et al., 2015). For cultures in monolayer, flasks or plates were pre-coated with collagen I (2.2%) (5005-B, Advanced BioMatrix). The medium used for culturing the cell lines was H14 (Blaschke et al., 1994), an enriched serum-free medium based on DMEM:F12 in which growth factors were added (insulin, transferrin, EGF, sodium selenite (NaSel), estradiol, hydrocortisone, prolactin).

Primary endothelial cells were also used in this project. Endothelial cells isolated from healthy breast tissue (BRENCs) from reduction mammoplasties following a previously published protocol (Sigurdsson et al., 2006). For other experiments, endothelial cells derived from the umbilical cord vein (HUVECs) are used. They were obtained from the National University Hospital (Landspitali), Reykjavik, Iceland, with informed consent and approved by the Landspitali ethical committee (No. 35/2013). Endothelial cells were cultured in EGM-2 (CC-3162, Lonza) +30% FBS in the first passage and later maintained in EGM-2 +5% FBS (further referred to as EGM-5).

In 3D cultures, cells were cultured in 300 µL reconstituted basement membrane, rBM, purchased as Matrigel (354230, Corning) per well, in 24-well plates (353047, Corning). In monoculture, 20.000 cells were embedded in Matrigel (354230, Corning) with 500µL of H14 media on top, while in co-cultures 500 cells of D492 or its sublines were co-cultured with 150.000-200.000 HUVECs, embedded in Matrigel, with 500µL of EGM-5 media on top.

Another modality of 3D cell culturing performed in this project was 3D cell cultures when cells were seeded on top of Matrigel. In this case, 25.000 cells per well were cultured in an 8-well µ-slide (Ibidi), 200µL of Matrigel (354230, Corning) per well were added and 200µL of the corresponding media on top (H14/ EGM-5), when cells were already settled.

Additional breast cancer cell lines were used in this project. The corresponding media to each cell line/ primary cells are referred to in table 1.

Table 1. Cell lines and primary cells used in this project. List of the cell lines and primary cells used and generated in this project with their correspondent culture media. References to the origin of the cell lines/ primary cells are included.

Cell lines	Medium	Reference
D492	H14	Gudjonsson et al., 2002
D492M	H14	Sigurdsson et al., 2011
D492HER2	H14	Ingthorsson et al., 2015
D492HER2 YKL-40 KD	H14	Article I
D492 YKL-40 OE	H14	Article I
D492M YKL-40 OE	H14	Article I
D492HER2 ECM1 KD	H14	Article II
D492 ECM1 OE	H14	Article II
D492M ECM1 OE	H14	Article II
D492EGFR	H14	Ingthorsson et al., 2015
D492EGFR/HER2	H14	Ingthorsson et al., 2015
D492-HER2 OE	H14	Aspects on methodology and experimental approach
D492M-HER2 OE	H14	Aspects on methodology and experimental approach
MCF-7	DMEM:F12+10% FBS	Soule et al., 1973
MDA-MB-231	RPMI+10% FBS	Cailleau et al., 1974
Primary cells	**Medium**	**Reference**
BRENCs	EGM-2+5% FBS	Sigurdsson et al., 2006
HUVECs	EGM-2+5% FBS	Provided by Dr Haraldur Halldórsson

3.2 Expression levels of genes and miRNAs by qRT-PCR analysis

Total RNA was isolated with cold Tri-Reagent (AM9738, Life Technologies). RNA precipitation was done using isopropanol and followed by high-speed centrifugation at 14.000 rpm for 20 min. Afterward, RNA was washed with ethanol a couple of times. The RNA pellet was diluted in RNAse free water. The absorbance of RNA was measured in the NanoDrop® ND-1000 UV/Vis-Spectrophotometer (Thermo Fisher Scientific) at 260 nm wavelength to assess the concentration and at 260/280 nm and 260/230 nm ratios to assess the purity. To write the cDNA, SuperScript IV (18090-200, Thermo Fisher

Scientific) and random Hex primers were used.

Table 2. qRT-PCR primers used in this project. List of the primers used for qRT-PCR in this project. Catalog number and company are included.

Primers	Cat. Number	Company
miR-200c	YP00204482	Qiagen
miR-203	205914	Exiqon
miR-205	204487	Exiqon
VEGF-A	Hs.PT.58.21234833	IDT
VEGF-C	Hs.PT.58.14602240	IDT
GUCA1C	Hs.PT.58.680712	IDT
CITED1	Hs.PT.58.1567731	IDT
MYBPH	Hs.PT.58.39772389	IDT
YKL-40/ CHI3L1	Hs.PT.58.22570467	IDT
KCNQ1OT1	Hs.PT.58.4572396.g	IDT
ERBB2	Hs.PT.58.1330269	IDT
GPR27	Hs.PT.58.38722549.g	IDT
S100A9	Hs.PT.58.20989743	IDT
DLK1	Hs.PT.58.40622309	IDT
GDF6	Hs.PT.58.20193545	IDT
PXDN	Hs.PT.58.630748	IDT
TLR4	Hs.PT.58.38700156.g	IDT
BMP4	Hs.PT.56a.3848863	IDT
CDH2	Hs.PT.58.26024443	IDT
VCAM1	Hs.PT.58.20405152	IDT
ECM1	Hs.PT.58.20438560	IDT
FABP4	Hs.PT.58.20106818	IDT
NOTCH1	Hs.PT.58.23074795	IDT
NOTCH3	Hs.PT.58.38492200	IDT

To quantify the expression level of genes, TaqMan probes (M3004L, New England Biolabs, NEB) or SYBR Green dye (M3003L, NEB) were used to detect gene expression. Comparative Ct values were determined using an ABI 7500 instrument (Applied Biosystems). All used primers are listed in table 2. GAPDH was used as the reference gene.

For miRNAs, total RNA was extracted with Tri-Reagent (AM9738, Thermo Fisher Scientific) as well but the RNA was reverse transcribed using miRCURY LNA RT Kit (339340, Qiagen) for cDNA synthesis reactions. Quantitative RT-

PCR analysis of miRNAs was performed using miRCURY LNA SYBR Green PCR Kit (339346, Qiagen). Relative expression was calculated with the $2\Delta\Delta Ct$ method. All used primers are listed in table 2. Normalization was done with U6 (203907, Exiqon).

3.3 Western Blot (WB)

Protein isolation was done using RIPA buffer and the concentration was measured by the colorimetric Bradford method. An equal amount of protein was loaded for each sample (5 µg) in precast NuPAGE 10% Bis-Tris gels (Invitrogen). Proteins were detected using IRDye secondary antibodies on an Odyssey imaging system (Li-Cor Biosciences). Antibodies used for WB are listed in table 3. Loading controls against actin or tubulin were used for quantification.

Table 3. Antibodies used in this project. List of the antibodies used in this project for Western Blot (WB) and immunofluorescence (IF). The dilutions used for each assay are included, as well as catalog number and company.

Antibodies	Assay	Cat. Number	Company	Dilution
Actin	WB	ab3280	Abcam	1:5000
Axl	IF	CS8661	Cell Signaling	1:100
CK14	WB	ab15461	Abcam	1:1000
CK19	IF	ab7754	Abcam	1:100
E-cadherin	WB	610182	BD Transduction Labs	1:1000
Tubulin	WB	ab6046	Abcam	1:5000
HER2	WB	CS2165	Cell Signaling	1:1000
Vimentin	WB	M0725	DAKO	1:1000
YKL-40/ CHI3L1	IF	H00001116 -B01P	Abnova	1:100
YKL-40/ CHI3L1	WB/ Neutralization	MABC196	Millipore	1:500
ECM1	IF	HPA027241	Sigma Aldrich	1:100
NOTCH1	IF	sc-376403	Santa Cruz	1:100
NOTCH3	IF	ab23426	Abcam	1:100

3.4 Immunofluorescence staining (IF)

Immunofluorescence staining (IF) was used to detect proteins and visualize their subcellular location. Cells were fixed with 3,7% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100. Blocking with FBS was done prior

to incubation with primary antibodies. Incubation was done overnight at 4ºC and followed by incubation of secondary antibodies that were conjugated with fluorochromes Alexa Fluor-488, 546 or 647 for one hour at room temperature. For nuclei staining DAPI was used. Imaging was done using an EVOS FL Auto 2 Cell Imaging System (Thermo Fisher Scientific) or FV1200 Olympus inverted confocal microscope. Antibodies used for IF are listed in table 3.

3.5 Enzyme-Linked ImmunoSorbent Assay (ELISA)

In order to detect the YKL-40 secreted protein, the Enzyme-Linked ImmunoSorbent Assay (ELISA) was performed. For this procedure, the kit Quantikine ELISA for Chi3L1 (DC3L10, R&D systems) was pursued and the specific protocol was followed. Briefly, a primary monoclonal antibody specific for YKL-40 was already pre-coated on the plate of the kit and when CM is added, YKL-40 is detected by the secondary antibody that was conjugated with peroxidase enzyme. Afterward, the substrate solution was added, and the colorimetric reaction took place. Optical density was measured at 540 nm, and 570 nm for correction. Quantification of the total secreted protein concentration was done using *Elisaanalysis* software.

3.6 Transcriptome data analysis and classification

Total RNA sequence profiles for D492, D492M and D492HER2 were obtained from normal cell cultures and treated following the protocol detailed in (Halldorsson et al., 2017).

Differentially expressed genes between cell lines were further classified into categories to identify enriched biological processes. Using a differential range from 2-fold change, genes were analyzed with the PANTHER analysis database (www.pantherdb.org) following instructions of (Mi et al., 2013).

3.7 Secretome data analysis

For mass spectrometry, D492 and its sublines were grown in T175 flasks (353112, Corning) until a confluence of 70-80% when the conditioned medium (CM) was collected. The CM was centrifuged at 2000 rpm for 3 minutes, sterile filtered through a 0.22 µm filter unit and concentrated for 55 minutes using EMD Millipore Amicon™ Ultra-15 Centrifugal Filter Units (UFC900324, Merck Millipore) followed by buffer exchange to 100 mM TRIS/ HCl buffer. Triplicate samples were stored at -80°C. Label-Free relative protein quantification by nLC MS/MS after trypsin digestion was performed at the FingerPrints Proteomics Facility, University of Dundee, UK, and raw data was analyzed

using MaxQuant software (version 1.6.2.1). Quantitative and statistical analyses were performed using XLStat (version 2018.1). Data were P-value corrected (significance level 0.05) and sorted based on ≥2-fold higher secretion (LFQ intensity) by D492HER2 compared to D492M (Paper I) and D492HER2 compared to D492 (Paper II).

3.8 Migration and invasion assays

The migration and invasion assays were done in 24-well plates with transwell filter inserts (353097, Corning) of 8 µm size pore diameter. Transwells in the migration assay were pre-coated with collagen I (2,2%) (5005-B, Advanced BioMatrix) in Paper I and in the invasion assay, they were pre-coated with a dilution of 1:10 Matrigel (354230, Corning) in H14. 50.000 cells/transwell were seeded on the upper chamber in normal medium. In the bottom chamber, the H14 medium was supplemented with 10% FBS as a chemoattractant (Fig. 6). A cotton swab was used to remove non-migrated and non-invaded cells after 24 h and after 48 h, respectively. Thereafter, cells were fixed with 3,7% PFA and stained with crystal violet (10%) or DAPI (1:5000 dilution) to count them. Three random pictures were taken per well and the number of cells was quantified. For DAPI stained samples, images were converted to 8-bit in *ImageJ* (version 2.0.0), threshold-adjusted and binary-converted, and migratory/ invasive cells were counted using the *analyze particles* function.

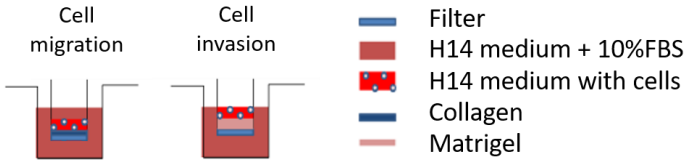

Figure 6. Schematic setup of the transwells used for migration and invasion assays *in vitro*. Components of the transwells setup in migration and invasion assays. In the migration assay, filters are pre-coated with collagen in Paper I, while in the invasion assay, filters are pre-coated with Matrigel (1:10). 10% FBS is used as chemoattractant for cells to migrate /invade through the transwell filters.

3.9 Proliferation assays

In order to test proliferation, cells were seeded in a 24-well plate at a density of 10.000 cells/well in triplicates in H14 (D492 cell lines) or EGM-5 (HUVECS). Daily (or every 2 days in the case of HUVECs), cells were fixed and stained with crystal violet (10%). Crystal violet was diluted with acetic acid and the optical density (OD) at 540 nm wavelength was measured.

In addition, cell viability was assessed using PrestoBlue™ Cell Viability Reagent (Thermo Fischer Scientific). Cells were seeded in a 96-well plate at a

density of 3000 cells/well in H14 media and cultured for 4 days. PrestoBlue™ was added at a concentration of 1:10 to each well and incubated for 4 h. Absorbance was measured on a plate reader at 570 nm and 595 nm.

3.10 Apoptosis assay

Apoptosis was induced by incubating cells with 10 µM camptothecin (CPT) for 24 h. To quantify apoptosis, cleavage of Caspase 3/7 was measured by a luciferase assay (ApoTox-Glo™ Triplex Assay, Promega), following the protocol of the manufacturer Briefly, after cellular lysis, cleavage of caspase 3/7 was exposed and acted as a substrate for luciferase reactions. Subsequently, luminescence was produced and measured with a microplate reader Modulus TM II (Turner Biosystems).

3.11 Glucose consumption and lactate production measurements

Glucose uptake was measured using the Glucose Uptake-Glo™ kit (J1341, Promega) following the manufacturer's protocol. Briefly, the analog of glucose, 2-deoxyglucose (2DG), was added to the media and taken up by cells. When transported into cells, 2DG was phosphorylated to 2-deoxyglucose 6-phosphate (2DG6P) and further metabolization stimulated luciferase reactions and luminescence was measured by the microplate reader Modulus TM II (Turner Biosystems).

Glucose consumption and lactate production were measured from the collected media when cells were grown in high confluency. Metabolites are measured at the Analyzer machine (ABL90 FLEX Analyzer, Radiometer) at the Blood Bank of Landspitali (Reykjavik, Iceland).

3.12 Neutralization assay of YKL-40 protein

Monoclonal antibody against YKL-40 (mA^{YKL-40}) (MABC196, Millipore) was used to block the secretion of YKL-40 in D492HER2 cells. The antibody was diluted in fresh H14 medium at a concentration of 10 µg/mL and cells were treated for 24 h. Medium from cells incubated with mA^{YKL-40} was collected and medium from non-treated D492HER2 cells was used as control. CM was used for tube formation assays (described below).

3.13 Tube formation assay of endothelial cells (angiogenesis assay *in vitro*)

To simulate angiogenesis in vitro, 10.000-12.000 endothelial cells were

seeded on top of 10 µL Matrigel in 96-well angiogenesis plates (89646, Ibidi). Controls included BRENCs or HUVECs cultured in EGM-5 media and a dilution of 1:1 EGM-5 and conditioned media (CM). Recombinant protein of YKL-40 (YKL-40^{r}) (11227H08H5, Thermo Fisher Scientific) (Paper I) was added to the medium at a final concentration of 100 ng/mL and cells were incubated overnight.

A similar strategy was used in Paper II when recombinant ECM protein (rECM1) (TP723147, Origene) was added to the ECs culture at the concentration of 60 ng/mL. For endothelial NOTCH inhibition, gamma-secretase inhibitor DAPT (D5942, Sigma-Aldrich) was added to the media in a final concentration of 20 µM.

The endothelial network was imaged with the EVOS FL Auto 2 Cell Imaging System. Analysis and quantification of parameters were done using the *Angiogenesis analyzer* plug-in on *ImageJ* software (version 2.0.0) (Fig. 7).

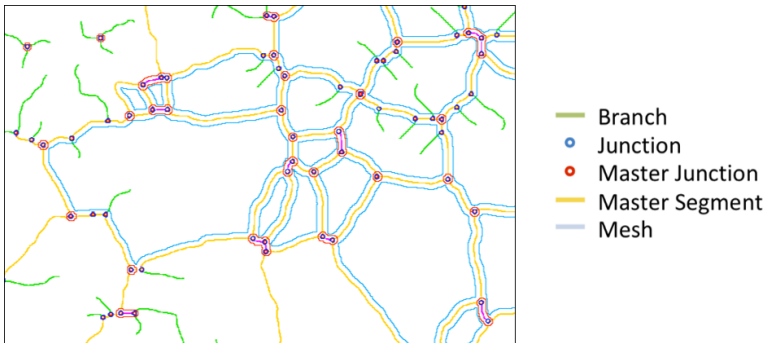

Figure 7. Representative picture of the parameters measured by *Angiogenesis analyzer* plug-in on *ImageJ* software (version 2.0.0). Description of the parameters: Branch: element delimited by a junction and one extremity. Junction: node (circular dot composed of pixels with 3 neighbors) or a group of combining nodes. Master junctions: junctions that link at least three master segments. Two close fusing master junctions can be counted as only one master junction. Master segments: pieces delimited by two junctions and none of them are exclusively implicated with one only branch (which are master junctions). Total master segments length: the addition of the length of all master segments. Meshes: areas enclosed by segments or master segments.

3.14 Knock-down/ Overexpression of target genes

Transient transfection of YKL-40 (Paper I) and ECM1 (Paper II) to knock-down genes in D492HER2 by siRNAs was performed in the thesis. To get permanent changes in expression, for both, knock-down and overexpression of YKL-40 and ECM1, stable cell lines were created by different strategies using CRISPR technologies. Stable overexpression of HER2 in D492 and D492M was carried out by lentiviral transduction.

3.14.1 Knock-down of YKL-40 and ECM1 by siRNA transient transfection

Pre-designed siRNAs (Silencer® Select Pre-Designed, Validated and Custom siRNA, Life Technologies) against YKL-40 (Paper I) or ECM1 (Paper II) were used at a concentration of 10nM in D492HER2 cells to down-regulate YKL-40 or ECM1, respectively. First, cells were seeded on a 6-well plate (353046, Corning) and, after 24 h, cells were transfected using a chemical transfection with lipofectamine® RNAiMAX (13778150, Life Technologies). For each experiment, siRNAs against YKL-40 or ECM1 and a siRNA negative control have been used. The siRNAs are listed in table 4. After 48 h, the knock-down was confirmed by qRT-PCR and WB.

Table 4. siRNAs used in this project. List of the siRNAs used to transiently knock-down (KD) YKL-40 and ECM1, respectively. Catalog number and company are included.

siRNAs	Product name	Cat. Number	Company
Neg Ctrl siRNA	Silencer® Select Negative Control No. 1 siRNA	4390843	Thermo Fisher Scientific
YKL-40 siRNA 1	Silencer® Select CHI3L1 siRNA s2998	AM16708 (ID 119124)	Thermo Fisher Scientific
YKL-40 siRNA 2	Silencer® Select CHI3L1 siRNA s3000	AM16708 (ID 119126)	Thermo Fisher Scientific
ECM1 siRNA	Silencer® Select ECM1 siRNA s4441	4392420	Thermo Fisher Scientific

3.14.2 Generation of stable cell lines with the knock-down of YKL-40 in D492HER2 by CRISPR

Specific gRNAs to knock-down YKL-40 were designed with the publicly available online ATUM CRISPR tool (https://www.atum.bio/). Annealing of the forward and reverse gRNA oligos was followed by ligation with the backbone vector pMLM3636. High-efficiency competent bacteria (C2987H, NEB) were transformed and confirmation of successful DNA insertion was done by colony PCR. Plasmids were sequenced to confirm correct gRNA sequences. Transfection of D492HER2 was done with four plasmids gRNAs each and a plasmid with only a Cas9 cassette (pST1374) as control using Lipofectamine® 3000 (Thermo Fisher Scientific). The control cell line was generated by transfecting the cells with only Cas9 (without gRNA). The selection of cells with the insertion of Cas9 was done using blasticidin. The knock-down of YKL-40 in D492HER2 was confirmed by qRT-PCR, WB and ELISA. Subsequently, the cell line that gave the best efficiency of knock-down was selected for further work. The sequence of this gRNA is in table 5. It should be noted that

D492HER2 is a cell line not suitable for single-cell cloning, therefore a pool of cells was used for confirmation of knock-down and further experiments (see Aspects on methodology and experimental approach chapter).

3.14.3 Generation of stable cell lines overexpressing YKL-40/ ECM1 in D492 and D492M by CRISPRa

Overexpression of YKL-40 (Paper I) and ECM1 (Paper II) in D492 and D492M was carried out using a variant of the CRISPR technology, CRISPR activation (CRISPRa) (Zhang et al., 2015). Two specific SAM gRNAs for YKL-40 or ECM1 and one empty control have been pursued from Genscript containing zeocin resistance cassette. The gRNA sequences are listed in table 5. Transfection of HEK293T cells was done to produce viral particles containing gRNAs. Viruses collected were used to infect D492 and D492M cells that were previously transfected with dCas9 VP64 to activate the promoter and later induce overexpression of the selected gene. Transduction with gRNAs was performed and the use of the two gRNAs at the same time showed the highest efficiency in overexpression. Selection was done with Zeocin (R25005, Thermo Fisher Scientific). Confirmation of overexpression of YKL-40 in D492 and D492M was done by qRT-PCR, WB, IF and ELISA (see Aspects on methodology and experimental approach chapter).

Table 5. gRNAs used in this project. List of the gRNAs and their sequences used in this project to knock-down (KD) or overexpress (OV/ OE) YKL-40 and ECM1, respectively, by CRISPR technology.

gRNAs	Sequence	Company
YKL-40/ CHI3L1 KD	CCGCCATTTCTGCGCACCCA	Manufactured
YKL-40/ CHI3L1 SAM OE 1	AGTTTTGAAAACTTTGGGTC	Genscript
YKL-40/ CHI3L1 SAM OE 2	CTGCCAGCAGAAGAGCCACT	Genscript
ECM1 KD 1	GTGGTCAGTTGCCCCAGGAT	Genscript
ECM1 KD 2	GCCGGCCACTGAAGCTTGTC	Genscript
ECM1 SAM OE 1	CATCTACAGGCTGCCTTCTG	Genscript
ECM1 SAM OE 2	GAAACTGAGGCACAAACTAG	Genscript

3.14.4 Generation of stable cell lines with knock-down of ECM1 in D492HER2 by CRISPRi

CRISPR interference (CRISPRi) system was selected to inhibit ECM1 in D492HER2. Following a similar strategy to CRISPRa, gRNAs pre-designed for KD ECM1 was purchased from Genscript (the gRNA sequences are listed in table 5) and cloned into a sgRNA(MS2)_zeo plasmid. As negative control, an

empty plenti sgRNA(MS2)_zeo plasmid was utilized (Genscript). For the production of lentiviral particles, HEK293T cells were used and viruses were collected 48 h and 72 h after transfection. D492HER2 were transduced before with pHR-SFFV-KRAB-dCas9-P2A-mCherry plasmid for repression and sorted for mCherry. Transduction with lentivirus containing gRNA plasmids for knocking-down (D492HER2 containing dCas9-KRAB cassette) was performed and cells were selected with Zeocin (Invitrogen). ECM1 KD was confirmed by qRT-PCR.

3.14.5 Generation of stable cell lines overexpressing HER2 in D492 and D492M by lentiviral transduction

Stable cell lines overexpressing HER2 in D492 and D492M by lentiviral transduction were generated. HER2 construct (16257) was purchased from Addgene and inserted in lentiviral GFP-positive vectors (Ingthorsson et al., 2015). Viral particles were produced in HEK293T cells after transfection with TurboFect. The supernatant containing viruses was collected after 48 and 72 hours. Target cells D492 and D492M were transduced with HER2 lentiviral plasmids and empty vector viruses as control. Successfully transduced cells were GFP-positive and were sorted by flow cytometry in the cell sorter (SH800 Cell Sorter, SONY). The confirmation of HER2 overexpression was tested by WB.

3.15 RNA microarray of endothelial cells

For RNA microarray analysis, D492, D492M and D492HER2 cells with and without ECM1 overexpression/ knock-down were grown until 80% confluence. CM was collected after 48 h and mixed with fresh EGM-5 (ratio 1:1) and added to HUVECs that were cultured to 40% confluence. After 24 h, RNA was extracted using the RNeasy Mini Kit (Qiagen). Sample concentration and quality were analyzed using the Agilent 2100 Bioanalyzer System (Agilent). As RNA microarray, Affymetrix human Clariom S Assay was performed at the sequencing core facility of the German Cancer Research Center (DKFZ) Heidelberg, Germany. Microarray candidates were validated using qPCR.

3.16 Statistical analysis

Data were presented as means of triplicate experiments with triplicate samples with standard deviations (SD) that were represented as error bars, unless stated otherwise. Student two-tailed T-test was used to analyze statistical differences between two population samples and ANOVA analysis was

performed among more than 2 population samples. P-values below 0.05 were considered significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

4 Results and discussion

In this chapter, I will discuss the main results I have collected during my Ph.D. project. Initially, I will present some unpublished data that were the basis of my thesis regarding heterotypic interactions between D492, D492M and D492HER2, and endothelial cells.

Secondly, I will discuss the results included in the papers and unpublished data that form the core of my thesis. In Paper I, I phenotypically and functionally compared D492M (endothelial-induced EMT) and D492HER2 (oncogene-induced EMT) cell lines. Subsequently, in order to explain the differences regarding tumorigenesis between the cell lines, I identified an interesting protein, YKL-40, secreted by D492HER2, that may support cancer progression and induces angiogenesis in ECs. In Paper II, I will present ECM1 as a relevant intermediary of the crosstalk between tumor cells and ECs supporting cancer progression and angiogenesis, similar to YKL-40. I will conclude this chapter with a discussion of some unpublished data about the relation between YKL-40 and the tumorigenic properties provided by HER2.

4.1 Crosstalk between endothelial cells and isogenic D492 cell lines (unpublished data)

Our group and others have previously shown that endothelial cells can induce cell proliferation, increased colony formation and branching morphogenesis of breast epithelial cells (Ingthorsson et al., 2010; Shekhar et al., 2000; Sigurdsson et al., 2011). Moreover, it has been observed that epithelial cells in contact with endothelial cells can undergo EMT (Briem et al., 2019b; Sigurdsson et al., 2011; Zhang et al., 2014). These effects were detected in co-cultures of D492 with endothelial cells (Sigurdsson et al., 2011). With this in mind, I was interested in studying whether endothelial cells had a similar effect on cells that had already undergone EMT. Indeed, 3D cell co-cultures with breast endothelial cells (BRENCs) and D492M or D492HER2 cell lines resulted in increased colony size. Furthermore, BRENCs facilitated clonal growth of the cell lines and the required number of cells for the colony growth was remarkably reduced, suggesting an increase in cell survival, not only in D492 but also in D492M and D492HER2 (Fig. 8A).

Previous experiments in our laboratory showed that indirect co-cultures with BRENCs in the upper chamber of transwells and D492 in the bottom

compartment had similar effects on D492 as with direct co-cultures (Sigurdsson et al., 2011). In order to investigate if the contact between cells was required to see effects, collected medium (or conditioned medium, CM) from BRENCs was added to D492M and D492HER2 cultures seeded beforehand on top of Matrigel (Fig. 8B). When D492M and D492HER2 were treated with CM from BRENCs, the 3D structures started to grow earlier than those treated with only fresh media. This indicated that the heterotypic interactions are not merely mediated by direct contact, also secreted factors from endothelial cells induce morphogenesis of the isogenic D492 cell lines *in vitro*. In addition, D492HER2 showed more 3D cell structures than D492M, suggesting that D492HER2 has a stronger interaction with BRENCs than D492M.

Analysis of the time-lapse video of direct co-cultures using fluorescent-labeled cells placed on top of Matrigel led to the same conclusion (Fig. 8C). The crosstalk between endothelial cells and the cell lines was reciprocal and stronger between BRENCs and D492HER2 than D492M. Collectively, these data showed crosstalk between ECs and D492M and D492HER2 cell lines, with the greatest effects seen between ECs and D492HER2.

Primary BRENCs were successfully isolated for these studies following the protocol that was previously established in the lab (Sigurdsson et al., 2006). However, the isolation process was time-consuming and similar results were obtained using human umbilical endothelial cells (HUVECs), therefore, alternatively, we used HUVECs in the rest of the experiments conducted in my thesis. The formation of the different 3D structures in co-cultures using BRENCs and HUVECs (in low passages) were similar for each cell line in-lab work (data not shown and no breast-endothelial specific effects were mentioned in (Sigurdsson et al., 2011)) and the capillary-like networks using both ECs demonstrated similar effects, showing the same behavior after treatments/ stimulus used in Paper I (data not shown).

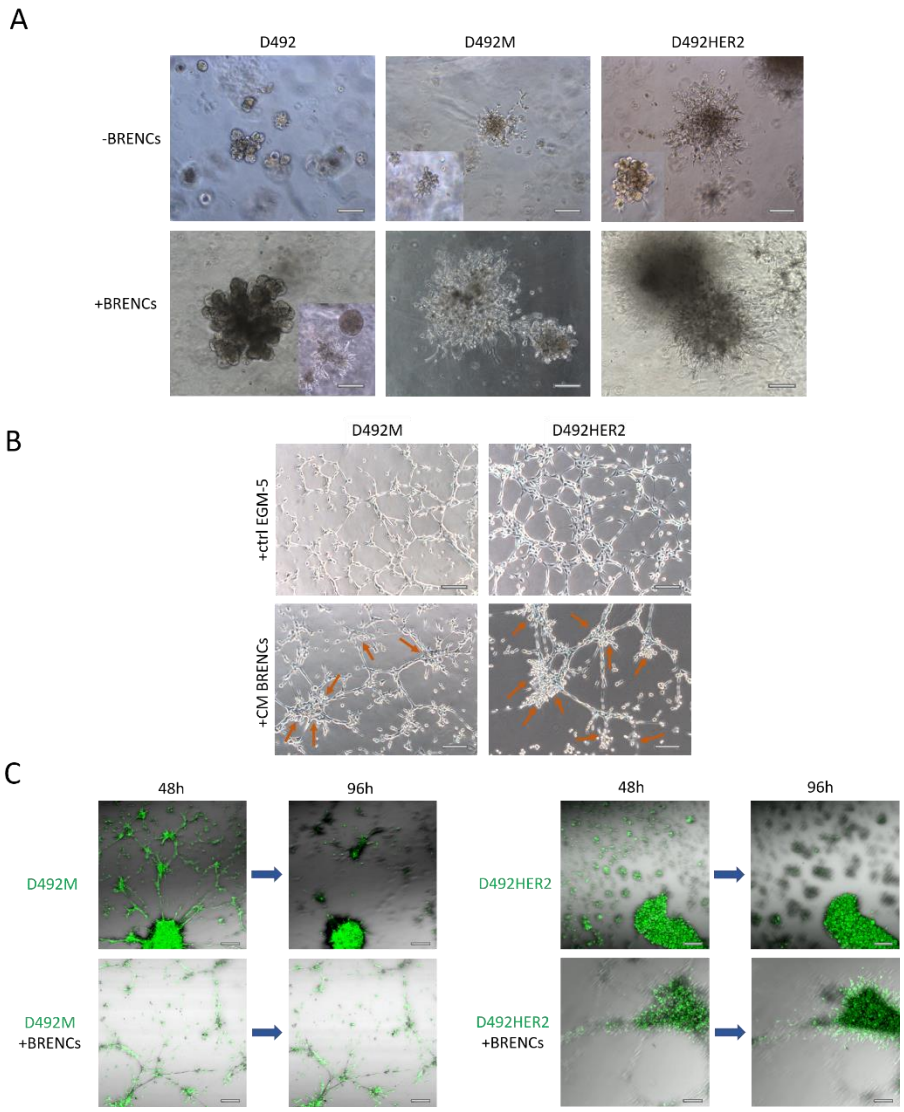

Figure 8. Interactions between the D492 sublines and endothelial cells (ECs) (phase-contrast pictures). A. The 3D cell cultures of D492 show TDLU-like structures (tree-like structures with polarized epithelial cells), while D492M cells form spindle-like structures (elongated stellate-like structures) in 3D and D492HER2 cells, spindle-like and grape-like structures (disorganized cells with poor cell-cell adhesion). Co-cultures in Matrigel of the cell lines with ECs increase the size of the 3D colonies of the cells. D492 cells form TDLU-like and round structures, but also some colonies undergo EMT in co-culture with BRENCs. *Scale bar = 200μm* in all images (including inserts). **B.** Conditioned medium (CM) of ECs accelerates the formation of the 3D structures of the cell lines in 24h. Arrows indicate 3D structures *Scale bar = 100μm.* **C.** Screenshots of a time-lapse video showing trajectories of the cell lines in co-culture with ECs on top of Matrigel. D492HER2 shows stronger effects due to ECs interaction than D492M. *Scale bar = 100μm.*

4.2 Paper I. YKL-40/ CHI3L1 facilitates migration and invasion in HER2 overexpressing breast epithelial progenitor cells and generates a niche for capillary-like network formation

In this article, I initially compared the phenotypic and functional differences between D492M and D492HER2 (having D492 as the reference cell line), positioning D492HER2 as a more partial-EMT cell line than D492M. Then, I identified YKL-40 as an important intermediary differently expressed in the D492 sublines and demonstrated its role in angiogenesis and tumorigenic properties. YKL-40, which was highly expressed in D492HER2, was essential for the ability of D49HER2 cells to migrate and invade. YKL-40, secreted by D492HER2 cells, induced the formation of capillary-like networks in endothelial cells. Furthermore, YKL-40 had an important role in the 3D phenotype of the cells when it was intrinsically expressed or added to the media.

4.2.1 Phenotypic and functional characterization of the isogenic D492 cell lines

D492M and D492HER2 are isogenic cell lines that share an EMT phenotype but are different in terms of their capability to form tumors in mice (Briem et al., 2019b; Ingthorsson et al., 2016; Sigurdsson et al., 2011). Initially, we wanted to explore phenotypically and functionally the differences between the two cell lines. D492M and D492HER2 were both derived from D492, a breast epithelial progenitor cell line, through endothelial-induced EMT and oncogene-induced EMT, respectively (Ingthorsson et al., 2016; Sigurdsson et al., 2011).

Reflecting their mesenchymal transition, D492M and D492HER2 have lost expression of some epithelial proteins such as E-cadherin, CK14 and CK19, and gained expression of some mesenchymal markers, vimentin and Axl, and in the case of D492M, N-cadherin (Fig. 9A and 9B). Moreover, microRNAs (miRs) such as miR-200c, miR-203 and miR-205 that are frequently downregulated in epithelial cells undergoing EMT (DeCastro et al., 2013; Hilmarsdottir et al., 2014; Hilmarsdottir et al., 2015; Moes et al., 2012; Wiklund et al., 2010) showed a high expression in D492 while they were greatly reduced in D492HER2 and almost absent in D492M (Fig. 9C). A paper from our laboratory demonstrated that the reintroduction of miR-200c-141 into D492M was sufficient to revert the mesenchymal phenotype to epithelial, albeit only to luminal epithelial lineage. The paper also showed that the basal cell transcription factor p63 was necessary to recover the basal/ myoepithelial phenotype. The co-transfection of both miR-200c-141 and p63 into D492M permitted the regain of the original bipotent phenotype of D492 (Hilmarsdottir

et al., 2015). Recently, another small RNA, miR203a, was overexpressed in D492M resulting in induced partial-MET. Consequently, proliferation, migration and invasion were reduced, but the expression of N-cadherin and Snai2 was partially reduced (Briem et al., 2019a).

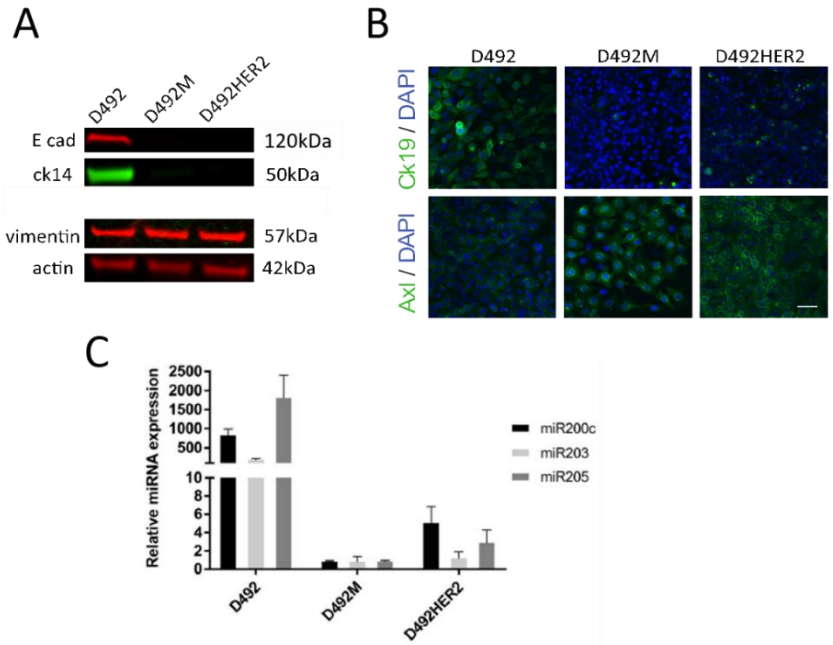

Figure 9. Expression of epithelial and mesenchymal markers in D492, D492M and D492HER2. A. Protein expression of E-cadherin (E-cad), cytokeratin 14 (CK14) and vimentin in the cell lines evaluated by WB. **B.** Protein expression of cytokeratin 19 (CK19) and Axl in the cell lines by IF. *Scale bar = 100µm*. **C.** MicroRNA expression of miR200c, miR203 and miR205 in the cell lines analyzed by qPCR. *Fig. 1B, 1C in Paper I.*

These differences in epithelial and mesenchymal marker expression between D492M and D492HER2 revealed that D492HER2 has a more intermediate EMT phenotype compared to D492M, placing D492HER2 as a cell line with a partial-EMT phenotype (Briem et al., 2019b; Ingthorsson et al., 2016). Very rarely, a complete EMT occurs, rather intermediate states are the most frequent phenotypes, which can also be referred to as cellular plasticity. Therefore, traces of epithelial traits usually remain, and a mesenchymal transition is often not complete, leading to a broad spectrum of EMT phenotypes (Nieto, 2013). D492M and D492HER2 shared the loss of E-cadherin and cytokeratins expression and the formation of a spindle-shaped phenotype in monolayer. However, D492M had a more fixed EMT phenotype with low or absent expression of epithelial markers, including microRNAs such

as miR-200c-141, miR-203 and miR-205, and gained expression of mesenchymal markers such as N-cadherin (Hilmarsdottir et al., 2015; Sigurdsson et al., 2011).

The functional characterization of the cell lines also demonstrated differences between them. Notably, D492HER2 cells migrated and invaded through transwell filters more efficiently than both D492M and D492 (Fig. 10A). The cell proliferation rate of D492HER2 cells was also significantly higher than in D492M and D492 cells (Fig. 10B). These are important properties in tumor growth (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011) that support the tumorigenic abilities of D492HER2. Despite this, D492HER2 cells were more susceptible to chemically induced apoptosis than D492 and D492M cells (Fig. 10C).

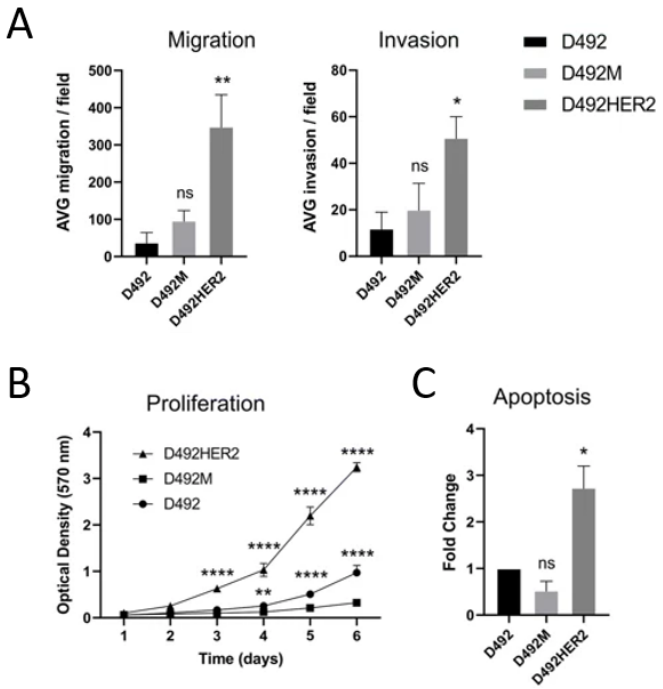

Figure 10. Functional characterization *in vitro* of D492, D492M and D492HER2. A. D492HER2 cells significantly migrate and invade more than D492 and D492M. Migration and invasion assays assessed *in vitro* in transwell filters. **B.** D492HER2 cells significantly proliferate more than D492 and D492M. Crystal-violet proliferation assay. **C.** D492HER2 cells are more susceptible to apoptosis than D492 and D492M. Apoptosis rate was assessed by caspase 3/7 luciferase activity that was measured by luminescence and normalized to D492. *Fig. 1D, 1E, 1G in Paper I.*

Tumor cells can develop drug resistance and are frequently resistant to induced-apoptosis (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011).

Camptothecin is a drug that targets the DNA topoisomerase I in the S-phase and, subsequently, the DNA replication. For this reason, highly proliferative cells, such as tumor cells, are susceptible to this drug (Legarza & Yang, 2006), which might be the case of D492HER2 cells.

In addition, supporting the intermediate EMT phenotype of D492HER2 is its glucose metabolism which is more comparable to D492 rather than D492M (Fig. 11A, 11B, 11C). The uptake of glucose in D492HER2 was more similar to the epithelial D492 cell line (Fig. 11A). Furthermore, there was an apparent shift from oxidative phosphorylation toward glycolysis in D492HER2 cells since they had higher glucose consumption and higher lactate production than either D492 and D492M cells (Fig. 11B and 11C). This glycolytic metabolism is often observed in tumor cells which demand more energy to support their increased proliferation (Carmeliet & Jain, 2000; Hanahan & Weinberg, 2011). By inducing glycolysis, glucose is broken down and the resulted pyruvate is converted into lactic acid in the process known as the Warburg effect (Hanahan & Weinberg, 2011; Pavlova & Thompson, 2016; Warburg, 1956). High glycolytic activity in D492HER2 compared to D492 (and D492M) may be explained by a higher proliferation rate and tumorigenic properties of D492HER2.

Figure 11. Glucose metabolism in D492, D492M and D492HER2. A. Levels of glucose uptake in the cells (measured by luciferase activity). **B.** Glucose consumption measuring glucose metabolites in CM by the ABL90 FLEX Analyzer (Radiometer). **C.** Lactate secretion measuring lactate metabolites in CM by the ABL90 FLEX Analyzer (Radiometer). *Supplemental data, Fig. S1 in Paper I.*

Previous studies described a glycolytic metabolism in D492 when it was compared to D492M metabolism (Choudhary et al., 2016; Halldorsson et al., 2017). The transcriptome analysis demonstrated that D492 had a higher expression of glycolytic genes than D492M (Choudhary et al., 2016; Halldorsson et al., 2017). Moreover, a functional characterization showed that D492 consumed more glucose and secreted more lactate than D492M,

indicating a shift toward glycolysis in D492 (Choudhary et al., 2016). This effect might be due to a higher proliferation rate in D492 compared to D492M.

Differences between the cell lines regarding glucose metabolism may be related to the differences between the cell lines in proliferation and tumorigenic properties, but also may be due to the EMT phenotype. In a previous study on breast cancer, it was shown when cancer cells underwent EMT, they also had increased glycolysis (Kondaveeti et al., 2015). However, a reduction in glycolysis in cells that have undergone EMT, as seen in D492M compared to D492, was observed in human non-small cell lung carcinoma (NSCLC) cells during EMT (Thomson et al., 2011). These data suggest that differences in the regulation of EMT may depend on the specific context of the microenvironment. Thus, a better understanding of glucose metabolism is needed for understanding EMT regulation and how this may contribute to aggressiveness and the dissemination of cells to distant organs. These studies may be of clinical relevance in cancer therapies.

4.2.2 Transcriptome and secretome analyses: Selection of YKL-40 as the candidate to explain differences between D492M and D492HER2 regarding tumorigenic properties

The functional differences between D492M and D492HER2 in terms of tumorigenicity prompted us to investigate the gene and protein expression of D492M and D492HER2 to search for potential candidates responsible for these effects. Subsequently, transcriptome analysis revealed differences in gene expression in the isogenic cell lines. Between D492M and D492HER2, there were more than 40.000 transcripts differently expressed that were sorted by different fold-change (Fig. 2A Paper I) and classified into enriched biological processes using the PANTHER analysis database (Mi et al., 2013) (Fig. 2B Paper I).

A gene that was highly expressed in D492HER2 compared to D492M was the Vascular Cell Adhesion Molecule 1 (VCAM1). VCAM1 was listed as enriched biological processes related to cell-cell and cell-substrate interactions, such as inflammatory response, cell chemotaxis, cytokine-mediated signaling pathway, cellular response to cytokine stimulus and defense response. VCAM1 was a relevant candidate due to its connection with the ECM modulation by the angiogenesis of the vascular network and EMT (Pein & Oskarsson, 2015), and with metastasis (Dokic & Dettman, 2006; Hoye & Erler, 2016; Pein & Oskarsson, 2015) in brain and bones during breast cancer (Lee et al., 2016; Lu et al., 2011). Moreover, VCAM1 is essential for the

adhesion of immune cells, such as leukocytes and lymphocytes, to the vascular endothelium (Fernandez-Borja et al., 2010).

The expression of VCAM1 was analyzed in the cell lines and was higher expressed in D492HER2 compared to D492M and D492 at RNA and protein levels (Fig. 12). However, the gene expression in D492HER2 was lower than in ECs (Fig. 12A), which suggested that VCAM1 expression in D492HER2 was not highly expressed at absolute value. Thus, I looked for a candidate whose expression could be more relevant.

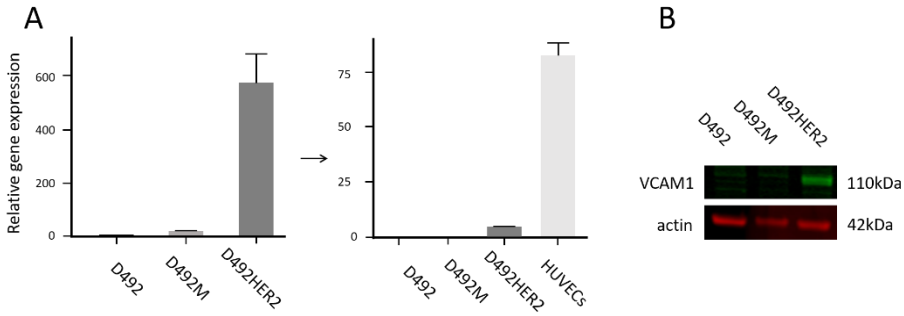

Figure 12. Gene and protein expression of VCAM1. A. Gene expression of VCAM1 in D492 sublines and HUVECs. D492HER2 revealed high expression compared to D492 and D492M by qPCR, but not to HUVECs. **B.** Protein expression of VCAM1 in D492 cell sublines by WB.

To find the most relevant and probable candidate to explain the differences in tumorigenicity between D492M and D492HER2, the focus was pointed to the 15 top candidates differently expressed. In Fig. 2A Paper I, gene expression was confirmed by qPCR for 10 genes highly expressed in D492HER2 compared to D492M and 5 genes highly expressed in D492M compared to D492HER2. As expected, HER2 (ErbB2) was highly expressed in D492HER2 cells due to its ectopic overexpression (Ingthorsson et al., 2016). However, the three top gene candidates in the transcriptome analysis (GUCA1C, CITED1 and MYBPH) did not show a higher expression by qPCR in D492HER2 compared to D492M. GUCA1C and CITED1 had almost undetectable expressions in D492HER2, and MYBPH had a similar expression compared to D492M. Consequently, these candidates were excluded as valid candidates and the study was focused on the next candidate, CHI3L1, also known as YKL-40.

I confirmed that YKL-40 was higher expressed in D492HER2 compared to D492M and D492 at RNA and protein levels (Fig. 13A). Moreover, the classification of the differentially expressed genes between D492M and D492HER2 with the PANTHER analysis database (Mi et al., 2013)

demonstrated that YKL-40 was listed in enriched biological processes related to cell-cell and cell-substrate interactions: inflammatory response, cell chemotaxis, cellular response to cytokine stimulus and defense response (Fig. 2B Paper I).

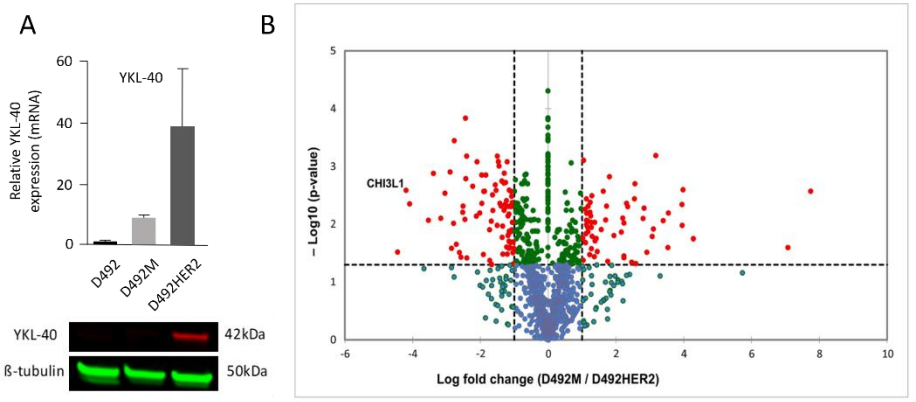

Figure 13. Gene and protein expression of YKL-40. A. Confirmation of gene expression (by qPCR) and protein expression (by WB) of YKL-40 in D492, D492M and D492HER2. **B.** Secretome data reveal that YKL-40 (also known as CHI3L1) is one of the proteins more secreted by D492HER2 compared to D492M. Data represented in a volcano plot. *Fig. 2C, 3A in Paper I.*

YKL-40 is a secreted glycoprotein that belongs to the chitinase family but lacks hydrolase activity (Renkema et al., 1998). It was first discovered in the CM of MG-63 cells, a human osteosarcoma cell line (Johansen et al., 1992). Several cell types, including macrophages, neutrophils, synovial cells, chondrocytes, epithelial cells, and smooth muscle cells, express YKL-40, but its function is still unclear. YKL-40 was a relevant candidate since it has been suggested to be involved in EMT (Jefri et al., 2015), angiogenesis (Faibish et al., 2011; Shao, 2013; Shao et al., 2009) and cancer progression (Cohen et al., 2017; Johansen et al., 2006; Libreros et al., 2012; Libreros & Iragavarapu-Charyulu, 2015).

Furthermore, YKL-40 is highly secreted under inflammatory conditions (Bonneh-Barkay et al., 2010; Johansen, 2006; Roslind & Johansen, 2009; Zhou et al., 2014) and malignant processes, such as breast cancer, melanoma, glioblastoma, colorectal cancer and small cell lung carcinoma (Cohen et al., 2017; Hamilton et al., 2015; Jefri et al., 2015; Johansen et al., 2006; Kawada et al., 2012; Libreros & Iragavarapu-Charyulu, 2015; Shao et al., 2011). Thus, to complement the gene expression data, mass spectrometry analysis of secreted proteins from D492, D492M and D492HER2 was performed (Fig. 13B). Ranking of the proteins differently secreted by D492M

and D492HER2 revealed that YKL-40 was highly secreted in D492HER2 cells (Fig. 13B). YKL-40 was the most differently secreted protein compared to D492M and the second most secreted protein at absolute values in D492HER2. Thus, YKL-40 became the selected candidate in Paper I for further comparative studies between D492M and D492HER2.

Additionally, in this process, other pro-angiogenic factors linked to tumorigenic properties were detected, enforcing the pro-angiogenic inducer role of D492HER2. For instance, ECM1 and VEGF-A were detected as highly secreted in D492HER2 compared to D492M and D492. ECM1 was the focus of Paper II that will be discussed in chapter 4.3 of this thesis. VEGF-A is a pro-angiogenic factor that is induced under hypoxic conditions after the activation of HIF-1α (Kim et al., 2006; Papandreou et al., 2006; Semenza, 2002, 2003). Interestingly, HER2 tumors show increased angiogenesis promoted by the upregulation of HIF-1α and, consequently, VEGF-A which increases the aggressiveness of the tumors (Laughner et al., 2001) and may facilitate metastasis (Wu et al., 2018b).

Further analysis of the transcriptome and secretome data revealed the upregulation of glycolytic genes/proteins in D492HER2. The PANTHER classification of genes differentially expressed between D492M and D492HER2 in the transcriptome analysis pointed out the enrichment of D-aspartate and organic anion transports in D492HER2 (Fig. 2B Paper I). D-aspartate transport occurs in the malate-aspartate shuttle which transports glycolytic products to and from the mitochondria (Baggetto, 1992) and several genes in the organic anion transport classification were associated with glucose metabolism, particularly CLIC4 and SLC16A7. These are transporters in glucose metabolism that might be relevant for future studies since they are linked to cancerous processes (Caruso et al., 2017; Ulmasov et al., 2009; Yao et al., 2009).

Interestingly, CLIC4 was also found highly secreted by D492HER2 compared to D492M in the secretome analysis. Furthermore, proteins involved in glycolysis and cancer, especially LDHA and ALDH1A3, were found to be highly secreted by D492HER2 cells. LDHA is the lactate dehydrogenase, also called the “Warburg enzyme” because it reduces pyruvate to lactate in glycolysis (Baumann et al., 2009; Eichenlaub et al., 2018; Fiume et al., 2014; Walenta & Mueller-Klieser, 2004). LDHA has been linked to increased tumor aggressiveness and poor outcome in cancer patients (Baumann et al., 2009; Eichenlaub et al., 2018; Walenta & Mueller-Klieser, 2004). ALDH1A3 oxidizes intracellular aldehydes and has been associated with LDHA in breast cancer

(Manerba et al., 2018). Moreover, ALDH1A3 is the predominant aldehyde dehydrogenase isoform in breast cancer stem cells (Duan et al., 2016; Marcato et al., 2011).

High expression of glycolytic genes/proteins corroborated the glycolytic metabolism of D492HER2 which might be involved in malignancy. This opens a new field of investigation to explore differences between the cell lines regarding tumorigenesis. In addition to comparative studies of KD and OE of the candidates in the D492 cell lines, I suggest future studies including inhibitors of glycolysis, particularly targeting the LDH activity that has been confirmed to reduce aggressiveness in tumors (Fiume et al., 2014; Pelicano et al., 2006). Moreover, it would be interesting to explore the behavior of the cell lines under hypoxic conditions to study changes in their metabolism and aggressiveness.

4.2.3 YKL-40 expression in D492HER2 is linked to cell migration and invasion

To explore the functional role of YKL-40 in migration and invasion, transient transfection was conducted in Paper I. YKL-40 was successfully knocked-down (KD) with two different siRNAs in D492HER2 cells. Consequently, the number of cells that migrated and invaded through the transwell filters was significantly reduced in YKL-40 KD D492HER2 cells (Fig. 14A).

To further corroborate the role in migration and invasion of YKL-40, CRISPR cell lines with stable KD of YKL-40 in D492HER2 and overexpression of YKL-40 in D492 and D492M were generated. Subsequent migration and invasion assays *in vitro* with D492HER2 YKL-40 KD demonstrated similar effects to the transient KD (Fig. 14B). On the other hand, overexpression of YKL-40 in D492 and D492M increased the migratory and invasive rates of cells (Fig. 14C and 14D).

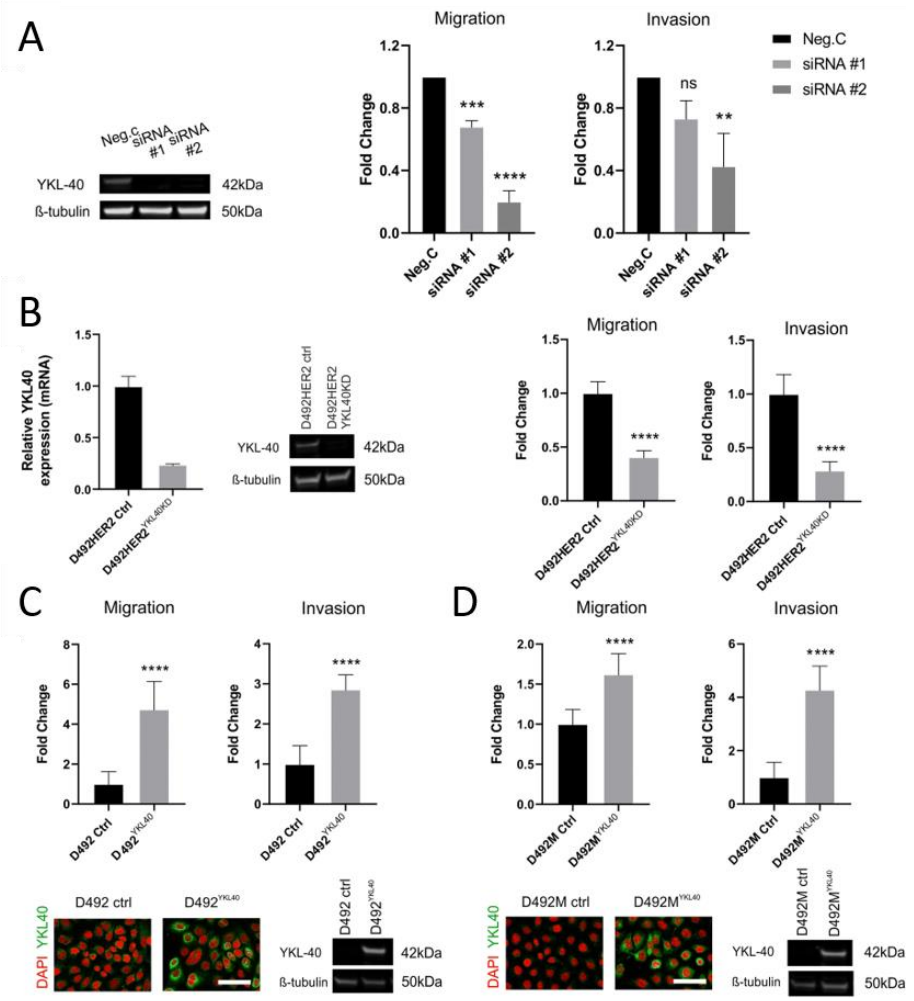

Figure 14. YKL-40 expression is linked to cell migration and invasion. A. Transient KD of YKL-40 reduces YKL-40 expression (protein expression measured by WB) and migration and invasion of D492HER2 cells. **B.** Stable KD of YKL-40 by CRISPR system reduces YKL-40 expression and migration and invasion of D492HER2 cells (gene expression measured by qPCR and protein expression measured by WB). **C and D.** Overexpression of YKL-40 by CRISPRa increases YKL-40 expression in D492 (**C**) and D492M (**D**), respectively, and their migration and invasion abilities. Protein detection by IF, *scale bar = 100µm* and measured by WB. All migration and invasion assays were assessed *in vitro* in transwell filters. *Fig. 3B-3G in Paper I.*

These results demonstrated that YKL-40 expression resulted in increased cellular migration and invasion, which are essential processes that support cancer development and tumor growth (Hanahan & Weinberg, 2011). YKL-40 has previously been linked to migration and invasion of cancer cells, such as prostate cancer cells (Hao et al., 2017), non-small lung carcinoma (Jefri et al.,

2015) and glioma cells (Singh et al., 2011). Also, YKL-40 is suggested to play a role in migration in stromal cells associated with malignant processes, particularly macrophages associated with colorectal cancer cells (Kawada et al., 2012), cancer-associated fibroblasts (CAFs) in breast cancer (Cohen et al., 2017) and endothelial cells in angiogenesis in breast cancer and colorectal cancer (Shao et al., 2009). In conclusion, YKL-40 may play a key role in the invasiveness and dissemination of cancer cells and, subsequently, a role in cancer progression and metastasis.

To verify if the role of YKL-40 in migration and invasion is linked to tumorigenesis and metastasis, experiments in animal models are required. Using the CRISPR cell lines with YKL-40 KD in D492HER2 might reduce tumorigenesis and, even, metastasis that might be induced afterward. CRISPR cell lines overexpressing YKL-40 in the non-tumorigenic D492 and D492M cell lines might induce malignant processes. Nevertheless, this needs to be tested *in vivo* before any conclusion can be made.

4.2.4 YKL-40 expression in D492HER2 is linked to vascular network formation

As previously stated, D492HER2 showed stronger effects on endothelial cells compared to D492M and D492 (chapter 4.1). Moreover, YKL-40 was linked to cell-cell interaction processes according to the PANTHER classification (Fig. 2B Paper I). Therefore, I wanted to investigate if YKL-40 was able to stimulate angiogenesis to contribute to D492HER2 tumorigenicity.

YKL-40 has previously been described to induce angiogenesis *in vitro* in breast and colon cancer cell models that ectopically expressed YKL-40 (Shao et al., 2011; Shao et al., 2009). In the D492HER2 cell line, YKL-40 was endogenously expressed and its pro-angiogenic ability was tested *in vitro*. By using a blocking antibody against YKL-40 (mA^{YKL-40}) directly on D492HER2 cells, the ability of the CM to induce the formation of capillary-like networks in ECs was significantly reduced. This blocking effect was reverted by adding recombinant YKL-40 protein ($YKL-40^{r}$) to ECs (Fig. 15A and 15B).

To confirm the role of YKL-40 in angiogenesis, CM from the CRISPR cell lines was collected and added to ECs seeded on top of Matrigel. The CM from D492 and D492M stably overexpressing YKL-40 increased endothelial network formation, whereas, on the contrary, the CM from the D492HER2 with the stable YKL-40 KD reduced angiogenesis *in vitro* (Fig. 15C).

These facts verified that the secretion or addition of YKL-40 stimulated ECs for capillary-like network formation, suggesting a role of YKL-40 as a potent

inducer of angiogenesis. This is in agreement with previously published results where YKL-40 has been linked to inducing tumor angiogenesis (Faibish et al., 2011; Shao, 2013; Shao et al., 2009). Moreover, the correlation between YKL-40 and vessel density has been observed (Francescone et al., 2011) and the correlation between YKL-40 and other pro-angiogenic factors has been suggested in glioblastoma (Faibish et al., 2011; Francescone et al., 2011). In the described work in Paper I, a synergetic pro-angiogenic collaboration involving YKL-40 was confirmed. When YKL-40 was stably KD in D492HER2, the expression of VEGF-A and VEGF-C was relevantly reduced (Fig. 15D).

Tumor cells and tumor-associated stromal cells secrete pro-angiogenic factors, such as YKL-40 and VEGF subtypes, contributing to tumor development. VEGF-A induces angiogenesis under hypoxic conditions while VEGF-C acts independently to oxygen concentrations (De Palma et al., 2017; Joukov et al., 1996; Semenza, 2002, 2003). These pro-angiogenic factors activate the VEGF receptor 2 (VEGFR-2, Flk-1/KDR) in ECs, which, consequently, activates the transcriptional machinery to promote angiogenesis (Carmeliet & Jain, 2000; Harper & Bates, 2008). Indeed, YKL-40 has been shown to activate VEGFR-2 to induce angiogenesis. Blocking YKL-40 abolishes the YKL-40-induced activation of VEGFR-2, which inhibits the activation of the extracellular signal-regulated kinase (Erk) 1 and Erk 2 and, subsequently, affects the intracellular mitogen-activated protein kinase (MAPK) signaling pathway (Faibish et al., 2011).

Furthermore, here I demonstrated that YKL-40 increased proliferation rates of ECs. When YKL-40^{r} was added to the media, ECs proliferated more than in control conditions (Fig. 15E). This fact has also been linked to induced angiogenesis by the activation of VEGFR-2 (Waltenberger et al., 1994), corroborating the inducer role of YKL-40 in the VEGFR-2 pathway. Further investigation of the VEGFR-2 activation is needed to properly confirm the YKL-40 inducer role in D492HER2. Quantification of the VEGFR-2 expression in the ECs in angiogenesis assays after the YKL-40 stimulation by staining or performing WB with an anti-VEGFR-2 antibody would be required. Also, the use of specific inhibitors of VEGFR-2 would show if YKL-40 is able to activate only this receptor or also others. Moreover, intermediate members of the angiogenic YKL-40-activation might be identified by performing RNA-seq in the stimulated ECs or using pro-angiogenic inhibitors.

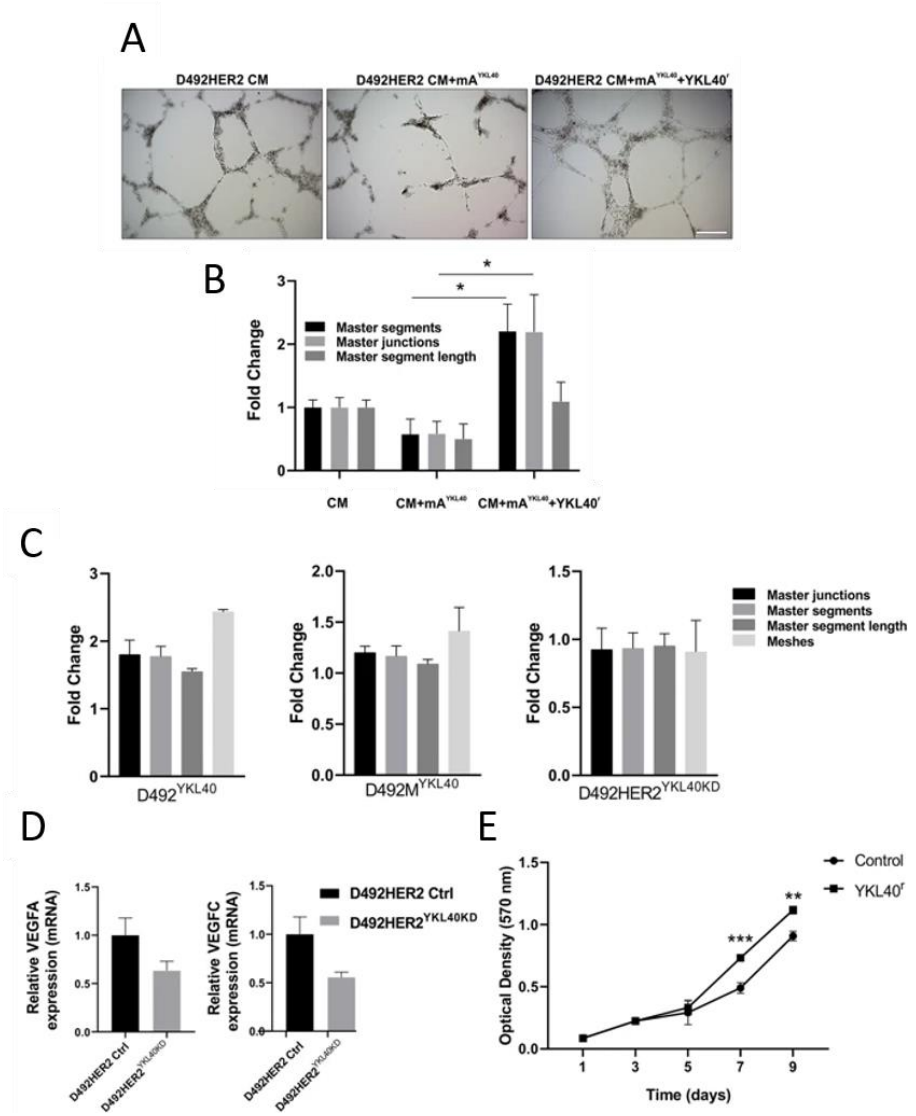

Figure 15. Role of YKL-40 in angiogenesis *in vitro*. A. Phase-contrast pictures of ECs on Matrigel developing capillary-like networks with different treatments: control, +monoclonal antibody (mA^{YKL-40}) and +recombinant YKL-40 protein ($YKL-40^r$). When YKL-40 is blocked by mA^{YKL-40} in D492HER2, the capacity to induce capillary-like network is compromised. When $YKL-40^r$ is added, the angiogenic effect is rescued. *Scale bar = 200µm.* **B.** Quantification by *ImageJ Angiogenesis analyzer* plug of A. **C.** Quantification of the angiogenic parameters by *ImageJ Angiogenesis analyzer* plug of the CRISPR cell lines when YKL-40 is KD or OE in comparison with their controls. **D.** KD of YKL-40 reduces the expression of VEGF-A and VEGF-C, suggesting a synergetic effect between the angiogenic factors. Gene expression measured by qPCR. **E.** $YKL-40^r$ increases the proliferation of ECs (measured by crystal-violet proliferation assay). *Fig. 4A-4D in Paper I.*

To determine whether the YKL-40-induced angiogenesis is involved in

tumorigenesis, *in vivo* studies are needed. By comparing the angiogenic effects in D492HER2 cells and D492HER2 YKL-40 KD in animal models, if YKL-40 had a role in tumor angiogenesis, interactions between YKL-40 and ECs might show a reduction in angiogenesis and, consequently, in tumor growth and aggressiveness. In contrast, D492 and D492M overexpressing YKL-40 cells might increase angiogenesis and malignancy. Moreover, the YKL-40 contribution to angiogenesis might increase the dissemination of malignant cells and induce metastasis. By using specific inhibitors of YKL-40, angiogenesis and aggressiveness might be reduced. In addition, blocking other pro-angiogenic factors, such as VEGF-A and VEGF-C, could improve effectiveness in tumor angiogenesis therapies.

4.2.5 YKL-40 affects the phenotype of D492 cell lines when cultured in 3D culture

YKL-40 has previously been linked to EMT in non-small cell lung cancer (Jefri et al., 2015), yet in my study, no phenotypic changes were observed in monolayer cell cultures when YKL-40 was downregulated in D492HER2 by transient nor stable transfection. In contrast, 3D cultures of stable YKL-40 KD in D492HER2 revealed differences regarding the phenotype.

As previously described, D492HER2 formed spindle-like and grape-like structures in 3D cell cultures (Fig.1A Paper I) (Ingthorsson et al., 2016). When YKL-40 was knocked-down in D492HER2, the grape-shaped structures were dramatically reduced in number (Fig. 16A), increasing the number of spindle-like structures, typically seen in D492M cells cultured in 3D (Sigurdsson et al., 2011). However, when YKL-40^{r} was added to the media of D492HER2 KD YKL-40, the grape-like structures were rescued and increased in number.

To confirm the connection between YKL-40 in media and the grape-like phenotype, D492M cells stably overexpressing YKL-40 were cultured in 3D. The entire cell population presented grape-like structures and lost the ability to form spindle-like structures (Fig. 16B).

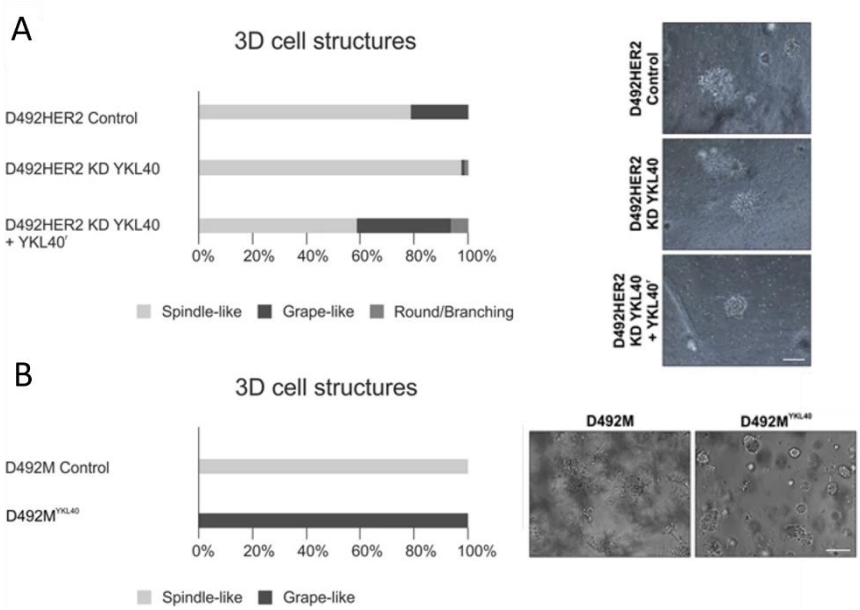

Figure 16. 3D phenotype in the YKL-40 CRISPR cell lines. A. When YKL-40 is stably KD in D492HER2, the grape-like phenotype is reduced in proportion. This phenotype is rescued when recombinant YKL-40 protein (YKL-40^r) is added to the medium. Phase-contrast pictures were taken and all 3D structures were counted and classified. **B.** The overexpression of YKL-40 in D492M swifts the typical spindle-like phenotype in 3D to the grape-shaped, only shown before by D492HER2. All phase-contrast pictures at *scale bar = 200µm. Fig. 5A, 5B in Paper I.*

The relation between YKL-40 and the EMT phenotype in 3D suggests a possible role of YKL-40 in plasticity. This observation is interesting since spindle-shaped colonies have been associated with the phenotype in 3D of triple-negative breast cell lines, which are more prominent in EMT (Han et al., 2010). In contrast, the grape-like structures have been connected with HER2 and aggressiveness (Holliday & Speirs, 2011; Kenny et al., 2007). Nevertheless, whether the grape-like phenotype is more migratory and invasive than the spindle-shaped, it is currently not known.

However, YKL-40 has been reported to induce migration in mammary epithelial cells (Scully et al., 2011). Scully and co-workers revealed that YKL-40 inhibited epithelial differentiation and reduced E-cadherin expression and increased MMP-9 expression in 3D cell cultures. Cell culture in more similar conditions to *in vivo* may be beneficial for the changes in gene expression derived from the gene-editing modifications, thus further 3D cell culture studies in EMT markers in our cell model might show differences in their expression.

Moreover, the shift toward grape-shaped structures induced by YKL-40 expression or addition of YKL-40^r to the medium may reflect cellular plasticity

which might provide adaptive benefits for tumorigenic purposes, but further investigation *in vivo* is required.

4.2.6 Conclusion

In Paper I, I compared two isogenic cell lines, the endothelial-induced EMT D492M cell line and the oncogene-induced EMT D492HER2 cell line. Results revealed that D492HER2 cells had increased abilities to proliferate, migrate, and invade compared to D492M (and D492). However, D492HER2 cells were more susceptible to apoptosis induction and the glucose metabolism was more similar to the epithelial D492 cells. Here, I demonstrated that D492HER2 cells have a more partial-EMT phenotype than D492M which may increase plasticity in D492HER2 cells and might improve their adaptation to the tumor microenvironment. This adaptation was triggered as well by modulating tumor-associated cells, in particular, endothelial cells that had been stimulated to increase angiogenesis.

In the study, I identified YKL-40 as a potential inducer of tumor angiogenesis in D492HER2. To my best knowledge, D492HER2 is the first described breast tumorigenic cell line that highly expresses and secretes YKL-40 endogenously. Also, CRISPR technology was used for the first time to knock-down and overexpress YKL-40 in cells. The establishment of stable cell lines modifying YKL-40 expression demonstrated the role of YKL-40 in migration and invasion as well as angiogenesis.

Although a connection between YKL-40 and VEGF-A has previously been suggested (Faibish et al., 2011; Francescone et al., 2011), no molecular studies were performed in a cell line that endogenously expressed YKL-40. In this article, this relation was confirmed and, also, a synergetic pro-angiogenic effect between YKL-40 and VEGF-A and VEGF-C. In addition, YKL-40 has been shown to increase the proliferation of ECs, which was not verified before.

Interestingly, the knock-down of YKL-40 in D492HER2 showed a change in the 3D EMT phenotype. The typical grape-like structures of D492HER2 cells shifted toward the typical spindle-shaped morphology of D492M. Conversely, when YKL-40 was overexpressed in D492M or added to the medium in D492HER2 KD YKL-40, cells shifted toward the grape-like phenotype. This study described for the first time grape-shaped colonies in D492M, a shift due to changes in YKL-40 expression. YKL-40 might reflect cellular plasticity that is believed to improve the adaption of cells, but further research is required to fully establish this.

Collectively, this study evidenced the potential role of YKL-40 to increase tumor properties, by increasing migration and invasion of breast HER2 overexpressing tumor cells and modulating the surrounding stromal cells. YKL-40 may be of clinical relevance acting as a novel target in breast cancer, but its pathway and tumorigenic mechanisms still require further investigation.

4.3 Paper II. ECM1 secreted by HER2-overexpressing breast cancer cells promotes the formation of a vascular niche accelerating cancer cell migration and invasion

In this paper, we focused on heterotypic interactions between the breast isogenic cell lines D492 and D492HER2 and endothelial cells. The extracellular matrix protein 1 (ECM1), which is highly expressed and secreted by D492HER2, was identified as an important effector of these interactions by inducing capillary-like network formation in ECs. Furthermore, feedback through ECM1-stimulated ECs increased migration and invasion of D492HER2 cells.

4.3.1 Identification of ECM1 as a pro-angiogenic candidate in D492HER2 secretome

Heterotypic interactions between ECs and D492HER2 cells have shown effects on both cell types. The CM collected from D492HER2 promoted angiogenesis *in vitro* in Paper I and the CM from stimulated ECs induced feedback toward D492HER2 cells resulting in increased migration and invasion (Fig. 1 Paper II). Thus, the aim of this study was to identify inducers of these effects.

Secreted proteins from D492HER2 were analyzed by mass spectrometry and compared to the proteins found in the CM from D492 and D492M. Herein 77 candidates showed significant differences between D492 and D492HER2. Classification of the candidates was done using the PANTHER database with a differential range from 2-fold change and the angiogenesis regulation process was listed as enriched. From the list of candidates related to angiogenesis, those that had the highest expression in D492HER2 were CHI3L1 and ECM1. CHI3L1, also known as YKL-40, was discussed in Paper I (above and enclosed) and ECM1 was the focus of Paper II.

ECM1 is a secreted glycoprotein that was discovered in the mouse osteogenic stromal MN7 cell line for the first time (Mathieu et al., 1994). It is involved in the proliferation and differentiation of epidermal keratinocytes, and basement membrane reconstitution in the skin (Oyama & Merregaert, 2017). The function of ECM1 on cell proliferation has also been described in the

chorioallantoic membrane of chicken embryos and blood vessel formation (Han et al., 2001).

ECM1 was an interesting candidate due to its previous link to angiogenesis and cancer progression (Han et al., 2001; Lee et al., 2015b; Lee et al., 2014; Oyama & Merregaert, 2017; Sercu et al., 2008a; Sercu et al., 2008b; Wang et al., 2003). ECM1 interacts with ECs to induce angiogenesis but also with most of the proteins that are present in the ECM regulating inflammation and immune response in many tissues (Chan, 2004; Li et al., 2011). A recent article has described a novel role of ECM1 in cardiac tissue in which immune and inflammatory cells secrete ECM1 to stimulate fibroblasts. In this context, ECM1 may act as a pro-fibrotic effector (Hardy et al., 2019).

In breast cancer, ECM1 has been linked to aggressiveness in breast epithelial tumors (Wang et al., 2003) and the breast cancer cell line MDA-MB-231 (Gomez-Contreras et al., 2017; Wu et al., 2018a). Moreover, ECM1 has been suggested to increase resistance to trastuzumab in HER2-positive breast cancer patients (Lee et al., 2014). In Fig. 2 of Paper II, we showed that ECM1 was associated with stromal cells in breast cancers and a decrease in distant-metastasis-free-survival (DMFS) in HER2-positive BC patients. Thus, ECM1 was selected as a relevant candidate for further studies.

4.3.2 ECM1 enhances endothelial network formation and induces endothelial feedback toward D492HER2

In order to explore the role of ECM1 in angiogenesis, capillary-like network formation assays were performed *in vitro* using ECs treated with CM from D492 cell lines with modified ECM1 expression and the addition of recombinant ECM1 protein (rECM1).

The expression of ECM1 was downregulated in D492HER2 by transient transfection with a siRNA and by CRISPR interference (CRISPRi) to KD the expression stably. When CM from D492HER2 KD ECM1 was collected and added to ECs on top of Matrigel, the formation of the capillary-like network was not increased; indeed, it was reduced compared to the effect of CM from D492HER2 control (Fig. 17A, 17B), suggesting similar results with both KD strategies.

On the other hand, CM from ECM1 overexpressing D492 and D492M cell lines (generated by CRISPR activation, CRISPRa) showed an increase in angiogenesis parameters in comparison with D492 and D492 (Fig. 17A, 17B). The addition of ECM1 recombinant protein (rECM1) to the medium of ECs also increased the tube formation of ECs (Fig. 3 Paper II).

Furthermore, we found that ECM1 plays a role in a feedback mechanism between stimulated ECs and D492HER2. To demonstrate this, CM from stimulated ECs was collected and used as an attractant in migration and invasion assays *in vitro.* D492HER2 cells increased their migration and invasion (Fig. 3 and Suppl. data Paper II), however, this feedback was not observed when ECM1 was KD in D492HER2 (Fig. 17C, 17D), establishing that this effect was dependent on ECM1.

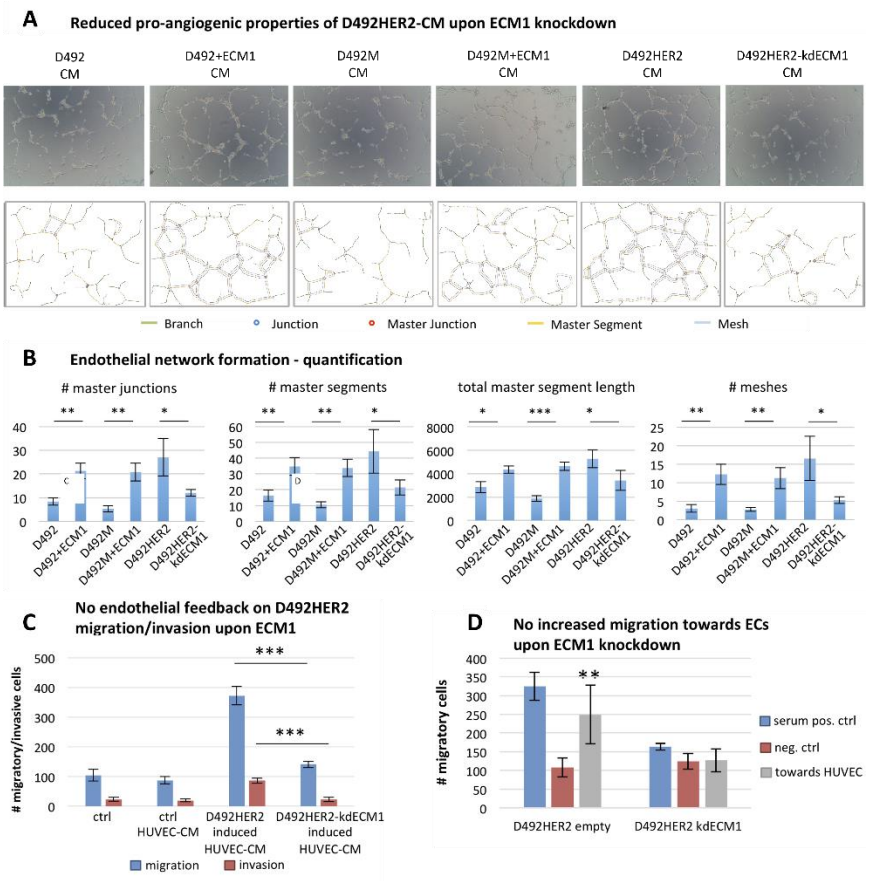

Figure 17. CM with ECM1 KD reduces the induction of angiogenesis by D492HER2 and the feedback toward D492HER2. A. The KD of ECM1 in D492HER2 reduces the properties of D492HER2 to induce angiogenesis in ECs. Phase-contrast upper pictures (10x) and ImageJ Angiogenesis analyzer pictures below. *Scale bar = 100µm.* **B.** Quantification with *ImageJ Angiogenesis analyzer* of A. **C.** The KD of ECM1 does not induce feedback through ECs to increase migration and invasion in D492HER2. **D.** The KD of ECM1 does not induce feedback through indirect co-culture toward ECs. All migration and invasion assays were assessed *in vitro* in transwell filters. *Fig. 4A, 4B, 4D, 4F in Paper II.*

Endothelial cells within an enriched angiogenic tumor microenvironment have been shown to increase aggressiveness in cancer (Cai et al., 2015; Hida et al., 2018). Also, tumor-associated endothelial cells (TECs) regulate the infiltration of other cells interfering with the protective immune cells and contributing to the dissemination of cancer cells during metastasis (Buckanovich et al., 2008; Pein & Oskarsson, 2015; Potente et al., 2011). Therefore, the increase in migration and invasion in D492HER2 cells after treatment with CM from stimulated ECs might reflect an increase in the aggressiveness but further *in vivo* studies in tumorigenesis and metastasis are needed to shed light on the role of ECM1 in aggressiveness and metastasis.

Future studies are also needed to unravel further feedback toward D492HER2 cells through ECM1- or D492HER2-stimulated ECs. The molecules that are secreted in response by stimulated ECs are not identified yet. A secretome analysis on CM of stimulated ECs in comparison with the non-stimulated ECs might shed light on these possible intermediaries.

4.3.3 ECM1 induces upregulation of the NOTCH signaling pathway in ECs

To investigate the differences regarding gene expression in stimulated ECs treated with CM from D492HER2 control and CM from D492HER2 ECM1 KD, a microarray assay was performed (Fig. Suppl. 7 Paper II). The results revealed that the expression of Fatty Acid Binding Protein 4 (FABP4) was lower in the EC stimulated with CM from D492HER2 ECM1 KD compared to ECs treated with CM from D492HER2 control. Although this was not confirmed, this candidate focused our attention on the NOTCH signaling pathway as FABP4 is regulated by NOTCH1 in a delta ligand-like 4 (DLL4)-dependent manner in angiogenesis (Harjes et al., 2014). Previous publications have also connected the NOTCH signaling pathway with angiogenesis in normal conditions and malignant disorders in the retina (Lobov et al., 2007), cutaneous melanoma (Murtas et al., 2015) and myeloma (Guo et al., 2013).

The expression of receptors included in the NOTCH family was analyzed on ECs treated with CM from D492HER2 control, CM from D492HER2 KD ECM1 and rECM1, resulting in increased gene and protein expression of NOTCH1 and NOTCH3 when ECs were treated with CM from D492HER2 control and rECM1 but not with CM from D492HER2 KD ECM1 (Fig. 18A), suggesting that the NOTCH pathway is involved in the angiogenesis that is induced by ECM1 in ECs.

By using a specific inhibitor of the NOTCH signaling, the gamma-secretase inhibitor DAPT, a connection between ECM1 and the NOTCH pathway was

verified. The DAPT treatment in ECs previously treated with rECM1 reduced the ECM1-induced capillary-like networks in tube formation assays (Fig. 18B and 18C), suggesting that the NOTCH signaling pathway was involved in the ECM1-induced angiogenesis. The inhibition of NOTCH was also able to block the ECM1-induced feedback toward D492HER2 cells (Fig. 18E).

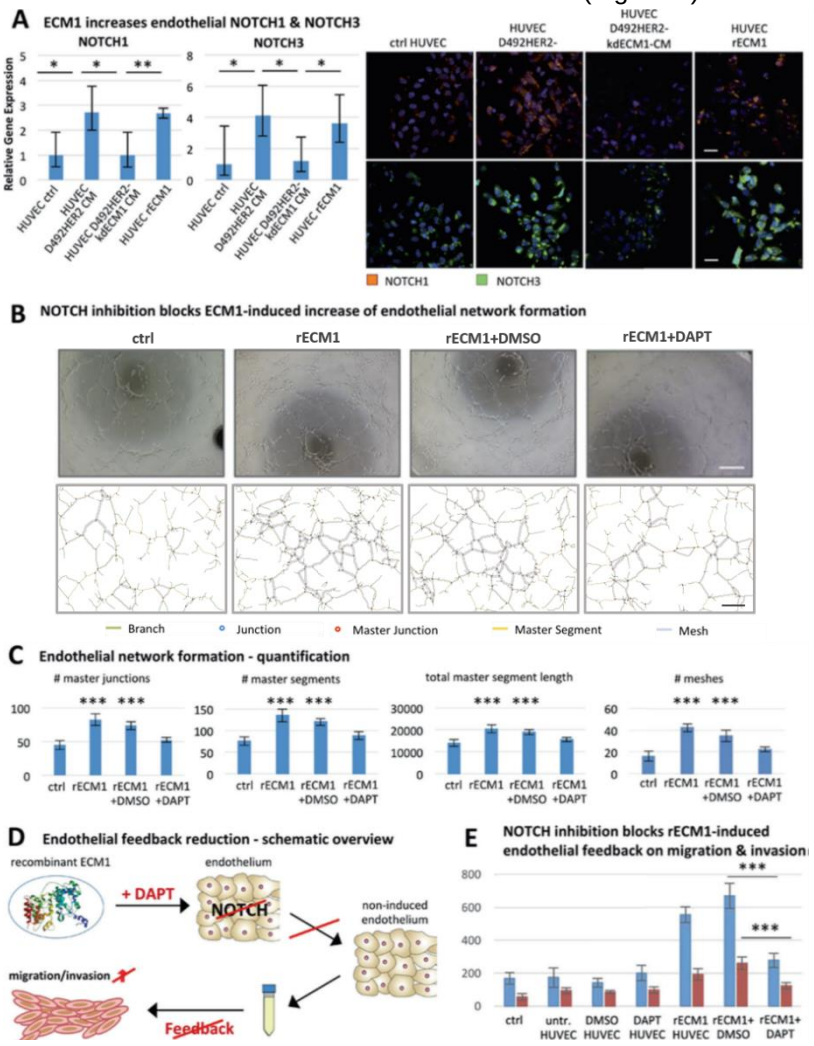

Figure 18. ECM1 stimulates the NOTCH pathway in ECs. A. The gene and protein expressions (measured by qPCR and IF, respectively) of NOTCH1 and NOTCH3 are increased in ECs when rECM1 is added to the media and is reduced when ECM1 is KD. *Scale bar = 50μm.* **B.** Inhibition of NOTCH blocks the induction of angiogenesis by ECM1. Phase-contrast pictures and *ImageJ Angiogenesis analyzer* pictures. *Scale bar = 100μm.* **C.** Quantification with *ImageJ Angiogenesis analyzer* of B. **D.** Schematic overview of the NOTCH inhibition. **E.** Inhibition of NOTCH blocks the ECM1 induction of feedback toward D492HER2. Migration and invasion assays were assessed *in vitro* in transwell filters. *Fig. 5A-5E in Paper II.*

The NOTCH signaling pathway is a complex pathway composed of members that interplay at multiple levels with other signaling pathways. The NOTCH signaling pathway is believed to act independently to VEGFR-2 in angiogenesis (Benedito et al., 2012), which suggests that ECM1 may activate a different pathway in ECs compared to YKL-40. Moreover, unlike YKL-40, ECM1 was not found to have a synergetic relationship with other pro-angiogenic factors or increase ECs proliferation, corroborating an alternative pathway to VEGFR-2.

The ECM1 signaling pathway in the cells where ECM1 is expressed has not been identified yet. It is believed that the STAT family and GATA-3 regulate ECM1 in T helper cells (He et al., 2018; Li et al., 2011). Nonetheless, further research on the ECM1 molecular pathway in D492HER2 and tumor cells would elucidate how ECM1 is regulated. The gene expression analysis of the cell lines with modifications in ECM1 expression compared with their control cell lines in 2D and 3D might reveal differences in EMT phenotype and differentiation. Moreover, functional assays, such as those regarding migration, invasion and proliferation might provide insight into whether ECM1 could be linked to other biological processes, including malignant processes.

Due to the potential inducer role of ECM1 in tumor angiogenesis, further assays in tumorigenesis and metastatic *in vivo* might confirm whether the expression and secretion of ECM1 are able to induce malignant processes and contribute to the dissemination of the tumor cells. These studies could contribute to developing therapies to treat tumors that express ECM1.

4.3.4 Conclusion

ECM1 secreted by D492HER2 cells was found to induce angiogenesis. ECM1 had previously been suggested to induce angiogenesis and cancer progression (Han et al., 2001; Lee et al., 2015b; Lee et al., 2014; Oyama & Merregaert, 2017; Sercu et al., 2008a; Sercu et al., 2008b; Wang et al., 2003). However, here, we demonstrated for the first time that ECM1 induced angiogenesis by activating the NOTCH signaling pathway. Moreover, ECM1-stimulated ECs promoted feedback toward D492HER2 cells which increased their migration and invasion. These findings identify ECM1 as an interesting candidate for further investigation as a novel target for cancer therapies, particularly in HER2+ breast cancer patients since ECM1 reduced their survival, probably increasing the resistance to trastuzumab in HER2 tumors (Lee et al., 2014).

4.4 Unpublished data. The relation between YKL-40 and the tumorigenic properties provided by HER2

In this chapter, I will discuss the unpublished data of my thesis that are open to further investigation. The data included in this chapter focus on the relationship between YKL-40 and the tumorigenic properties provided by HER2 in the D492 cell lines and open new perspectives for future studies. In Paper I, YKL-40 was described as an important mediator for angiogenesis and other biological processes, such as migration, invasion and plasticity that may be involved in tumorigeneses in D492HER2. In the current chapter, other relevant implications of YKL-40 in malignant processes will be discussed.

4.4.1 Modification of the YKL-40 expression induces changes in HER2 expression in D49HER2 and D492M

Several studies have tried to correlate YKL-40 expression in breast cancer with specific grades and distinct subtypes, but these correlations were based on cohorts and, to my best knowledge, no molecular studies have been performed (Jensen et al., 2003; Kang et al., 2014; Kim et al., 2007; Shao et al., 2011). Nevertheless, a relation between YKL-40 and poor prognosis in breast cancer has been established (Kang et al., 2014; Shao et al., 2011) and a link between YKL-40 and HER2 has also been suggested (Shao et al., 2011). Therefore, I wanted to explore the molecular relationship between YKL-40 and HER2.

First, I used the D492HER2 cell line to unravel this relation, since D492HER2 cells overexpress both YKL-40 and HER2. I succeeded in knocking-down YKL-40 by using both transient siRNA and stable CRISPR in D492HER2 cells (Morera et al., 2019). Interestingly, using both knock-down methods not only YKL-40 expression was reduced, but also HER2 expression (Fig. 19A and 19B). This provided a good starting point for further research into the molecular relation between HER2 and YKL-40.

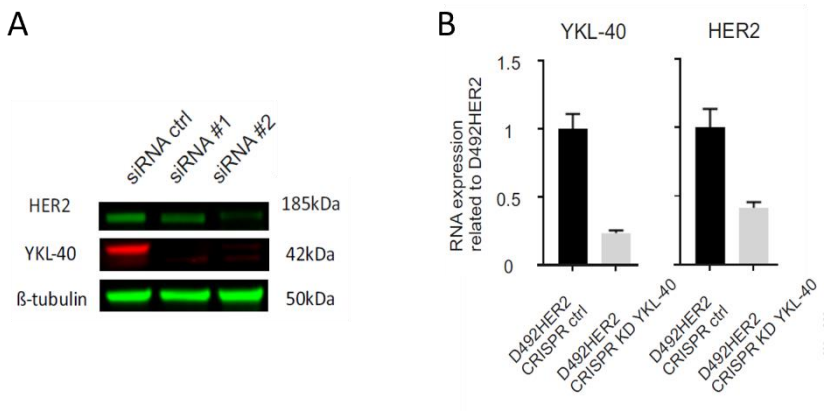

Figure 20. Expression of YKL-40 and HER2 when YKL-40 is KD in D492HER2. A. Transient KD of YKL-40 in D492HER2 reduces the expression of YKL-40 and HER2 as well. Protein expression measured by WB. **B.** The same effect is observed when YKL-40 is stably KD by CRISPR technology. Gene expression measured by qPCR.

The next step was to overexpress HER2 in D492M by lentiviral transduction. The overexpression of HER2 was confirmed and the expression of YKL-40 was analyzed (Fig. 20B). Interestingly, YKL-40 expression was increased in the D492M overexpressing HER2 cell lines. Moreover, these cell lines presented differences in the phenotype which showed a more elongated morphology than usual (Fig. 20A). Whether this phenotype is more aggressive or not has not been confirmed yet. Further characterization of these cell lines is required to explore if the overexpression of HER2 and upregulation of YKL-40 in D492M might provide tumorigenic properties and cellular plasticity. Phenotypic and functional studies *in vitro* are needed as well as tumorigenesis assays *in vivo*.

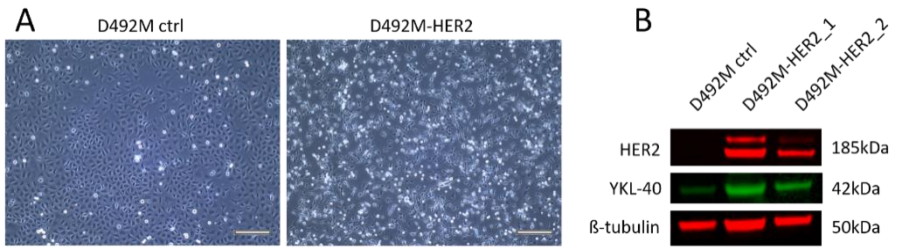

Figure 19. D492M overexpressing HER2 cell lines. A. Phenotype of D492M ctrl and overexpressing HER2 cell lines. Phase-contrast pictures. *Scale bar = 100µm.* **B.** Confirmation of the HER2 overexpression and increase in expression of YKL-40 by WB in the D492M overexpressing HER2 cell lines.

4.4.2 Inhibition of HER2 decreases YKL-40 expression in D492HER2

To continue exploring the relationship between YKL-40 and HER2, inhibition of HER2 with different strategies was tested and, subsequently, the expression of YKL-40 was analyzed. In a previous work of our lab, it was demonstrated that when EGFR is co-transfected with HER2 in D492, EGFR partially restores the epithelial phenotype and acts as a tumor suppressor, since tumors formed by D492$^{HER2/EGFR}$ cells were reduced and less aggressive rather than those formed by D492HER2 cells (Ingthorsson et al., 2016). By using cell lysates from D492 cell lines overexpressing HER2, EGFR and both HER2/EGFR, I analyzed the YKL-40 expression by WB (Fig. 21). YKL-40 was not detected in the non-tumorigenic cell lines, D492 and D492^{EGFR}. However, YKL-40 was highly expressed in the highly tumorigenic D492HER2 and lower expressed in D492$^{HER2/EGFR}$ which has mild tumorigenic properties.

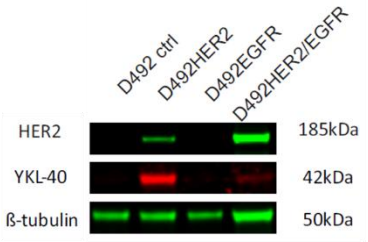

Figure 21. Expression of YKL-40 and HER2 in D492, D492HER2, D492EGFR and D492HER2/EGFR. YKL-40 and HER2 are expressed in D492HER2. The expression of YKL-40 is reduced when D492 is co-expressing HER2 and EGFR. Protein expression measured by WB.

Lysates from xenograft tumors derived from D492, D492HER2, D492^{EGFR} and D492$^{HER2/EGFR}$ cells were used to analyze the expression of HER2 and YKL-40 (Fig. 22). HER2 was expressed in the xenografts derived from D492HER2 and D492$^{HER2/EGFR}$, but YKL-40 was only detected in the D492HER2-derived tumors, which were the most aggressive and largest in size. This indicates that YKL-40 might have a role in the tumorigenic properties provided by HER2.

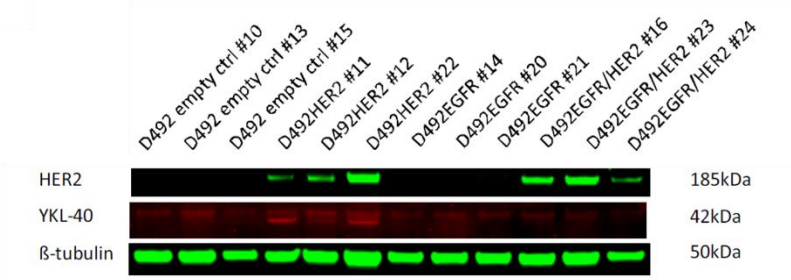

Figure 23. Expression of YKL-40 and HER2 in the xenografts derived from the D492 sublines. Expression of YKL-40 and HER2 in xenografts samples from different mice (3 for each kind) injected with cells from D492, D492HER2, D492EGFR and D492HER2/EGFR. YKL-40 is expressed in the xenografts derived from D492HER2. Protein expression measured by WB.

At the cellular level, there are specific inhibitors that block the tyrosine kinase pathway HER2 with good results. CP724,714, a small-molecule inhibitor of HER2 that selectively binds the intracellular domain of HER2, was used in 3D cell cultures of D492HER2 and resulted in a decrease in the percentage of 3D EMT-like structures including the grape-shaped, typically expressed in D492HER2 cells (Ingthorsson et al., 2016). By using lysate protein samples of the treated and untreated D492HER2 cells with the inhibitor, the expression of YKL-40 was found to be reduced in the treated cells (Fig. 23). This suggests a relation between YKL-40 and HER2 in D492HER2. Whether this connection is direct or indirect is currently unknown.

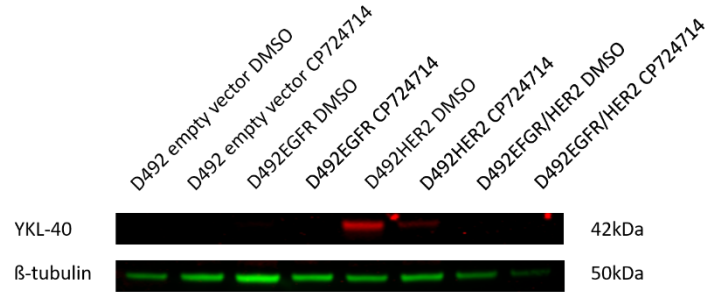

Figure 22. Expression of YKL-40 when D492HER2 is treated with HER2 inhibitor. Expression of YKL-40 is reduced when the specific inhibitor CP724,714 inhibits the action of HER2. Protein expression measured by WB.

Future studies about the pathway that might connect HER2 and YKL-40 are needed. Treatments with activators and inhibitors of the HER2 signaling pathway at different points of the phosphorylation cascade might locate YKL-40 in the pathway. YKL-40 has been linked to MAPK and PI3K-AKT pathways in U87 brain tumor cells (Faibish et al., 2011; Francescone et al., 2011). In breast cancer, HER2 has also been suggested to activate these pathways

(Gallardo et al., 2012; Nahta & Esteva, 2006), hence this might be a possible common pathway. Interestingly, YKL-40 has also been suggested to be linked to the EGFR signaling pathway in lung epithelial cells under mechanical stress (Park et al., 2010), suggesting that YKL-40 might be connected with the members of the EGFR family in a complex manner.

Future analysis of YKL-40 expression in other HER2-positive BC cell lines would be relevant. For instance, MDA-MB-453 cells develop grape-like structures in 3D cell cultures, which are reduced in number when HER2 is blocked (Holliday & Speirs, 2011; Kenny et al., 2007), similar to D492HER2 (Ingthorsson et al., 2016). Also, the comparative analysis of HER2-positive tumors treated and untreated with trastuzumab might reveal differences in YKL-40 expression in comparison with untreated tumors. Moreover, due to the inducer role of YKL-40 in angiogenesis (Faibish et al., 2011; Morera et al., 2019; Shao, 2013; Shao et al., 2009) and the activation of pro-angiogenic factors in HER2-positive tumors (Laughner et al., 2001), it would be relevant to explore the correlation between YKL-40 and blood vessel density.

Finally, only by performing experiments in animal models, the role of YKL-40 in tumorigenesis could be fully revealed. Future experiments *in vivo* might contribute to understanding YKL-40 mechanisms in malignancy and metastasis. By inhibiting YKL-40, tumorigenesis of D492HER2 and other tumor cells might be reduced. This could be of clinical relevance in treating HER2-positive BC tumors, probably in combination with conventional therapies.

5 Aspects on methodology and experimental approach

In this chapter, I will discuss some of the techniques and methods I have used during my study. I will use this section to discuss the technical problems I encountered and explain how they were solved. I will discuss these problems both in terms of success but also with a retrospective view on other approaches that could have been used.

5.1 Issues of culturing the isogenic D492 cell lines

In this project, I have mainly used the D492, D492M and D492HER2 cell lines. These cell lines share genetic patterns but differ in the EMT phenotype and their ability to form tumors. I also generated D492 cell lines in which the expression of YKL-40 was modified. Therefore, I have worked with several isogenic cell lines and it was essential to know them well and reduce the risk of cross-contamination.

Cross-contaminations are one of the most problematic issues in cell culture laboratories (Capes-Davis et al., 2010; Horbach & Halffman, 2017; Neimark, 2015). A single cell of a different kind inserted in the cell culture can outgrow and take advantage of the original culture (Neimark, 2015). It is believed that between 20-36% of all cell lines are cross-contaminated or misidentified (Horbach & Halffman, 2017; Neimark, 2015). A well-known example of cross-contamination in cell cultures is from the HeLa cell line (Horbach & Halffman, 2017). A major issue with cross-contaminations is that they are almost impossible to remove completely and it is difficult to determine when the contamination started. Thus, to diminish cross-contaminations, it is necessary to use aseptic conditions, conduct DNA fingerprinting regularly, and have a good knowledge of the particular cell lines.

During my project, the use of isogenic cell lines made it difficult to discriminate between them by DNA fingerprinting; therefore, it was imperative to learn how to culture and monitor them regarding morphology, proliferation and spreading. Furthermore, fluorescent-labeling with GFP or RFP in cells proved to be very useful not only for the selection of positive-cells in the creation of new cell lines but also for the confirmation of suspected cross-contamination when there was strange cellular morphology or behavior. For instance, D492HER2 is a GFP-positive and highly proliferative cell line. When

there was a suspicion of cross-contamination involving D492HER2, D492HER2 cells were easily detected using a fluorescence microscope.

However, the fluorescent-labeling in D492HER2 cells was also an issue in the performance of some experiments, since the permanent GFP emission limited the antibody staining in the green range. This forced me to find alternative methods to compare D492, D492M and D492HER2 in terms of apoptosis. I could not use the apoptosis assay that was frequently used in our laboratory which quantifies fluorescence emission by flow cytometry after the staining of Annexin V FITC-488 nm and propidium iodide (PI)-547 nm. Alternatively, I used a luciferase-based kit that measured the cleavage of caspase 3/7 to quantify apoptosis (see Material and methods chapter for protocol and reference).

5.2 Technical considerations regarding 3D cell cultures

In my Ph.D. project, I have set up 3D cell cultures, but herein I experienced several challenges. Initially, I started using hydrogels made either of collagen I or Matrigel but those performed with Matrigel were more successful for the 3D cell culturing experiments I performed. Matrigel was more stable and 3D cell structures developed faster than in collagen gels, presumably as a consequence of the composition and consistency of Matrigel that probably recapitulates the microhistology of the basement membrane (BM) better.

Matrigel is composed of a heterogeneous mix of proteins secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. These proteins are extracellular matrix (ECM) proteins, principally components of the BM (Kleinman & Martin, 2005). Therefore, Matrigel is often referred to as a reconstituted basement membrane (rBM). Proteins in the rBM, such as laminin and collagen IV (Kleinman & Martin, 2005), are potent inducers of morphogenesis which make Matrigel an excellent tool for studying tissue morphogenesis of normal and cancer cells *in vitro*.

There are several aspects to be considered when working with Matrigel. Assays need to be planned with proper time in advance since Matrigel requires to be thawed in cold conditions. Moreover, Matrigel gels are fragile, so they need careful handling. Nevertheless, the most challenging issue of using Matrigel is to find suitable production lots for cell culture. The composition of Matrigel varies between production lots, which can affect cell survival and colony growth. During my project, I experienced that composition differences from batch to batch resulted in time-consuming optimization steps, in particular, regarding the appropriate number of cells for the survival of the

culture that was needed to validate for each lot. Furthermore, the development of the 3D structures may be impeded by non-suitable protein concentration, endotoxin levels, or density of the material. The variation between batches affected more the D492 and D492M cells rather than D492HER2 cells, probably, due to the high proliferation rate of D492HER2.

Alternatively, I could have used other rBMs, such as Cultrex (Trevigen) or Geltrex (Thermo Fischer Scientific), that have a comparable composition of proteins secreted by EHS mouse sarcoma cells. Nonetheless, other rBMs have not been tested, therefore if they are suitable for survival and growth of the D492 cell lines in 3D and if the composition of the material varies less between batches than in Matrigel, it is currently unknown.

I also applied Matrigel to seed cells on top of its surface, instead of embedding them into the gel (Lee et al., 2007). The advantage of this approach was that the cellular behavior was less affected by differences between lots, and it saved material and time. The 3D cell structures of D492 cell lines were visible within a shorter time than those embedded into Matrigel and time-lapse imaging was easier as colonies grow in a single plane. Moreover, this experimental setup was also successful for mimicking angiogenesis *in vitro* in endothelial cells in tube formation assays.

5.3 Gene-editing tools to knock-down and overexpress target genes

In this subchapter, I will describe the difficulties that I experienced during the performance of different methods to create stable cell lines with modifications in the expression of target genes and the solutions that I found to solve them. Nowadays, different techniques can be used for genome editing. It is, therefore, crucial to select a suitable method for the gene target and the experiments that will be conducted on the new cell lines.

As a side-project of my main Ph.D. project, we decided to overexpress HER2 in D492M cells to induce malignant properties and in D492 cells to confirm our previous results in the D492HER2 cell line. I used the same plasmids to overexpress HER2, but instead of double transduction in D492HER2 (Ingthorsson et al., 2016), I performed single transduction to reduce cellular stress.

Lentiviral transduction is a common gene-editing technique that is widely used because of its high efficiency in a relatively short period. Lentiviruses have a high ability to infect and integrate the DNA of the plasmid in the host cells. Nonetheless, as a consequence of this high virulence, these lentiviral

transductions need to be done in biosafety level 2 (BSL2) laboratories. Furthermore, every single step is crucial for the correct lentiviral transduction, cells need to be in optimum conditions and there is a schedule that needs to be respected (see Material and Methods for the detailed protocol).

I established the stable D492M and D492 overexpressing HER2 cell lines and I observed differences in the morphology in comparison to their control cell lines (Fig. 24A, 24B). D492 overexpressing HER2 cells showed a spindle mesenchymal-like phenotype and D492M overexpressing HER2 had a more elongated morphology than usual. However, due to time limitations, I did not have time to characterize them entirely and analyze tumorigenicity in animal models, but further characterization is being conducted in the lab.

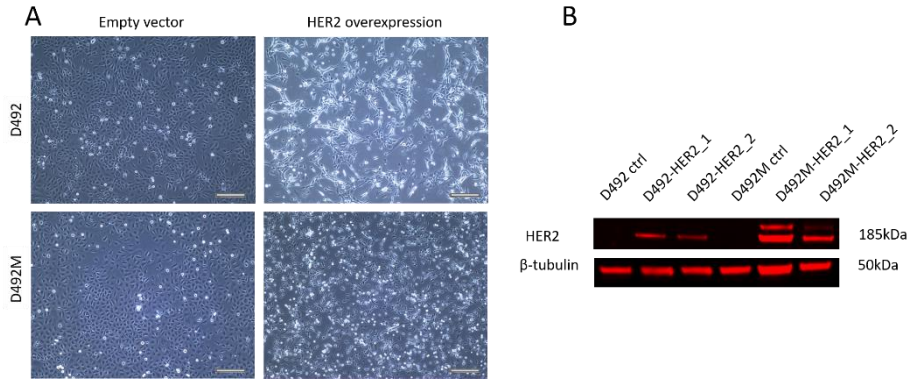

Figure 24. D492 and D492M overexpressing HER2 oncogene cell lines. A. D492 overexpressing HER2 cells showed a spindle mesenchymal-like phenotype and D492M overexpressing HER2 presented a more elongated morphology than usual. Whether these phenotypes are more aggressive has not been confirmed yet. Phase-contrast pictures. *Scale bar = 100μm.* **B.** Confirmation of the HER2 overexpression by WB.

On the other hand, I used the Clustered Regularly Interspaced Short Palindromic Repeats (worldwide known as CRISPR) technology to modify the expression of YKL-40 in the D492 cell lines and, in a retrospective view, I could have alternatively used it to overexpress HER2 in D492 and D492M.

The CRISPR technology is a powerful tool to edit gene expression that is based on a natural system used by microorganisms as an immune defense to cleave foreign genetic information by RNA-guided nucleases (Garneau et al., 2010; Horvath & Barrangou, 2010; Jansen et al., 2002; Mojica et al., 2005). This method is becoming more and more relevant in daily work in molecular and cell biology laboratories and, currently, the variety of modifications in the technology is increasing.

This technique is more specific than lentiviral transductions as it is not possible to know exactly where the lentiviral insertion is integrated into the genome. Moreover, by using the CRISPR technology instead of lentiviral transductions, I could avoid the use of viruses, which always implies some handling risk. Even if there are more steps to follow in the CRISPR protocol, for me it worked at the first attempt and I had no problems in the transfection. Collectively, learning the CRISPR technique took time but it was accurate in gene-editing. In addition, this technique is giving good results in other projects in our lab when working with non-coding RNAs (work in progress). However, it is important to take into account the risk of off-targets and the knock-down (or other modifications) need to be confirmed regularly.

Although the CRISPR protocol that I used to knock-down (KD) YKL-40 in D492HER2 cells was to knock-out (KO) YKL-40, it was not possible to perform a complete knock-out due to the difficulty of single-cell cloning in the D492HER2 cell line. Single-cell clones were not able to survive when I seeded only one D492HER2 cell in each well of a 96-well plate. In a different attempt, seeding D492HER2 cells at clonal dilutions in a big plate was also unsuccessful.

Thinking in a retrospective view, it may be possible to improve this protocol with different cell density seeding to have cells closer or using the conditioned media from other D492HER2 cells that could increase the survival of the cells. It has been previously shown at the laboratory that endothelial cells increase survival, growth and morphogenesis of breast epithelial cells (Ingthorsson et al., 2010; Sigurdsson et al., 2011) and cells with different EMT profiles (chapter 4.1). Based on this, one method I could have set up was single-cell cloning of D492HER2 cells by seeding them into 48- or 96-well plates and putting transwell filters on top with pre-seeded endothelial cells in each well. Endothelial cells may condition the medium and facilitate the growth of D492HER2 cells. However, this would be a time-consuming experiment to do.

Nevertheless, working with the pool of D492HER2 cells has shown high efficiency in the knock-down of YKL-40. It is a general rule to confirm the efficiency of the KD, but, in this case, it was imperative to do it by different techniques because the generated cell lines were not pure populations. The knock-down of YKL-40 was confirmed by qPCR, WB and ELISA. The cell line with the lowest expression of YKL-40 in all techniques was selected for further characterization and experiments.

To overexpress YKL-40 in D492 and D492M cells, I used a variant of the CRISPR technology, the CRISPRa (CRISPR activation) (Zhang et al., 2015).

A major advantage of this method is that the performance of single-cell cloning is not required, which saves time and some cell lines are problematic for this procedure. Also, the activation of the transcription of an endogenous gene instead of an ectopically inserted gene may reduce the cellular stress caused by the modification of gene expression. However, the creation of new cell lines involves two transfections at different times; first, the dCas9 plasmid is inserted in the genome of the desirable cell line and, then, the gRNA plasmids need to be encapsulated in virus particles for the following transduction of the cells. This system is time-consuming but, in the end, it has provided excellent results in YKL-40 overexpression and other target genes used in colleagues' projects, such as ECM1.

6 Concluding remarks and future perspectives

In my Ph.D. thesis, I compared the breast epithelial progenitor D492 cell line and the derived EMT phenotype D492M and D492HER2 cell lines. These isogenic D492 cell lines share their genetic patterns but differ in the EMT phenotype and their ability to form tumors. The fact that D492HER2 is tumorigenic, whereas D492M is not, may reside in the plasticity of D492HER2 cells since D492HER2 has a more partial-EMT phenotype than D492M. D492HER2 has reduced the expression of epithelial markers and gained expression of mesenchymal proteins, but this is not as extensive as in D492M. Furthermore, D492HER2 has increased abilities to proliferate, migrate and invade compared to D492M, but is more susceptible to apoptosis and its glucose metabolism is more similar to the metabolism in the epithelial D492 cell line.

Differences in the EMT phenotype between D492M and D492HER2 were not visible in monolayer cell cultures, as they shared a spindle-shaped phenotype in 2D (Briem et al., 2019b; Morera et al., 2019). Nevertheless, there are evident phenotypic differences between these two cell lines when cultured in 3D. D492M cells form exclusively spindle-shaped EMT-like colonies in contrast to D492HER2 cells, which form a mixture of spindle-shaped and grape-like colonies. This highlights the need to implement 3D cultures in the characterization of cell lines.

Research into the regulators of EMT has been useful for the understanding of the plasticity of EMT states (Dvinge et al., 2013; Korpal et al., 2008; Lim et al., 2013). In Paper I, I demonstrate that miR200c-141 and miR203a are differently expressed in D492, D492M and D492HER2. These microRNAs are higher expressed in D492HER2 than in D492M, but in D492 they are greatly expressed. The miR200c-141 and the miR203a have been overexpressed in D492M in previous studies in our lab resulting in the induction of partial-MET since the epithelial phenotype was partially recovered (Briem et al., 2019a; Hilmarsdottir et al., 2015). Therefore, it would be interesting to overexpress these microRNAs in D492HER2 to study if the epithelial phenotype is reestablished entirely or, at least partially, both, in monolayer and 3D cultures, and if the tumorigenic abilities are reduced.

Interestingly, in a recent paper, Kröger *et al.* demonstrated that basal breast cancer cells that express a hybrid epithelial and mesenchymal state show

tumorigenic abilities only when they remain in an intermediate EMT state. This partial-EMT status seems to be crucial for the maintenance of malignant properties because when cells are forced to a complete mesenchymal phenotype by ectopically adding ZEB1, they become considerably less aggressive (Kröger et al., 2019). Following this strategy, it would be interesting to induce the expression of ZEB1 and other EMT-TFs in D492HER2 and analyze the effects regarding cellular plasticity and tumorigenic abilities.

To explore further EMT plasticity and tumorigenesis in the D492 cell lines, it would be relevant to induce malignant properties to the fixed EMT phenotype D492M cell line. This was the aim of overexpressing the HER2 oncogene in D492M. However, due to time limitations (see Aspects on methodology and experimental approach chapter), it was not possible to entirely characterize this cell line. Differences regarding the phenotype have been noticed but future research should further develop the characterization and confirm the possibility of tumorigenic abilities.

In my Ph.D. project, I have also studied the importance of the heterotypic interactions between stromal cells and the D492 cell lines with different EMT profiles. The crosstalk between endothelial cells (ECs) and epithelial cells, as previously described, induces epithelial branching and EMT (Ingthorsson et al., 2010; Shekhar et al., 2000; Sigurdsson et al., 2011; Zhang et al., 2014), like in the D492 cell line (Briem et al., 2019b). In my thesis, I have also shown that ECs increase survival and clonal growth of D492M and D492HER2 cells, which are EMT phenotype cell lines. Interestingly, ECs have a stronger interaction with D492HER2 in comparison to D492M. These different effects toward these two cell lines gave me the reason to believe that these could be due to secreted factors from D492HER2 cells.

Therefore, one of the main aims of my project was to identify possible effectors of the interactions between endothelial cells and the D492 cell lines. In Paper I, the comparisons of gene expression and protein secretion between D492M and D492HER2 identified YKL-40 as a candidate that may explain the differences in malignancy and plasticity between the cell lines. This study revealed that YKL-40 is a potential inducer of angiogenesis. Likewise, in Paper II, ECM1 was identified when the comparative analyses between the cell lines were focused on the D492 and D492HER2 cell lines.

The role of YKL-40 in angiogenesis when it is secreted by D492HER2 cells has been confirmed using a monoclonal antibody anti-YKL-40 that blocks YKL-40 in D492HER2 and, subsequently, the capillary-like network formation was decreased. Similar effects were obtained after transient and stable KD of YKL-

40 in D492HER2. The pro-angiogenic inducer power of D492HER2 was rescued by adding recombinant YKL-40 protein in ECs media. Moreover, the overexpression of YKL-40 in D492 and D492M increased the induction of angiogenesis in ECs considerably.

Similar to YKL-40, the secretion of ECM1 induced pro-angiogenic effects in ECs. However, further characterization of the CRISPR cell lines revealed that YKL-40 and ECM1 followed different pathways to activate angiogenesis in the endothelial machinery (Fig. 25). While YKL-40 is able to activate VEGFR-2, which induces not only angiogenesis but also proliferation in ECs, and works collaboratively in a synergetic relationship with other pro-angiogenic inducers, such as VEGF-A and VEGF-C; ECM1 stimulates the NOTCH signaling network in ECs and no relation with other pro-angiogenic factors was noticed. Nevertheless, these pathways and the synergetic collaboration between pro-angiogenic agents are not well understood yet, thus further investigation on the subject would clarify the action of YKL-40 and ECM1 in angiogenesis. In addition, animal experiments would contribute to understanding and development of effective treatment in tumor angiogenesis *in vivo*. It would also be worth blocking YKL-40 and ECM1 simultaneously, since blocking the activation of two different angiogenic pathways in ECs might be more efficient.

My study indicates that YKL-40 may be related to cellular plasticity (Fig. 25). Even if there were no differences in the phenotype and expression of EMT markers in the comparison of D492HER2 and D492HER2 YKL-40 KD in monolayer cell cultures, there were differences in the 3D EMT phenotypes. When YKL-40 is knocked-down in D492HER2, the grape-shaped colonies are reduced in number, shifting toward a phenotype similar to D492M, with only spindle-like colonies. Likewise, when YKL-40 is overexpressed in D492M, colonies shift toward the grape-shaped form in 3D cell cultures. The same effect occurs when the recombinant YKL-40 protein is added to the media. This indicates that YKL-40 may be an important regulator of cellular plasticity. In future research, more investigation is needed to understand the purpose of these changes in phenotype and if they provide tumorigenic abilities. Furthermore, it would be interesting to explore if ECM1 has a role in plasticity similar to YKL-40 and shows changes in the 3D phenotype and expression of EMT markers.

In Paper I, I have revealed that YKL-40 has a role in cellular migration and invasion (Fig. 25). When YKL-40 is knocked-down in D492HER2, the migration and invasion abilities of the cells are reduced. In contrast, if YKL-40 is overexpressed in D492 and D492M cell lines, migration and invasion are

increased. These properties are essential to support cancer development and tumor growth (Hanahan & Weinberg, 2011). It would also be relevant to investigate whether ECM1 has similar migrative and invasive properties in D492HER2 cells and cell lines that overexpress ECM1.

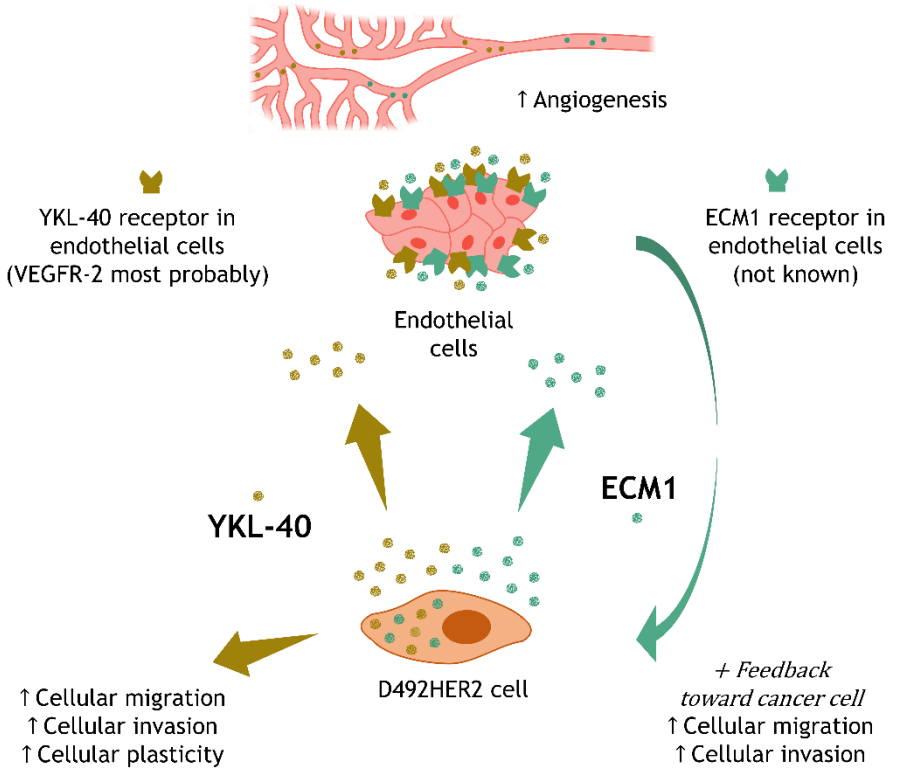

Figure 25. **Overview of the effects of YKL-40 and ECM1 in D492HER2 cells.** D492HER2 cells have high expression of YKL-40 and ECM1. YKL-40 increases migration, invasion and plasticity in the tumorigenic cell line. When YKL-40 and ECM1 are secreted into the ECM, they induce increased angiogenesis stimulating different receptors in endothelial cells. The most probably receptor of YKL-40 is VEGFR-2 (Faibish et al., 2011) and the receptor of ECM1 is unknown yet, however the NOTCH signaling pathway may be upregulated. When ECs are stimulated with ECM1, there is a feedback toward D492HER2 cells that increases their migration and invasion (Morera et al., 2019; Steinhaeuser et al., 2020).

The correlation between YKL-40 and HER2 regarding poor prognosis in breast cancer has been previously suggested (Kang et al., 2014; Shao et al., 2011). However, the relation between YKL-40 and HER2 has not been molecularly confirmed. Here, I demonstrate that transient and stable KD of YKL-40 in D492HER2 leads to a reduction in HER2 expression. Also, the inhibition of tumorigenic properties of HER2 in D492HER2 reduced YKL-40 expression. Further investigation of the connection between YKL-40 and HER2

is needed. YKL-40 may be related to the tumorigenesis provided by HER2 and might be a novel potential target with therapeutic applications in HER2-positive breast cancer patients. Likewise, a connection between ECM1 and HER2 was suggested in Paper II, but no molecular tests have been performed yet to support this theory. Thus, further research on the ECM1 molecular pathway in D492HER2 and other tumor cells would elucidate how ECM1 intervenes in malignant processes and could contribute to therapies to treat those tumors.

Interestingly, studies have shown that YKL-40 not only interacts with ECs but also with fibroblasts and macrophages which may contribute to increased aggressiveness and dissemination of the malignant cells toward metastasis. YKL-40 interconnects cancer cells, cancer-associated fibroblasts (CAFs), macrophages and ECs (Fig. 26). CAFs upregulate and secrete YKL-40, which induces the recruitment and reprogramming of macrophages (Cohen et al., 2017). Macrophages switch to tumor-associated macrophages (TAMs), which produce YKL-40 as well (Shao, 2013). Together with YKL-40 secreted by tumor cells, YKL-40 derived from the cancer-associated stromal cells promotes tumor development and dissemination of tumor cells toward metastasis (Cohen et al., 2017; Shao, 2013). However, these interactions are not completely understood and have not been confirmed *in vivo*. Therefore, a better understanding of the role of YKL-40 in stromal cells may constitute the object of future studies in breast cancer and metastasis. Furthermore, ECM1 has been shown to induce angiogenesis and feedback toward the tumorigenic D492HER2 from ECs in Paper II, therefore it would be relevant to analyze tumor growth and aggressiveness in addition to interactions between tumor cells and stromal cells. Hopefully, tumorigenesis and metastasis assays *in vivo* will be conducted in the future using the cell lines that were created modifying the expression of YKL-40 and ECM1, and analysis of the results will contribute to understanding the complex roles of YKL-40 and ECM1.

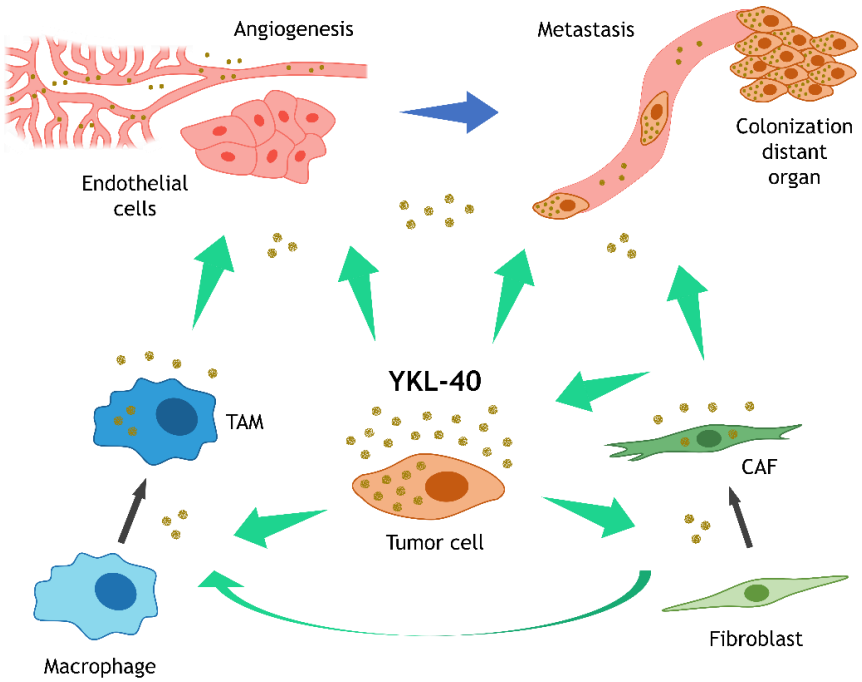

Figure 26. Overview of the YKL-40 interactions in breast cancer. YKL-40 has a complex role that involves tumor cells and stromal cells. Tumor cells secrete YKL-40, which contributes to macrophage and fibroblasts differentiation. Malignant macrophages and fibroblasts secrete YKL-40 as well. YKL-40 stimulates endothelial cells to induce angiogenesis. In the last instance, YKL-40 facilitates cancer progression and, most probably, metastasis (Morera *et al*., 2019, Cohen *et al*., 2017, Shao, 2013).

Collectively, in my studies, the phenotypic and functional comparisons between the isogenic breast epithelial D492, the endothelial-induced EMT D492M and the oncogene-induced EMT D492HER2 cell lines have resulted in a deeper understanding of how these cell lines behave in terms of EMT and their interactions with the microenvironment. Moreover, I have identified candidates that may explain the differences between the cell lines concerning angiogenesis, migration and invasion: YKL-40 (Paper I) and ECM1 (Paper II). YKL-40 and ECM1 have complex roles that involve interactions between tumor cells and the surrounding stromal cells. These signals induce changes within tumor cells to facilitate migration and invasion but also induce feedback from the stromal cells to support cancer progression, tumor growth, and, most likely, cancer dissemination toward metastasis. However, the role of YKL-40 and ECM1 in tumorigenicity in animal models and cancer patients is still unclear. Further studies are required to understand the function of YKL-40 and ECM1 completely and unravel the signaling pathways that these proteins may use. The knowledge of YKL-40 and ECM1 functions and effects is of clinical

relevance since new approaches targeting YKL-40 or ECM1, or both can result in novel treatments in breast cancer and metastasis.

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Original publications

Paper I

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YKL-40/CHI3L1 facilitates migration and invasion in HER2 overexpressing breast epithelial progenitor cells and generates a niche for capillary-like network formation

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Abstract
Epithelial to mesenchymal transition (EMT) is a developmental event that is hijacked in some diseases such as fibrosis and cancer. In cancer, EMT has been linked to increased invasion and metastasis and is generally associated with a poor prognosis. In this study, we have compared phenotypic and functional differences between two isogenic cell lines with an EMT profile: D492M and D492HER2 that are both derived from D492, a breast epithelial cell line with stem cell properties. D492M is non-tumorigenic while D492HER2 is tumorigenic. Thus, the aim of this study was to analyze the expression profile of these cell lines, identify potential oncogenes, and evaluate their effects on cellular phenotype. We performed transcriptome and secretome analyses of D492M and D492HER2 and verified expression of selected genes at the RNA and protein level. One candidate, YKL-40 (also known as CHI3L1), was selected for further studies due to its differential expression between D492M and D492HER2, being considerably higher in D492HER2. YKL-40 has been linked to chronic inflammation diseases and cancer, yet its function is not fully understood. Knock-down experiments of YKL-40 in D492HER2 resulted in reduced migration and invasion as well as reduced ability to induce angiogenesis in an in vitro assay, plus changes in the EMT-phenotype. In summary, our data suggest that YKL-40 may provide D492HER2 with increased aggressiveness, supporting cancer progression and facilitating angiogenesis.

Keywords YKL-40/CHI3L1 · Epithelial to mesenchymal transition (EMT) · Migration · Invasive breast cancer · Angiogenesis

Introduction

Epithelial to mesenchymal transition (EMT) is a developmental process that describes the plasticity of epithelial cells to change phenotype from compact adherent epithelium to mesenchymal-like cells that have lost their polarity and cell-cell adherence (Thiery *et al*. 2009). This is an important event in normal development, during gastrulation, neural crest formation, and wound healing, but also takes place in malignant processes like fibrosis and cancer (Thiery *et al*. 2009; Nieto 2013). In cancer, EMT is associated with increased aggressiveness and it is believed to be necessary for migration and invasion of cancer cells for the initial steps in the metastatic processes (Thiery *et al*. 2009).

The EMT process is accompanied by increased expression of mesenchymal markers such as vimentin, N-cadherin, α-smooth muscle actin, fibronectin, and/or Axl, as well as decreased or absent expression of epithelial markers like cytokeratins, yet the most referenced change in EMT is the loss of E-cadherin (Peinado *et al*. 2007; Gjerdrum *et al*. 2010). EMT is known to be induced by a number of factors. It can take place due to intrinsic factors such as genetic mutations or epigenetic changes or extrinsic factors like hypoxia or inflammation that stimulate the release of signals from the stroma (Yang *et al*. 2008; Kalluri and Weinberg 2009; Polyak and Weinberg 2009). Transforming growth factor beta (TGF-β) has been

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Table 1. List of primers used in this study. List of gene IDs for all target genes used and their corresponding primer IDs and supplier

Primers	Cat. number	Company
miR-200c	No. YP00204482	Qiagen
miR-203	No. 205914	Exiqon
miR-205	No. 204487	Exiqon
VEGF-A	Hs.PT.58.21234833	IDT
VEGF-C	Hs.PT.58.14602240	IDT
GUCA1C	Hs.PT.58.680712	IDT
CITED1	Hs.PT.58.1567731	IDT
MYBPH	Hs.PT.58.39772389	IDT
YKL-40 /CHI3L1	Hs.PT.58.22570467	IDT
KCNQ1OT1	Hs.PT.58.4572396.g	IDT
ERBB2	Hs.PT.58.1330269	IDT
GPR27	Hs.PT.58.38722549.g	IDT
S100A9	Hs.PT.58.20989743	IDT
DLK1	Hs.PT.58.40622309	IDT
GDF6	Hs.PT.58.20193545	IDT
PXDN	Hs.PT.58.630748	IDT
TLR4	Hs.PT.58.38700156.g	IDT
BMP4	Hs.PT.56a.3848863	IDT
CDH2	Hs.PT.58.26024443	IDT

shown to be a powerful inducer of EMT in a number of cell types (Kasai *et al*. 2005; Willis and Borok 2007; Kalluri and Weinberg 2009). Tyrosine kinase receptors and their ligands like EGF, PDGF, FGF, and HGF have also been shown to induce EMT (Kalluri and Weinberg 2009). Recently, regulatory functions carried out by non-coding RNAs have attracted the attention of researchers. Downregulation of miRNAs, especially members of the miR-200 family, miR-203, and miR-205, have been linked to EMT (Wiklund *et al*. 2010; Moes *et al*. 2012; DeCastro *et al*. 2013; Hilmarsdottir *et al*. 2014, 2015). In epithelial cells, the expression of these miRs is high but is commonly reduced or absent during EMT. Interestingly, rescue of these miRs can revert the EMT phenotype in the process mesenchymal to epithelial transition (MET) (Burk *et al*. 2008; Korpal *et al*. 2008; Hilmarsdottir *et al*. 2014). Furthermore, EMT in cells is often accompanied by increased ability to migrate and invade, as well as increased resistance to apoptosis (Thiery *et al*. 2009). For this reason, EMT has been related to aggressiveness and metastasis in cancer (Thiery 2002; Nieto 2013; Tan *et al*. 2014). The concept of cancer stem cells (CSCs) has also been linked to EMT, where EMT is an integral part of CSC plasticity and survival (Mani *et al*. 2008). Like somatic stem cells, CSCs have the capability to self-renew and their EMT phenotype is believed to be protective against external damages, which could lead to resistance to cancer therapies (Al-Hajj *et al*. 2003; Stingl 2006). In addition, CSCs as a cell population could be the cells responsible for the dissemination of cancer cells and the heterogeneity evidenced in metastasis (Brabletz 2012). Therefore, it is vital to elucidate the role of CSCs in cancer progression in order to improve efficient treatments and targeted therapy.

In breast cancer, the subtype basal-like breast cancer (BLBC) is identified as having the most undifferentiated cells and heterogeneity, which has been linked to CSCs and interestingly has a higher incidence of EMT (Mani *et al*. 2008; Morel *et al*. 2008; Sarrio *et al*. 2008). However, EMT is not exclusively found in this subtype. In luminal and HER2 breast cancers, there is an EMT-derived phenotype, although at a lower percentage (Yu *et al*. 2013; Tan *et al*. 2014).

Resistance to drugs in breast cancer may be associated with EMT-phenotype and heterogeneity of cells in the tumor. It is important to note that states of EMT are not merely defined as completely epithelial or mesenchymal; there are intermediate states of EMT that provide plasticity and advantages to cells to adapt to their microenvironment. This program is referred as partial-EMT (p-EMT) (Huang *et al*. 2013; Nieto 2013; Tam and Weinberg 2013; Yu *et al*. 2013).

The epithelial cells that reside in the breast gland are under continuous remodeling due to hormonal cycling and interactions with the surrounding stroma. This crosstalk is required in normal development and differentiation, and is utilized by tumor cells during cancer progression. Resident fibroblasts and macrophages in the stroma are able to produce growth

Table 2. List of antibodies used in this study. List of primary antibodies (protein IDs, dilution, company, and order IDs) for western blotting and IF staining for candidate proteins

Antibodies	Assay	Cat. number	Company	Dilution
Actin	WB	ab3280	Abcam	1:5000
Axl	IF	CS no. 8661	Cell Signaling	1:100
CK14	WB	ab15461	Abcam	1:1000
CK19	IF	ab7754	Abcam	1:100
E-cadherin	WB	610182	BD Transduction Labs	1:1000
Tubulin	WB	ab6046	Abcam	1:5000
Vimentin	WB	M0725	DAKO	1:1000
YKL-40 /CHI3L1	IF	MABC196	Millipore	1:100
YKL-40 /CHI3L1	WB	MABC196	Millipore	1:500

Table 3. siRNAs used for transient YKL-40 knockdown. Catalog numbers of siRNAs used as negative control and transient knock-down of YKL-40

siRNAs	Product name	Cat. number	Company
Neg Ctrl siRNA	Silencer® Select Negative Control No. 1 siRNA	4390843	ThermoFisher Scientific
YKL-40 siRNA 1	Silencer® Select CHI3L1 siRNA 1	AM16708 (ID 119124)	ThermoFisher Scientific
YKL-40 siRNA 2	Silencer® Select CHI3L1 siRNA 2	AM16708 (ID 119126)	ThermoFisher Scientific

factors and pro-inflammatory molecules, but also pro-angiogenic molecules that support the creation of new blood vessels. Fibroblasts not only interact with epithelial cells, they also activate the action of immune cells like macrophages and promote angiogenesis by cell communication with endothelial cells. The vascular endothelial growth factor (VEGF) and TGF-β are secreted by fibroblasts and macrophages, and they are able to stimulate angiogenesis. TGF-β induces the expression of VEGF that is secreted to the ECM and stimulates endothelial cells to initiate angiogenesis (Relf *et al*. 1997; Carmeliet and Jain 2000).

There is also direct interaction between epithelial cells and endothelial cells. The vascular niche plays an important role in transporting oxygen and nutrients and releasing signals for its correct morphogenesis and development. Indeed, it has also been observed that endothelial cells increase growth and branching morphogenesis of breast epithelium (Shekhar *et al*. 2000; Sigurdsson *et al*. 2006; Ingthorsson *et al*. 2010) and induce EMT (Sigurdsson *et al*. 2011). In the context of cancer, the high proliferation rate of tumor cells can lead to a reduction in oxygen concentration resulting in acidification of the surrounding stroma. In order to survive, cancer cells are able to secrete signals that induce angiogenesis, such as VEGF (Hanahan and Weinberg 2011). Receptors on endothelial cells, such as VEGFR2, are induced to trigger activation of the transcriptional machinery of angiogenesis that involves the hypoxic inducible factor 1 (HIF1) and other factors like nuclear factor κB (NF-κB) (Vegran *et al*. 2011; Sonveaux *et al*. 2012). The increase in angiogenesis ensures oxygen and a nutrient supply and therefore results in a worse prognosis of the cancer, making it an important factor to study.

D492 is a breast progenitor epithelial cell line that was established by immortalization of a suprabasal subpopulation of breast tissue from a healthy donor (Gudjonsson *et al*. 2002). As reviewed in Briem *et al*. (2019), D492 can generate both luminal and myoepithelial cells, and in 3D culture forms branching terminal ductal lobular units (TDLU) like structures (Briem *et al*. 2019). We have previously demonstrated that when D492 is co-cultured with breast endothelial cells (BRENCs), a subpopulation of cells undergoes EMT. One such subpopulation was isolated, giving rise to the D492M cell line, which has a fixed mesenchymal phenotype (Sigurdsson *et al*. 2011). In addition, we have shown that D492 cells with forced overexpression of the HER2 oncogene (D492HER2) have lost their epithelial phenotype and gained a mesenchymal one (Ingthorsson *et al*. 2015).

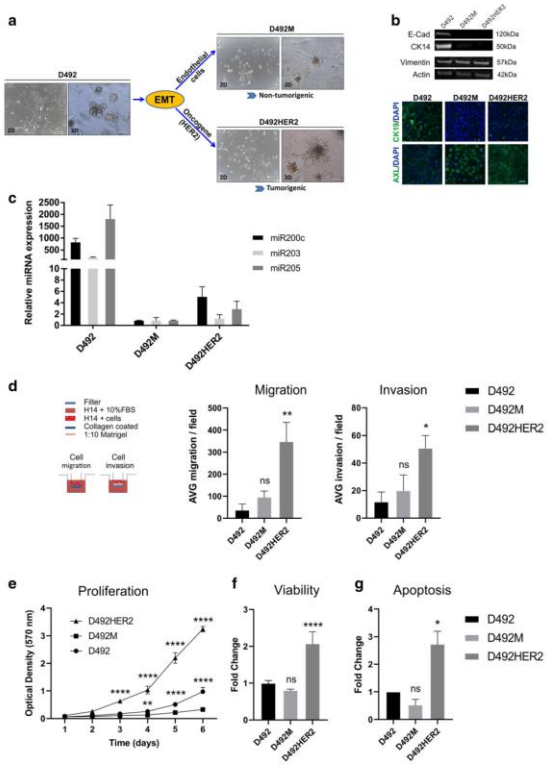
In this study, we have compared functional and phenotypic differences between D492M and D492HER2. We show here that D492HER2 proliferates, migrates, and invades faster than D492M. Furthermore, glucose metabolism is more dependent on glycolysis than oxidative phosphorylation in D492HER2. The comparative analysis of transcriptome revealed that a glycoprotein, YKL-40, also known as CHI3L1, is highly upregulated in D492HER2 and may contribute to the differences in tumorigenicity between the cell lines. YKL-40 has previously been linked to chronic inflammation diseases and cancer (Libreros *et al*. 2012; Jefri *et al*. 2015; Libreros and Iragavarapu-Charyulu 2015; Cohen *et al*. 2017); however, its function is not clearly understood yet. Functional studies using knockdown of YKL-40, recombinant protein, and overexpression experiments reveal that YKL-40 influences migration, invasion, and angiogenesis and induces changes in the EMT phenotype.

Material and Methods

Cell culture (2D and 3D) The main cell lines used in this project were D492 (Gudjonsson *et al*. 2002) and its EMT-phenotype-derived cell sublines D492M (Sigurdsson *et al*. 2011) and D492HER2 (Ingthorsson *et al*. 2015). For monolayer culture, flasks or plates were pre-coated with collagen I (2.2%) (no. 5005-B, Advanced BioMatrix, Carlsbad, CA). The medium used for culturing the cell lines was H14 (Blaschke *et al*. 1994), an enriched serum-free medium based on DMEM:F12 in which growth factors are added (insulin,

Table 4. gRNAs used for generating stable cell lines. Names and sequences of gRNAs used to either knockdown or overexpress YKL-40

gRNAs	Sequence	Action	Company
YKL-40/CHI3L1 KD	CCGCCATTTCTGCGCACCCA	Knockdown	
YKL-40/CHI3L1 SAM OV 1	AGTTTTGAAAACTTTGGGTC	Overexpress	Genscript
YKL-40/CHI3L1 SAM OV 2	CTGCCAGCAGAAGAGCCACT	Overexpress	Genscript



transferrin, EGF, sodium selenite (NaSel), estradiol, hydrocortisone, prolactin).

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords obtained from Landspitali University Hospital, Reykjavik, Iceland, with informed consent and approved by the Landspitali ethical committee (No. 35/2013). HUVECs were cultured in EGM2 + 5% FBS (no. CC-3162, Lonza).

In 3D cultures, cells were cultured in 300 μL of Matrigel (no. 354230, Corning, Corning, NY) per well, in 24-well plates (no. 353047, Corning, Corning, NY). In monoculture, 20,000 cells were embedded in reconstituted basement membrane, rBM, purchased as Matrigel (Corning no. 354230), with 500 μL of H14 media on top, while in co-cultures, 500 cells of D492 or its sublines were co-cultured with 150,000–200,000 HUVECs, embedded in Matrigel, with 500 μL of EGM2 + 5% FBS media on top.

Gene expression levels by qRT-PCR Total RNA was isolated with cold Tri-Reagent reagent (no. AM9738, Life Technologies, Carlsbad, CA). RNA precipitation was done using isopropanol and centrifugation at 14,000 rpm for 20 min.

◀ **Figure 1.** Phenotypic and functional characterization of D492M and D492HER2 cell lines. (*a*) D492M and D492HER2 are isogenic EMT-derived sublines of D492, a breast epithelial cell line with progenitor properties, which in 3D cell culture forms branching structures, resembling the TDLUs of the breast. D492M and D492HER2 were generated when D492 underwent endothelial-induced and oncogene (HER2)-induced EMT, respectively. D492M and D492HER2 show similar EMT-phenotype in 2D, but in 3D, D492M generate spindle-like colonies whereas D492HER2 generate spindle-like and grape-like colonies. D492M and D492HER2 also differ in their ability to generate tumors, as only D492HER2 is able to form tumors. *Scale bar* = 200 μm. (*b*) D492M and D492HER2 have lost expression of epithelial markers (E-cadherin, cytokeratin-14, and cytokeratin-19) and gained expression of mesenchymal markers (Axl and vimentin) as shown by western blotting and immunofluorescence. *Scale bar* = 100 μm. (*c*) D492M and D492HER2 show reduction in the expression of epithelial microRNAs such as miR-200c, miR-203, and miR-205 compared to D492. The lowest levels of expression are found in D492M (mean ± SD, $n = 2$). (*d*) In transwell migration and invasion assays, D492HER2 has an increased ability to migrate and invade compared to D492 and D492M. Results are shown as average number of cells per field (mean ± SEM, $n = 3$). One-way analysis of variance (ANOVA) and Dunnett's multiple comparison test were used to test significance ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$). (*e*) D492HER2 cells proliferate at a higher rate than D492 and D492M cells as shown by staining with crystal violet. Results are shown as average of four replicates (mean ± SD). Statistical significance was assessed using multiple *t* tests (one per *row*) ($^{**}p \leq 0.01$, $^{****}p \leq 0.0001$). (*f*) Furthermore, increased proliferation rate of D492HER2 compared to D492 and D492M cells was demonstrated using PrestoBlue™ Cell Viability reagent. Results are shown as average of eight replicates normalized to D492 (mean ± SD). One-way analysis of variance (ANOVA) and Dunnett's multiple comparison test were used to test significance ($^{****}p \leq 0.0001$). (*g*) In addition, D492HER2 cells are more susceptible to chemically induced apoptosis as compared to D492 and D492M cells. Caspase 3/7 luciferase activity was measured by luminescence and normalized to D492 (mean ± SEM, $n = 2$). Significance was assessed with one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test ($^{*}p \leq 0.05$).

Afterwards, RNA was washed with ethanol two times. The RNA pellet was diluted in RNAse free water. Concentration of RNA was measured using a NanoDrop® ND-1000 UV/Vis-Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). For cDNA synthesis, SuperScript IV (no. 18090-200, Thermo Fisher Scientific) and random hexamer primers were used.

To quantify the expression level of genes, TaqMan (no. M3004L, NEB) or SYBR Green (no. M3003L, NEB) chemistries were used to detect gene expression. Comparative Ct values were determined using an ABI 7500 instrument (Applied Biosystems, Foster City, CA). All used primers are listed in Table 1. GAPDH was used as the reference gene.

Expression levels of miRNAs by qRT-PCR Total RNA was extracted with Tri-Reagent (no. AM9738, Thermo Fisher Scientific). The RNA was reverse transcribed using miRCURY LNA RT Kit (no. 339340, Qiagen, Hilden, Germany) for cDNA synthesis reactions. Quantitative RT-PCR analysis of miRNAs was performed using miRCURY LNA SYBR Green PCR Kit (no. 339346, Qiagen). Relative expression was calculated with the $2^{\Delta\Delta Ct}$ method. All used primers are listed in Table 1. Normalization was done with U6 (no. 203907, Exiqon, Vedbaek, Denmark).

Protein levels by Western Blot (WB) Protein isolation was done using RIPA buffer, and the concentration was measured by the colorimetric Bradford method. Equal amount of protein was loaded for each sample (5 μg) in precast NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, CA). Proteins were detected using IRDye secondary antibodies on an Odyssey imaging system (Li-Cor Biosciences, Lincoln, NE). Loading controls actin or tubulin were used for quantification. Antibodies used for WB are listed in Table 2.

Immunostaining Immunofluorescence (IF) was used to detect proteins and visualize their subcellular location. Cells were fixed with 3.7% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100. Blocking with FBS was done prior to incubation with primary antibodies. Incubation was done overnight at 4°C and followed by incubation of secondary antibodies conjugated to fluorochromes Alexa Fluor-488, Fluor-546, or Fluor-647 for 1 h at room temperature. For nuclei staining, DAPI was used. Imaging was done using an EVOS FL Auto 2 Cell Imaging System (Thermo Fisher Scientific) or FV1200 Olympus inverted confocal microscope. Antibodies used for IF are listed in Table 2.

Transcriptome data analysis and classification Total RNA sequence profiles for D492, D492M, and D492HER2 were obtained from normal cell cultures and treated following the protocol detailed in Halldorsson *et al*. (2017).

Differentially expressed genes between cell lines were further classified into categories to see the biological processes that were enriched. Using a differential range from two fold change, genes were analyzed by the PANTHER analysis database (www.pantherdb.org) following instructions of Mi *et al*. (2013).

Secretome data analysis For mass spectrometry, D492 lines were grown in T175 flasks (no. 353112, Corning) and conditioned medium (CM) was collected, concentrated for 55 min using EMD Millipore Amicon™ Ultra-15 Centrifugal Filter Units (no. UFC900324, Merck Millipore, Darmstadt, Germany) followed by buffer exchange to 100 mM TRIS/HCl buffer. Triplicate samples were stored at − 80°C. Label-free relative protein quantification by nLC MS/MS after trypsin digestion was performed at the FingerPrints Proteomics Facility, University of Dundee, Dundee, UK and raw data were analyzed using MaxQuant software (version 1.6.2.1). Quantitative and statistical analyses were performed using XLStat (version 2018.1). Data were *P* value corrected (significance level 0.05)

a

Feature ID	D492 HER2 vs D492M Fold change	Higher/Lower expression confirmed by qPCR
GUCA1C	594.2	Almost not expressed
CITED1	306.1	D492HER2 > D492M
MYBPH	304.1	Similar expression
CHI3L1	199.2	D492HER2 > D492M
KCNQ1OT1	175.8	D492HER2 > D492M
ERBB2	136.1	D492HER2 > D492M
LINC00958	114.2	No
GPR27	73.3	D492HER2 > D492M
S100A9	71.0	D492M > D492HER2
DLK1	68.7	D492HER2 > D492M
More than 3.000 transcripts		
BMP4	-105.0	D492M > D492HER2
CDH2	-111.6	D492M > D492HER2
TLR4	-149.0	D492M > D492HER2
PXDN	-219.4	D492M > D492HER2
GDF6	-835.8	Similar expression

b

Enriched PANTHER GO Biological Processes in D492HER2 compared to D492M	Fold enrichment	P-value
D-aspartate transport (GO:0070777)	100	3.05E-02
inflammatory response (GO:0006954)	3.16	4.10E-04
cell chemotaxis (GO:0060326)	3.01	1.03E-02
cytokine-mediated signaling pathway (GO:0019221)	2.79	3.15E-02
regulation of neurotransmitter levels (GO:0001505)	2.72	4.02E-02
cellular response to cytokine stimulus (GO:0071345)	2.55	1.58E-02
organic anion transport (GO:0015711)	2.35	4.22E-02
cell proliferation (GO:0008283)	2.17	5.71E-03
defense response (GO:0006952)	2.09	4.70E-03
vasculature development (GO:0001944)	-2.02	4.95E-02
skeletal system development (GO:0001501)	-2.1	1.83E-02
circulatory system development (GO:0072359)	-2.23	1.84E-02
regulation of synapse structure or activity (GO:0050803)	-2.69	1.00E-02
sensory organ development (GO:0007423)	-2.77	2.85E-03
sensory organ morphogenesis (GO:0090596)	-3.54	1.44E-02
regulation of cartilage development (GO:0061035)	-4.5	3.96E-02
eye morphogenesis (GO:0048592)	-4.67	1.10E-02
negative regulation of embryonic development (GO:0045992)	-11.37	3.05E-02

c

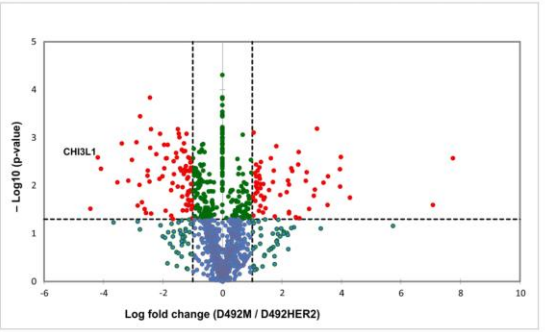


Figure 2. YKL-40 is enriched in D492HER2. *a* The analysis of transcriptome data comparing D492M and D492HER2 revealed more than 3000 transcripts differently expressed (more than two fold). YKL-40 (or CHI3L1) is more highly expressed in D492HER2 compared to D492M. *b* Classification of genes in biological processes shows an enrichment in D492HER2 compared to D492M in D-aspartate transport, inflammatory response, cell chemotaxis, cytokine-mediated signaling pathway, regulation of neurotransmitter levels, cellular response to cytokine stimulus, organic anion transport, cell proliferation, and defense response. YKL-40 is involved in the processes that require interaction with the stroma: inflammatory response, cell chemotaxis, cellular response to cytokine stimulus, and defense response (marked with *yellow*). *c* Mass spectrometry analysis of secreted proteins in CM revealed that YKL-40 is enriched in the secretome of D492HER2 compared to D492M (p value = 0.01).

and sorted based on ≥ 2-fold higher secretion (LFQ intensity) by D492HER2 compared to D492M.

Migration and invasion assays Migration and invasion assays were done in 24-well plates with transwell filter inserts (no. 353097, Corning) of 8 μm size pore diameter. Transwell inserts in the migration assay were pre-coated with collagen I (2.2%) and in the invasion assay; they were pre-coated with Matrigel diluted 1:10 in H14 media. Fifty thousand cells/transwell were seeded on the upper chamber in H14 media. In the bottom chamber, H14 was supplemented with 10% FBS as a chemoattractant. A cotton swab was used to remove non-migrated and non-invaded cells after 24 h and after 48 h, respectively. Thereafter, cells were fixed with 3.7% PFA and

stained with crystal violet (10%) or DAPI (1:5000 dilution) for 30 min. Three random pictures were taken per well and the number of cells was quantified. For DAPI-stained samples, images were converted to 8-bit in ImageJ (version 2.0.0), threshold-adjusted, and binary-converted and migratory/invasive cells were counted using the *analyze particles* function.

Proliferation assay Proliferation of cells was determined by seeding 10,000 cells/well in triplicate in 24-well plates in H14 (D492 cell lines) or EGM5 (HUVECS). Every day (2 d for HUVECs), cells were fixed and stained with crystal violet (10%). Crystal violet was diluted with acetic acid and the OD was measured at 570 nm wavelength. Alternatively, cell viability was assessed using PrestoBlue™ Cell Viability Reagent (ThermoFisher Scientific, Waltham, MA). Cells were seeded in H14 media in a 96-well plate at a density of 3000 cells/well and cultured for 4 d. PrestoBlue was added (1/10th of the total volume) to each well and incubated for 4 h, and absorbance was read on a plate reader at 570 nm and 595 nm.

Apoptosis assay To quantify apoptosis, cleavage of caspase 3/7 was measured by a luciferase assay (ApoTox-GloTM Triplex Assay, Promega, Madison, WI). Apoptosis was induced by incubating cells with 10 μM camptothecin (CPT) for 24 h according to the manufacturer's protocol. After cellular lysis, luciferase was measured with a microplate reader ModulusTM II (Turner Biosystems, Sunnyvale, CA).

Glucose consumption and lactate production measurements Glucose uptake was measured using Glucose Uptake-GloTM kit (no. J1341, Promega) following the manufacturer's protocol. Briefly, the analogue of glucose, 2-deoxyglucose (2DG), was added to the media and taken up by cells. When transported into cells, 2DG is phosphorylated to 2-deoxyglucose 6-phosphate (2DG6P) and further metabolization stimulates luciferase reactions and luminescence was measured by the microplate reader Modulus TM II (Turner Biosystems, Sunnyvale, CA).

Glucose consumption and lactate production were measured from the collected media when cells were in a high confluency. Metabolites were measured at the Analyzer machine (ABL90 FLEX Analyzer, Radiometer) at the Blood Bank of Landspitali (Reykjavik, Iceland).

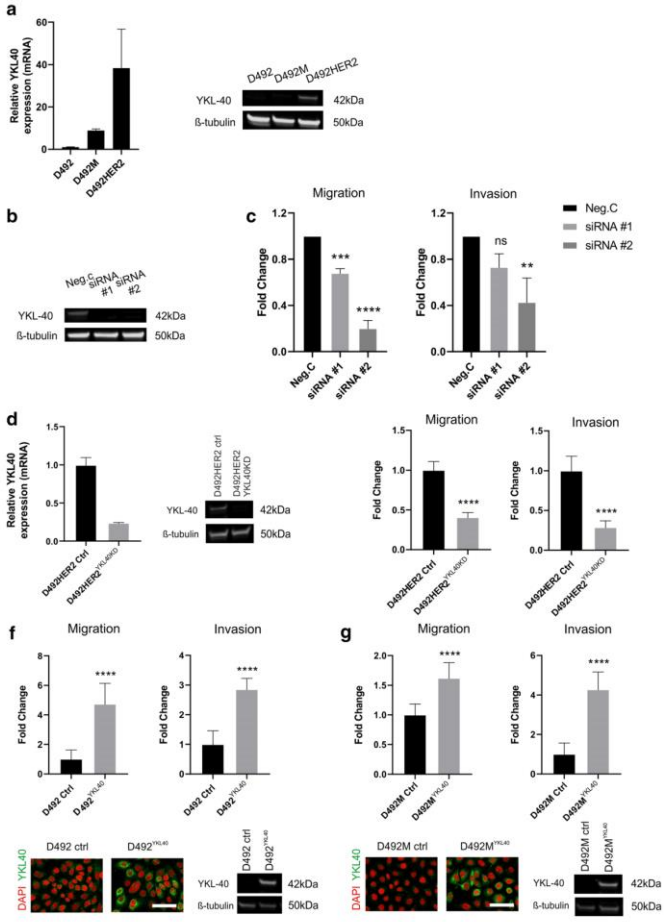
Neutralization assay of YKL-40 protein A monoclonal antibody against YKL-40 (mA^{YKL40}) (MABC196, Millipore) was used to block the secretion of YKL-40 in D492HER2. The antibody was diluted in fresh H14 medium at a concentration of 10 μg/mL. Medium from cells incubated for 24 h with mA^{YKL40} was collected, and medium from non-treated D492HER2 cells was used as control. Conditioned media (CM) were used for tube formation assays (described below).

Tube formation assay on endothelial cells (angiogenesis assay in vitro) To simulate angiogenesis in vitro, 10,000–12,000 HUVECs were seeded on top of 10 μL solidified rBM in a 96-well angiogenesis plate (no. 89646, Ibidi). Controls included HUVECs cultured in EGM5 media and a dilution of 1:1 EGM5 and conditioned media (CM). Recombinant YKL-40 protein ($YKL\text{-}40^{r}$) (no. 11227H08H5, Thermo Fisher Scientific, Waltham, MA) was added to the medium at a final concentration of 100 ng/mL. After incubation overnight, the endothelial network was imaged using the EVOS FL Auto 2 Cell Imaging System. Analysis and quantification were done using the *Angiogenesis analyzer* plug-in on ImageJ software (version 2.0.0).

Transient knockdown of YKL-40 by siRNA Pre-designed siRNAs (Silencer® Select Pre-Designed, Validated and Custom siRNA, Life Technologies) against YKL-40 were used at a concentration of 10 nM in D492HER2 cells to down-regulate YKL-40. First, cells were seeded on 6-well plate (no. 353046, Corning, Corning, NY) and after 24 h, cells were transfected with the siRNA using chemical transfection with Lipofectamine® RNAiMAX (no. 13778150, Life Technologies, Carlsbad, CA). For each experiment, two different siRNAs against YKL-40 and a negative control siRNA (no. 4390843, Life Technologies, Carlsbad, CA) were used. The siRNAs are listed in Table 3. After 48 h, the knockdown was confirmed by qRT-PCR and by WB.

Generation of stable cell line with knockdown of YKL-40 in D492HER2 by CRISPR Specific gRNAs to knockdown YKL-40 were designed with the publicly available online ATUM CRISPR tool (https://www.atum.bio/). Annealing of the forward and reverse gRNA oligos was followed by ligation with the backbone vector pMLM3636. High-efficiency competent bacteria (no. C2987H, NEB) were transformed, and confirmation of successful DNA insertion was done by colony PCR. Plasmids were sequenced to confirm correct gRNA sequences. Transfection of D492HER2 was done with four plasmid gRNAs and a plasmid with only a Cas9 cassette (pST1374) using Lipofectamine® 3000 (Thermo Fisher). The control cell line was generated by transfecting the cells with only the Cas9 cassette. Selection of cells with insertion of Cas9 was done using blasticidin. Knockdown of YKL-40 in D492HER2 was confirmed by qRT-PCR and WB. Subsequently, the cell line that gave the best efficiency of knockdown was selected for further work. The sequence of this gRNA is in Table 4. It should be noted that D492HER2 is a cell line not suitable for single-cell cloning; therefore, a pool of cells was used for confirmation of knockdown and further experiments.

Generation of stable cell lines overexpressing YKL-40 in D492 and D492M by CRISPRa Overexpression of YKL-40 in D492



and D492M was carried out using the modified CRISPR strategy, called CRISPRa (Zhang *et al*. 2015). Two specific SAM gRNAs for YKL-40 and one empty control were purchased from Genscript containing zeomycin resistance cassettes. The gRNA sequences are listed in Table 4. Transfection of HEK293T cells was done to produce viral particles including gRNAs. Viruses collected were used to infect D492 and D492M cells that were previously transfected with dCas9-

◀ **Figure 3.** YKL-40 affects migration and invasion in D492 cell lines. *a* YKL-40 is more highly expressed in D492HER2, compared to D492 and D492M, both at mRNA and protein level. *b*, *c* Transient siRNA knock-down of YKL40 reduced the ability of D492HER2 to migrate and invade in vitro (mean ± SD, $n = 3$). Statistical significance was tested using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test (**$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). *d*, *e* Knock-down of YKL-40 in D492HER2 in stable cell lines generated by CRISPR/Cas9 technology reduced the ability of D492HER2 to migrate and invade similarly to transient transfection. *f*, *g* Accordingly, overexpression of YKL40 in D492 and D492M using CRISPRa system increased their ability to migrate and invade. Overexpression of YKL-40 was confirmed by immunofluorescence staining (*Scale bar* = 100 μm) and western blotting. Migration and invasion assay results are shown as average of eight replicates (mean ± SD). Unpaired *t* test was used to test significance (**$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

VP64 to activate the promoter and later induce overexpression of the selected gene. Transduction with gRNAs was performed and selection was done with zeocin (no. R25005, Thermo Fisher Scientific). Confirmation of overexpression of YKL-40 in D492 and D492M was done by qRT-PCR, WB, and IF.

Statistical analysis One-way analysis of variance (ANOVA) using Dunnett's multiple comparison test or unpaired *t* tests were performed using GraphPad Prism to test significance. *P* values below 0.05 were considered significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

Results

Phenotypic and functional characterization of D492M and D492HER2 D492M and D492HER2 are isogenic cell lines that share a partial-EMT phenotype but are different in terms of their ability to form tumors in mice (Sigurdsson *et al*. 2011; Ingthorsson *et al*. 2015). D492M and D492HER2 are both derived from D492, a breast epithelial progenitor cell line, through endothelial-induced EMT and oncogene-induced EMT, respectively (Fig. 1*a*). D492M and D492HER2 have lost the expression of epithelial markers like E-cadherin, CK14, and CK19 and gained expression of some mesenchymal markers like vimentin and Axl, (Fig. 1*b*). MicroRNAs (miRs) such as miR-200c, miR-203, and miR-205 are frequently down-regulated in epithelial cells undergoing EMT, and this was the case for D492M and D492HER2. While D492 showed high expression of miR-200c, miR-203, and miR-205, these microRNAs were greatly reduced in D492HER2 and relatively absent in D492M (Fig. 1*c*). The difference in expression levels between D492M and D492HER2 may have been due to a more intermediate state of EMT for D492HER2. This intermediate state was also supported by the glucose metabolism (Fig. S1). These data show D492HER2 is more comparable in glucose uptake to D492 than D492M.

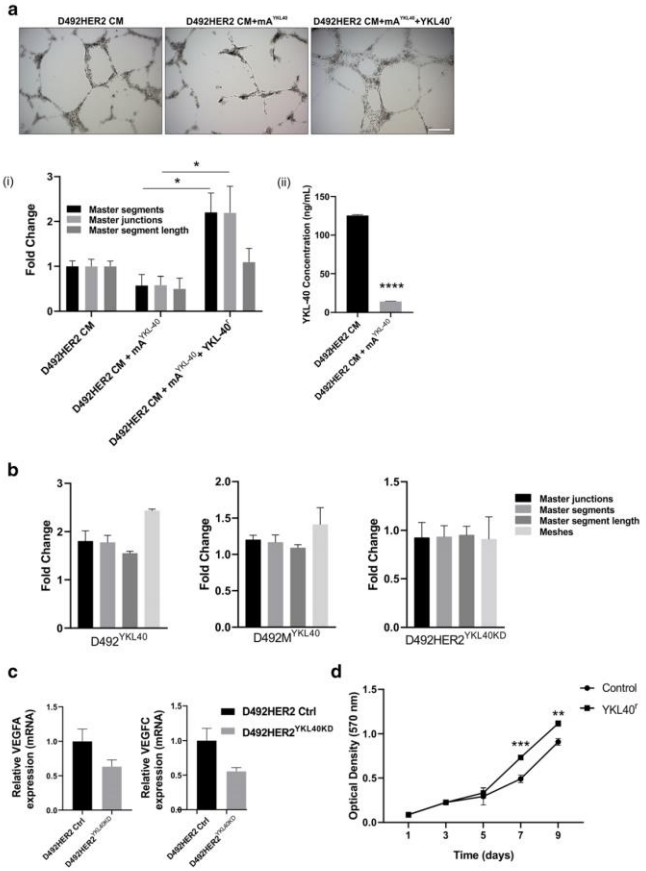
Further characterization demonstrated functional differences between the cell lines. Notably, D492HER2 cells migrated and invaded through transwell filters more efficiently than both D492M and D492 (Fig. 1*d*). The cell proliferation rate of D492HER2 cells was also significantly higher than in D492 and D492M cells (Fig. 1*e*, *f*). Furthermore, D492HER2 cells were more susceptible to chemically induced apoptosis than D492 and D492M cells (Fig. 1*g*), despite an apparent shift from oxidative phosphorylation to glycolysis as D492HER2 had higher glucose consumption and higher lactate production than either D492 or D492M cells (Fig. S1).

The functional differences between D492M and D492HER2 in terms of tumorigenicity prompted us to look into gene expression and secretome of D492M and D492HER2 to search for potential candidates responsible for these effects.

Transcriptome and secretome analyses show that YKL-40 is enriched in D492HER2 Next, we employed genome-wide analysis to reveal differences in gene expression in D492M and D492HER2. More than 40,000 transcripts were differentially expressed between D492M and D492HER2 and the most 15 differentially regulated genes were confirmed by qPCR (Fig. 2*a*). As expected, HER2 (ErbB2) has much higher expression in D492HER2 cells due to the ectopic overexpression of the gene (Ingthorsson *et al*. 2015). Although CITED1 was the first validated candidate, its absolute level of expression was low; thus, we focused our interest on a gene very highly expressed in D492HER2 compared to D492M (and also to D492), namely YKL-40 (also known as CHI3L1). The function of YKL-40 is not clearly understood but it has been suggested to be involved in cancer progression and other inflammation diseases (Libreros *et al*. 2012; Libreros and Iragavarapu-Charyulu 2015; Cohen *et al*. 2017) and even in EMT (Jefri *et al*. 2015).

Genes differentially expressed within the range of two fold change between D492M and D492HER2 were further classified using PANTHER analysis (Mi *et al*. 2013) in order to establish the biological processes that were enriched in each cell line. Genes more highly expressed in D492HER2 compared to D492M were shown to be involved in the following biological processes: D-aspartate transport, inflammatory response, cell chemotaxis, cellular response to cytokine stimulus, defense response, cellular response to cytokine stimulus, cell proliferation, and organic anion transport, among others (Fig. 2b). Interestingly, YKL-40 was found enriched in four of the 18 groups, as highlighted in yellow in Fig. 2b.

Complementing the gene expression data, we also measured by mass spectrometry the secretion of the proteins by D492M and D492HER2. Analysis of the total secreted



proteins in the conditioned media (CM) from D492M and D492HER2 revealed YKL-40 was found in much higher concentration in CM from D492HER2 (97.8 LFQ intensity) than D492M (5.4 LFQ intensity) (Fig. 2*c*). After these promising results, we strengthened our focus on YKL-40 as a firm candidate that could lead to differences between D492M and D492HER2.

Knockdown of YKL-40 in D492HER2 inhibits migration and invasion To explore the functional role of YKL-40 in

◀ **Figure 4.** YKL-40 expression in D492HER2 is linked to increased potential to stimulate angiogenesis. (*a*) Conditioned media (CM) from D492HER2 cells stimulates angiogenesis in an in vitro angiogenesis assay. This effect was abrogated upon YKL-40 neutralization by a monoclonal antibody (mA^{YKL40}) but rescued by the addition of recombinant YKL-40 protein ($YKL40^r$) (*Scalebar* = 200 μm). (i) Parameters of angiogenesis were measured and analyzed by ImageJ angiogenesis analyzer plug (mean ± SD) and significance was assessed with one-way analysis of variance (ANOVA) and Tukey's multiple comparison test ($*p \leq 0.05$). (ii) Decreased concentration of YKL-40 upon incubation of cells with mA^{YKL40} was verified with ELISA. Significance was tested with an unpaired t-test ($****p \leq 0.0001$). (*b*) When YKL-40 was overexpressed in D492 and D492M, there was an increase in the capillary network formation, whereas stable knockdown of YKL-40 in D492HER2 showed a tendency to reduced capillary network formation. (*c*) Knockdown of YKL-40 reduced the expression of other pro-angiogenic inducers, VEGFA and VEGFC. (*d*) Finally, recombinant YKL-40 protein ($YKL-40^r$) induced proliferation of endothelial cells when added to the media. Unpaired *t* test was used to test significance ($**p \leq 0.01$, $***p \leq 0.001$).

D492HER2, we did both transient and stable knockdown (KD). First, we confirmed the expression of YKL-40 at mRNA and protein level in D492, D492M, and D492HER2 demonstrating its abundant expression in D492HER2 (Fig. 3*a*). Transient knockdown with two different siRNAs greatly reduced YKL-40 expression in D492HER2 cells (Fig. 3*b*) and resulted in a significant decrease in migration and invasion (Fig. 3*c*). To further corroborate these effects, we generated stable YKL-40 KD using CRISPR technology in D492HER2 cells (Fig. 3*d*). Similar to transient KD of YKL-40, the stable KD cell line showed a reduction in migration and invasion compared to control D492HER2 cells (Fig. 3*e*). To analyze if overexpression of YKL-40 in D492 and D492M resulted in increased migration and invasion, we stably overexpressed YKL-40 in these cells and, indeed, this resulted in a large increase in both migration and invasion (Fig. 3*f*, *g*). No differences were seen with regard to cell proliferation regardless if YKL-40 was knocked down in D492HER2 or overexpressed in D492/D492M (data not shown). Collectively, these results demonstrate that in our cell system, YKL-40 increases migration and invasion.

YKL-40 expression in D492HER2 is linked to vascular network formation YKL-40 has previously been linked to angiogenesis (Shao *et al*. 2009, 2011). In order to see if increased expression of YKL-40 in D492HER2 was linked to increased potential to stimulate angiogenesis in an in vitro assay, we treated HUVECs on top of Matrigel with CM from D492HER2 with and without a functional blocking antibody against YKL-40 (mA^{YKL40}). Blocking YKL-40 directly reduced the ability of CM medium to induce HUVECs to form capillary-like networks. This blocking effect was reversed by adding recombinant YKL-40 protein ($YKL-40^r$) (Fig. 4*a*).

In agreement, CM from D492 and D492M stably overexpressing YKL-40 increased HUVEC network formation on top of Matrigel, whereas stable knockdown of YKL-40 in D492HER2 cells reduced HUVECs network formation (Fig. 4*b*). Additionally, the reduction of YKL-40 in the stable cell line revealed reduced expression of other pro-angiogenic genes, like VEGF-A and VEGF-C (Fig. 4*c*), suggesting a synergic pro-angiogenic effect induced in D492HER2. Furthermore, adding recombinant YKL-40 protein ($YKL-40^r$) to media not only stimulated tube formation on endothelial cells, but also increased proliferation rates of HUVECs (Fig. 4d).

YKL-40 affects phenotype of D492 cell lines when cultured in 3D culture When YKL-40 was downregulated in D492HER2 by transient or stable transfection, no phenotypic changes were seen in monolayer culture and EMT markers in immunostaining and western blot did not show any differences; thus, the EMT phenotype remained unaltered in 2D. Due to the transient nature of using siRNA, it was difficult to explore if there were any phenotypic changes occurring in 3D culture. In contrast, 3D culturing of cells with stable knockdown of YKL-40 revealed differences in phenotype of the D492 cell lines.

In 3D culture, D492HER2 formed two colony shapes, spindle-like and grape-like (Ingthorsson *et al*. 2015). Interestingly, when YKL-40 was knocked down, there was a dramatic change in the proportion of spindle-like and grape-like structures, with the proportion of grape-like structures being reduced from 21.4% to 1% (Fig. 5*a*). This subsequent change in the 3D phenotype of D492HER2 cells with knockdown of YKL-40 was similar to D492M cells that only showed a spindle-like phenotype (Sigurdsson *et al*. 2011). However, when the recombinant YKL-40 protein ($YKL-40^r$) was added to the media of D492HER2 with YKL-40 KD, the grape-like structures were rescued to 32.8% of the total 3D structures (Fig. 5*a*).

To confirm the shift to grape-like structures with the addition of YKL-40, D492M cells overexpressing YKL-40 were seeded in 3D cell culture. Remarkably, the entire cell population lost the ability to form spindle-like structures, and instead only grape-like structures were observed (Fig. 5*b*).

Discussion

In this study, we compared phenotypic and functional differences between two isogenic cell lines that share a partial-EMT phenotype but differ in terms of oncogenic properties. D492M is non-tumorigenic, whereas D492HER2 is tumorigenic. We identified YKL-40, also known as a chitinase-3-like protein 1 (CHI3L1), as a protein highly expressed and secreted in D492HER2 compared to D492M. Functional in vitro studies demonstrated that YKL-40 is involved in migration and invasion of D492HER2. Furthermore, blocking YKL-40 in conditioned media (CM) from D492HER2 reduced the ability of the

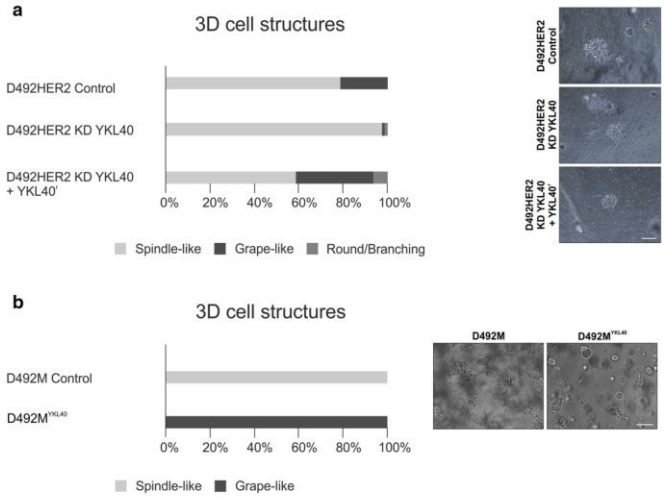


Figure 5. YKL-40 affects the phenotype of D492 cell lines in 3D. *a* CRISPR/Cas9-mediated knockdown of YKL-40 in D492HER2 cells, increased the number of spindle-like structures in a 3D culture, but grape-like structures were recovered with addition of recombinant YKL-40 protein (YKL40′) to the media (*Scalebar* = 200 μm). *b* Overexpression of YKL-40 in D492M shifted the phenotype from spindle-like structures to grape-like structures in 3D (Scalebar = 200 μm).

D492HER2-derived CM to induce formation of capillary like network in an in vitro angiogenesis assay, which conversely can be rescued with the addition of recombinant YKL-40 protein. Finally, KD of YKL-40 in D492HER2 changed the ratio of spindle- and grape-like structures in favor of spindle-like structures, which could be reverted when the recombinant protein was added to the KD cells in 3D culture.

The term EMT is used for describing phenotypic changes where epithelial cells lose and gain epithelial and mesenchymal traits, respectively. Very rarely, a complete EMT occurs, and rather intermediate states are the most frequent phenotypes. Thus, it is common that traces of epithelial traits remain and a full mesenchymal phenotype is often incomplete resulting in a large spectrum of EMT phenotypes (Nieto 2013).

D492M and D492HER2 share some EMT properties such as loss of E-cadherin and cytokeratins and formation of a spindle-shaped phenotype in monolayer. D492M has a more fixed EMT phenotype with low or absent expression of epithelial markers, including microRNAs, and gain of mesenchymal markers (Sigurdsson *et al*. 2011; Hilmarsdottir *et al*. 2015). In a recent paper, we demonstrated that reintroduction of miR-200c-141 into D492M was sufficient to revert the phenotype to epithelial lineage, albeit only to luminal epithelial cells. Further studies demonstrated that the basal cell transcription factor P63 was necessary to recover the basal/myoepithelial phenotype. Co-transfection of miR-200c-141 and P63 into D492M was sufficient to regain the original phenotype of D492 (Hilmarsdottir *et al*. 2015). On the other hand, D492HER2 shows more intermediate EMT phenotype than D492M and could be defined as a cell line with partial-EMT (Ingthorsson *et al*. 2015). Even though D492HER2 migrates, invades, and proliferates more, it is more susceptible to apoptosis, its glucose metabolism is more similar to the one in the epithelial cell line (D492), and the expression levels of the regulatory epithelial miRNAs are reduced but not as dramatic as in the case of D492M. Moreover, there are evident phenotypic differences between these two cell lines when cultured in 3D in reconstituted basement membrane matrix, as D492M forms exclusively spindle-shaped EMT-like colonies in contrast to D492HER2 that forms mixture of spindle-shape and grape-like colonies.

Triple-negative breast cancer cell lines are often spindle-shaped with more prominent EMT phenotype in 3D rBM while cell lines with high HER2 expression are associated with grape-like phenotype (Han *et al.* 2010). We have previously shown that D492M and D492HER2 form spindle-shaped and grape-like structures in 3D rBM, respectively (Sigurdsson *et al.* 2011; Ingthorsson *et al.* 2016). That overexpression of YKL-40 in D492M shifts the phenotype toward grape-like structures may indicate that YKL-40 confers increased plasticity to D492M. Further studies in our laboratory aim to unravel whether YKL-40 expression is associated with tumorigenic potential of D492M and D492HER2.

Comparing and analyzing gene-wide expression and protein-wide secretion of D492M and D492HER2, YKL-40 was the most relevant candidate to study the differences between the cell lines. YKL-40 is a secreted glycoprotein of a still unclear function. The protein belongs to the chitinase family, but it lacks hydrolase activity (Renkema *et al.* 1998). It is expressed by several cell types including macrophages, neutrophils, epithelial cells, synovial cells, chondrocytes, smooth muscle cells, and some cancer cells (Recklies *et al.* 2002; Johansen *et al.* 2006). It is suggested to be involved in inflammation and angiogenesis, as well as survival and growth in tumors (Johansen *et al.* 2006). It is highly secreted in inflammation diseases (Roslind and Johansen 2009; Bonneh-Barkay *et al.* 2010) and some cancers, such as breast cancer, melanoma, glioblastoma, or small cell lung carcinoma (Johansen *et al.* 2006; Shao *et al.* 2011; Jefri *et al.* 2015; Libreros and Iragavarapu-Charyulu 2015). Recently, it has been linked to idiopathic pulmonary fibrosis (Zhou *et al.* 2014) and immunosuppressive effects by cancer-associated fibroblasts in breast cancer (Cohen *et al.* 2017).

We have confirmed here that changes in intrinsic expression of YKL-40 in D492HER2 lead to differences in migration and invasion behavior of the cells. When YKL-40 is downregulated by transient or stable knockdown, D492HER2 becomes less migrative and invasive. Migration and invasion are important processes that support cancer development and progression (Hanahan and Weinberg 2011) what indicates that YKL-40 may play a key role in invasiveness and dissemination of cancer cells.

YKL-40 has been previously linked to EMT in non-small cell lung cancer (Jefri *et al.* 2015), yet in our study, when YKL-40 was downregulated in D492HER2, its EMT phenotype in monolayer remained unaltered and there were no differences in expression of EMT markers. Nevertheless, high expression of YKL-40 or the addition of the recombinant protein has been related to an increase in the grape-shaped colonies in 3D cell cultures of the EMT-derived cell lines. This fact suggests that YKL-40 may have been involved in the plasticity of the cells, as the phenotype in 3D was changed when its expression was reduced in D492HER2 and then rescued when the recombinant protein was added. This is also interesting, since the grape-like structures are often associated with HER2-expressing cell lines (Kenny *et al.* 2007).

A number of papers have shown association between YKL-40 and HER2 expression in breast cancer. Wang *et al.* showed by meta-analysis of existing databases that YKL-40 is associated with poor prognosis in breast cancer patients (Wan *et al.* 2017). Kang *et al.* have demonstrated that YKL-40 expression in breast cancer is associated with Her-2 and basal-like molecular subtype of breast cancer (Kang *et al.* 2014). Also, Shao *et al.* showed an association between YKL-40 and Her2 subtype (Shao *et al.* 2011). Although, the association between YKL-40 and HER2 has been demonstrated, it is, however, to our best knowledge, still not clear if there are any direct or indirect molecular interactions between these two proteins in breast cancer. Using the breast mark database (Madden *et al.* 2013), we were however not able to link high or low expression of CHI3L1 in distinct subtype of breast cancer to increased or reduced survival.

PANTHER classification of the differentially regulated genes showed that YKL-40 is in enriched in biological processes related to interactions with the microenvironment that can support the development of tumors by cancer cells (inflammatory response, cell chemotaxis, cellular response to cytokine stimulus, and defense response). We demonstrate here that YKL-40 interacts with the microenvironment and more concretely is contributing to the angiogenesis-inducing effect of D492HER2.

We have demonstrated that YKL-40 secreted by D492HER2 has a role in angiogenesis. When YKL-40 protein is reduced in the media by a specific YKL-40 blocking antibody or stable knockdown in D492HER2, angiogenesis is decreased. Conversely, increased levels of YKL-40 in the CM by the addition of recombinant YKL-40 protein or secreted by the cell line reverted the effects to standard tube formation parameters.

Interestingly, the reduction of YKL-40 in D492HER2 by the stable knockdown cell line revealed the reduction of the expression of other pro-angiogenic genes, including VEGF-A and VEGF-C. These genes encode protein ligands that bind and activate VEGF receptor 2 (VEGFR2, Flk-1/KDR), one of the main receptors implicated in the induction of angiogenesis in endothelial cells (Carmeliet and Jain 2000; Harper and Bates 2008). Indeed, it has been shown that a monoclonal antibody against YKL-40 abolishes YKL-40-induced activation of the membrane VEGF receptor 2 and intracellular signaling mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (Erk) 1 and Erk 2 (Faibish *et al.* 2011).

However, the role of YKL-40 in cancer progression may not be exclusively derived from cancer cells and not limited to promote interactions with endothelial cells. Recently, it has been described that YKL-40 is highly secreted by fibroblasts

associated to cancer (CAFs) in breast-promoting tumor growth and facilitating metastasis. Moreover, YKL-40 released by CAFs supports recruitment of macrophages and promotes the switch to tumor-associated macrophages (TAMs) (Cohen *et al*. 2017). Furthermore, TAMs in breast cancer express YKL-40 where the secreted protein increases inflammation and angiogenesis leading to a worse prognosis and conceivably to metastasis (Shao 2013). Collectively, this suggests that YKL-40 has a complex role in malignancy that may provide the signals for further progression involving changes within cancer cells to migrate and invade and interact with cells of the surrounding stroma. Moreover, it also may provide feedback from the microenvironment to support cancer development, tumor growth, and incrementation of dissemination of cancer cells in metastasis.

Conclusion

In conclusion, our data suggest that YKL-40 may provide D492HER2 with increased aggressiveness, evidenced by enhanced migration and invasion. Furthermore, YKL-40 may also support cancer progression by facilitating angiogenesis and may therefore be of interest as a potential novel therapeutic target in HER2-positive breast cancer.

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Paper II

Laboratory Investigation
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ARTICLE

ECM1 secreted by HER2-overexpressing breast cancer cells promotes formation of a vascular niche accelerating cancer cell migration and invasion

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Abstract

The tumor microenvironment is increasingly recognized as key player in cancer progression. Investigating heterotypic interactions between cancer cells and their microenvironment is important for understanding how specific cell types support cancer. Forming the vasculature, endothelial cells (ECs) are a prominent cell type in the microenvironment of both normal and neoplastic breast gland. Here, we sought out to analyze epithelial–endothelial cross talk in the breast using isogenic non-tumorigenic vs. tumorigenic breast epithelial cell lines and primary ECs. The cellular model used here consists of D492, a breast epithelial cell line with stem cell properties, and two isogenic D492-derived EMT cell lines, D492M and D492HER2. D492M was generated by endothelial-induced EMT and is non-tumorigenic while D492HER2 is tumorigenic, expressing the ErbB2/HER2 oncogene. To investigate cellular cross talk, we used both conditioned medium (CM) and 2D/3D co-culture systems. Secretome analysis of D492 cell lines was performed using mass spectrometry and candidate knockdown (KD), and overexpression (OE) was done using siRNA and CRISPRi/CRISPRa technology. D492HER2 directly enhances endothelial network formation and activates a molecular axis in ECs promoting D492HER2 migration and invasion, suggesting an endothelial feedback response. Secretome analysis identified extracellular matrix protein 1 (ECM1) as potential angiogenic inducer in D492HER2. Confirming its involvement, KD of ECM1 reduced the ability of D492HER2-CM to increase endothelial network formation and induce the endothelial feedback, while recombinant ECM1 (rECM1) increased both. Interestingly, NOTCH1 and NOTCH3 expression was upregulated in ECs upon treatment with D492HER2-CM or rECM1 but not by CM from D492HER2 with ECM1 KD. Blocking endothelial NOTCH signaling inhibited the increase in network formation and the ability of ECs to promote D492HER2 migration and invasion. In summary, our data demonstrate that cancer-secreted ECM1 induces a NOTCH-mediated endothelial feedback promoting cancer progression by enhancing migration and invasion. Targeting this interaction may provide a novel possibility to improve cancer treatment.

Introduction

Organ morphogenesis is dependent on heterotypic interactions between multiple cell types. In breast morphogenesis, the epithelial compartment generates branching ducts that result in terminal duct lobular units (TDLU) [1]. Ducts and TDLUs are surrounded by a basement membrane, embedded in stroma consisting of extracellular matrix (ECM) as well as multiple cell types including fibroblasts, immune cells, and endothelial cells (ECs) forming the microvessels [1, 2]. Formation of TDLU is highly dependent on heterotypic interactions between the epithelial cells and the surrounding vascular-rich stroma. Multiple studies have shown that the stromal compartment plays a fundamental role when it comes to epithelial morphogenesis [2–6]. For example, the ability of breast epithelial cells to form TDLU-like structures in a 3-dimensional environment in vitro is strongly enhanced by the presence of breast ECs [2]. In

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addition to its role in normal development, recent studies provide evidence that proliferation and migration of cancer cells is also largely dependent on interactions with the surrounding stroma [3, 7–9].

The tumor microenvironment is composed of a plethora of different cell types and ECM [10]. The interaction between cancer cells and cells of the microenvironment is a crucial determinant of cancer progression. Co-evolution of cancer cells and tumor stroma can lead to the generation of niches that support tumor growth, both at the primary site and at distant metastatic sites [11]. In this context, many studies have been focusing on identifying the role of tumor-associated macrophages and cancer-associated fibroblasts in cancer progression [12–16].

Recent research, however, suggests that ECs of blood vessels play a role in cancer progression that is far beyond delivering oxygen and nutrients [7, 17–20]. There is evidence that the endothelium can impact cancer progression by either preventing or supporting tumor growth and metastasis formation. An intact vasculature can keep metastatic cells in a dormant and non-proliferative state whereas a sprouting vasculature rather supports metastatic outgrowth [7, 21, 22]. Ghajar and colleagues found that endothelial-derived thrombospondin-1 induces cancer cell dormancy in breast cancer and therefore prevents outgrowth of metastasis in lung and bones [7]. However, in the presence of sprouting neovasculature this suppressive effect was lost and the endothelium now appeared to promote tumor outgrowth via TGF-b1 and periostin [7]. Therefore, an essential question to ask is how cancer cells interact with ECs in order to promote the generation of a vascular niche promoting tumor growth and cancer progression. Presently, a number of molecular mediators of angiogenesis have been identified [23]. The most commonly described candidates include vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), tumor necrosis factor alpha (TNF-a), transforming growth factor beta 1 (TGF-b1), platelet-derived growth factor (PDGF), as well as angiopoietins. Laughner et al. were able to show that HER2 signaling in breast cancer cells could increase HIF1a-mediated VEGF expression resulting in increased tumor angiogenesis [24]. Lee et al. showed that increased IL-6 secretion in breast cancer cells induces secretion of CCL5 in lymphatic endothelial cells (LEC), which in return enhances cancer cell migration [18]. Furthermore, cancer cell-secreted IL-6 increases VEGF expression in LECs, which enhances lung vascular permeability and lymph node angiogenesis and thereby promotes metastatic outgrowth [18, 19]. In addition, the NOTCH signaling pathway has recently been linked to angiogenesis [23, 25, 26]. Murtas et al. report increased NOTCH1 expression by tumor endothelium in cutaneous melanoma that was linked to increasing microvascular density [25]. This suggests that targeting the interaction between cancer cells and their niches may provide additional means to inhibit progression of cancer. However, our understanding of the generation of vascular niches and molecular interactions within the niches is generally still rudimentary.

We have previously established a breast epithelial cell line with stem cell properties, referred to as D492 [27–30]. D492 can generate both luminal and myoepithelial cells, and in 3D rBM (reconstituted basement membrane, matrigel) culture it forms branching structures reminiscent of TDLU in vivo. When D492 was co-cultured with ECs in an organoid culture, a subpopulation of D492 underwent EMT and such a structure isolated gave rise to D492M [27]. D492HER2 was generated by overexpressing the ErbB2/HER2 oncogene in D492, which then underwent oncogene-induced EMT [31]. In contrast to D492M, D492HER2 has tumorigenic properties, as evidenced by injection of cells in the mammary fat pads of NOD scid gamma (NSG) mice. The different tumorigenicities of these two isogenic EMT derivatives of D492 present an interesting research platform to study tumorigenicity, especially with regard to interaction with the endothelium.

In this study, we show that conditioned medium (CM) from D492HER2 increases endothelial network formation in vitro and induces an endothelial feedback promoting D492HER2 migration and invasion. Secretome analysis identified extracellular matrix protein 1 (ECM1) as potential pro-angiogenic factor in D492HER2. Recombinant ECM1 (rECM1) increases endothelial network formation while knockdown (KD) of ECM1 reduces the ability of D492HER2-CM to increase the endothelial network. ECM1 has previously been associated with decreased overall and distant metastasis-free survival when expressed in HER2+ breast tumors [32–34]. A role in angiogenesis and cancer cell migration and invasion has been suggested in laryngeal carcinoma, cholangiocarcinoma, gastric cancer, bladder cancer, and breast cancer [32, 35–38]. However, the mechanism through which ECM1 induces a pro-tumorigenic vascular niche promoting cancer progression is still unknown. Finally, increased expression of NOTCH1 and NOTCH3 in ECs treated with D492HER2-CM or rECM1 indicates a possible novel modulatory role of ECM1 in NOTCH signaling during angiogenesis and cancer.

Materials and methods

Cell culture

D492, D492M, and D492HER2 cells were maintained in H14 medium in tissue culture-treated T25 Falcon flasks (BD Biosciences (BD), Franklin Lakes, NJ, USA) coated with collagen I (Advanced Biomatrix, San Diego, CA, USA), as described previously [27, 31]. The cells were

grown at 37 °C and 5% CO_2 and subcultured into new flasks 1–2 times per week in a ratio of 1:10 (D492), 1:5 (D492M), or 1:15 (D492HER2). MDA-MB-231 was cultured in Gibco™ RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Invitrogen), further referred to as R10F, and MCF-7 in Gibco™ DMEM:F12 medium with 10% FBS. Both cell lines were subcultured 1–2 times per week at a ratio of 1:10. Primary human umbilical vascular ECs (HUVEC) were obtained from the National University Hospital (Landspitali) Reykjavik and cultured up to passage 8 in EBM2 medium (Lonza) supplemented with growth factors and 5% FBS, further referred to as EGM5. HUVECs were subcultured 1–2 times per week at a ratio of 1:10. Culture medium was changed three times per week and routinely checked for mycoplasma contamination.

CM and mass spectrometry

D492, D492M, and D492HER2 were grown in H14 medium until 70–80% confluence. Cells were washed with 1x PBS, and half of the original volume of fresh H14 was added in order to enrich for secreted factors. After 48 h incubation at 37 °C, CM was collected, centrifuged at 2000 rpm for 3 min and sterile filtered through 0.22 µm filter unit. For endothelial network formation in 3D culture, 10,000 HUVECs were seeded in freshly collected CM (or unconditioned control H14) and fresh EGM5 medium (ratio 1:1) in a 96 well angiogenesis plate (#89646, Ibidi) on top of 10 µl solidified rBM Matrigel (#354230, Corning). For recombinant protein experiments, rECM1 (#TP723147, Origene) was added to the medium in a final concentration of 15, 30, 60, or 120 ng/ml. 60 ng/ml was then chosen as concentration for further experiments. For endothelial NOTCH inhibition, gamma-secretase inhibitor tert-Butyl (2S)-2-[[(2S)-2-[[2-(3,5-difluorophenyl)acetyl]amino]propanoyl]amino]-2-phenylacetate (DAPT) (#D5942, Sigma-Aldrich) was added to the medium in a final concentration of 20 µM. Phase-contrast images of the establishing endothelial network were taken after 4, 24, and 48 h. Images were converted to RGB color and analyzed using ImageJ Angiogenesis Analyzer macro. For mass spectrometry, D492 lines were grown in T175 flasks and CM was collected as described above, concentrated for 55 min using EMD Millipore Amicon™ Ultra-15 Centrifugal Filter Units (#UFC900324, Merck Millipore) followed by buffer exchange to 100 mM TRIS/HCL buffer. Samples (triplicates) were stored at −80 °C. Label-free relative protein quantification (LFQ) by nLC MS/MS after trypsin digestion was performed at the FingerPrints Proteomics Facility, University of Dundee, UK, and raw data were analyzed using MaxQuant software (version 1.6.2.1). Quantitative and statistical analysis was performed using XLStat (version 2018.1). Data were p value corrected (significance level 0.05) and sorted based on greter than or equal to twofold higher secretion (LFQ intensity) by D492HER2 compared with both D492 and D492M. The resulting candidate list of 77 proteins was used for GO term analysis using PANTHER database (statistical overrepresentation test). As annotation dataset we used "GO biological process complete" with Bonferroni correction for multiple testing. To investigate feedback effects of conditioned/induced ECs, CM from D492 lines was added to 40–50% confluent HUVECs (as described above) and incubated for 48 h. Cells were washed with 1x PBS, and fresh EGM5 (half volume) was added to collect conditioned endothelial-secreted factors. After 48 h, CM from induced vs. non-induced HUVECs was collected, centrifuged (2000 rpm, 3 min) and filtered (0.22 µm) and added to 40% confluent D492HER2 together with fresh H14 (ratio 1:1) for 48 h.

Migration and invasion assay

Migration was analyzed using transwell-filter units for 24-well plates with 8 µm pore size (#353097, Corning). Thirty thousand cells were seeded on top of the filter in 250 µl culture medium. For the invasion assay, filters were pre-coated with 1:10 rBM in H14 for 30 min at 37 °C. For the migration assay, cells were incubated for 24 h and for 48 h in the invasion assay. rECM1 and NOTCH inhibitor DAPT were used in the concentrations as described before. For migration towards ECs, HUVECs were grown in 500 µl EGM5 to 80% confluence in 24-well plate. Then, medium was changed to H14+ EGM5 (1:1), and D492 line cells were seeded on top of filter units (triplicates) in H14+ EGM5 (1:1). As positive control, 10% FBS was added to the medium below the filter instead of HUVECs and as negative control, medium above and below the filter unit was identical (H14+ EGM5, 1:1). For migration and invasion of D492HER2 treated with CM of induced vs. non-induced ECs, 30,000 D492HER2 cells were seeded in 250 µl H14 on top of the non-coated or rBM-coated filter units (triplicates) and 500 µl H14 containing 10% FBS as attractant was added below. For MDA-MB-231, R10F was used instead of H14 while keeping the same experimental setup. After 24 h or 48 h, non-migratory or non-invasive cells were removed from the top of the filter using Q-tips, migratory cells below the filter were fixed for 10 min with 4% PFA (#252549, Sigma-Aldrich) and stained for 30 min with 1:5000 diluted DAPI nuclear staining (20 mg/ml) (#D9542, Sigma-Aldrich). Using an EVOS FL Auto 2 Cell Imaging System (Thermo Fisher Scientific), three images per replicate were taken at ×10 magnification. Images were converted to 8-bit in ImageJ (version 2.0.0), threshold-adjusted and binary-converted. Migratory cells were counted using the "analyze particles" function.

Transient KD using siRNA

KD was performed in 24-well plate using pre-designed Silencer® Select human ECM1 siRNA (#s4441, Thermo Fisher Scientific) compared with a scrambled negative control (Silencer Select negative control No. 1, Thermo Fisher Scientific, #4390843). Cells were reverse transfected for 48 h according to the manufacturer's protocol using a siRNA concentration of 10 nM. Proliferation was monitored using an IncuCyte ZOOM 2016B System. KD was confirmed using RT-qPCR and western blotting. After 48 h, medium was changed to normal H14 and incubated for another 48 h for collection of CM as described above. Endothelial network formation was analyzed as previously described for HUVECs treated with CM from D492HER2^{siECM1} compared with control D492HER2.

Generation of stable KD and overexpression cell lines using CRISPRi/CRISPRa

In order to generate stable ECM1 KD in D492HER2 and overexpression in D492 and D492M, we used the novel CRISPRi (inhibition) and CRISPRa (activation) system. sgRNA plasmids were purchased from Genscript; for ECM1 overexpression and KD, a combination of two sgRNAs was used. Overexpression sgRNAs were predesign and located between 1 and 200 bp upstream of the ECM1 transcription start site and KD sgRNAs custom-designed to bind between 1 and 200 bp downstream. sgRNAs were cloned into the plenti sgRNA(MS2)_zeo plasmid (Genscript). As negative control, an empty plenti sgRNA(MS2)_zeo plasmid was used (Genscript). For production of lentiviral particles, HEK293T cells were used and virus was collected 48 and 72 h after transfection. D492 and D492M were first transduced with plenti dCAS9-VP64-Blast plasmid for transcriptional activation and selected with blasticidin at a concentration of 2 µg/ml for 7 days. D492HER2 were transduced with pHR-SFFV-KRAB-dCas9-P2A-mCherry plasmid for repression and sorted for mCherry expression. Next, transduction with lentivirus containing sgRNA plasmids for overexpression (D492 and D492M containing dCas9-VP64 cassette) and KD (D492HER2 containing dCas9-KRAB cassette) was performed. For both KD and OE, two different gRNAs were pooled (KD: ECM1 custom gRNA 1 and 2 (GTGGTCA GTTGCCCCAGGAT, GCCGGCCACTGAAGCTTGTC) and OE: ECM1 SAM guide RNA 1 and 2 (CATCTACA GGCTGCCTTCTG, GAAACTGAGGCACAAACTAG)). Cells were selected with 400 µg/ml (D492, D492M) or 600 µg/ml (D492HER2) zeocin (Invitrogen) for 14 days. For enhancement of ECM1 OE, D492 and D492M were then transduced with lenti MS2-P65-HSF1 Hygro plasmid, containing enhancer domains MS2, P65, and HSP1 that bind to VP64 and enhance its activation activity. Cells were selected with 100 µg/ml hygromycin (Sellekchem, #S2908) for 10–12 days. ECM1 KD or OE were validated on gene expression level using RT-qPCR.

RNA microarray

For RNA microarray analysis, D492, D492M, and D492HER2 with and without ECM1 overexpression/KD were grown until 80% confluence, fresh H14 medium was added and CM was collected after 48 h. HUVECs were grown to 40% confluence, washed with 1x PBS and CM was added together with fresh EGM5 (ratio 1:1). After 24 h, RNA was extracted using the RNeasy Mini Kit (Qiagen). Sample concentration and quality was analyzed using the Agilent 2100 Bioanalyzer System (Agilent). As RNA microarray, Affimetrix Human Clariom S Assay was performed at the sequencing core facility, German Cancer Research Center (DKFZ) Heidelberg, Germany. Raw data were analyzed using Chipster high-throughput data analysis software v3.12.

RNA, cDNA, and RT-qPCR

Total RNA was extracted with Trizol (Life Technologies) and reverse transcribed with hexanucleotides using the SuperScript® IV First-Strand Synthesis System (Invitrogen). Resulting cDNA (10 ng per reaction) was used for quantitative real-time PCR, in master mix (Life Technologies) with predesigned primer pairs ECM1 (Hs.PT.58.20438560), NOTCH1 (Hs.PT.58.23074795), NOTCH3 (Hs.PT.58.38492200), and beta-2-microglobulin (B2M) as reference gene (Hs.PT.58v.18759587). Experiments were carried out in triplicate on the 7500 Real-Time PCR System (Life Technologies). Expression levels were normalized to the reference gene, and relative mRNA differences were calculated with the ΔΔCt method according to the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" (MIQE) guidelines [39].

Protein isolation and western blotting

Protein was isolated using RIPA lysis buffer supplemented with phosphatase and protease inhibitor cocktails (Life Technologies). For western blotting, 5–10 µg protein was used per lane, unless otherwise stated. Samples were denatured using 10% mercaptoethanol at 95 °C for 5 min and run on NuPage 10% Bis-Tris gels (Life Technologies) in 2-(N-morpholino)ethanesulfonic acid running buffer. Samples were then transferred to immobilon FL polyvinylidenfluorid (PVDF) membranes (Millipore). Membranes were blocked in Li-Cor blocking buffer, and primary antibodies (ECM1:

sc-515843 (Santa Cruz), beta tubulin: ab6046 (Abcam)) were incubated overnight at 4 °C. Near-infrared fluorescence visualization was measured using Odyssey CLx scanner (Li-Cor, Cambridge, UK).

In vivo tumor formation assay

To assess tumorigenicity of D492HER2 and D492HER2 with KD of ECM1, cells were injected subcutaneously into female NSG mice, bred at the Department of Comparative Medicine, Oslo University Hospital. The mice were kept in pathogen-free environment at a constant temperature (21.5 ± 0.5 °C) and humidity (55 ± 5%); 15 air changes/h and a 12 h light/dark cycle. The animals were 5–6 weeks old and their weight was 18–20 g before they were included in experiments. Anesthesia was obtained with 5% (v/v) Sevofluran along with 1 L oxygen and 3 L nitrous oxide, given with inhalation mask. Food and water were supplied ad libitum. 5×10^5 cells in 100 µl of 1:1 mixture of PBS and Matrigel (Corning, #354248) were injected on both flanks of the animal. Tumor growth was measured twice a week using a caliper, and the tumor volume was calculated according to the formula $0.5 \times \text{length} \times \text{width}^2$. Animals were sacrificed performing cervical dislocation at the end of the experiments, or when tumor volume reached 1500 mm^3, the weight loss exceeded 20% or when they became moribund. Tumors were dissected and from each tumor one part was frozen in liquid nitrogen and the other was fixed in PFA and embedded in paraffin. All experiments involving animals have been approved by the Norwegian Animal Research Authority (ethical approval FOTS ID: 12080) and conducted according to the regulations of the Federation of European Laboratory Animals Science Association (FELASA) [40].

Immunofluorescence (IF) staining

HUVECs were grown to 80% confluence on eight-well chamber slides (#354108, Falcon), fixed with 3.7% PFA, permeabilized with 0.1% Triton X-100, and unspecific background was blocked using 10% FBS in 1x PBS. Incubation with primary antibodies for ECM1 (Sigma-Aldrich, #HPA027241), NOTCH1 (#sc-376403, Santa Cruz), and NOTCH3 (#ab23426, Abcam) was done overnight at 4 °C, followed by incubation with secondary antibodies conjugated with Alexa Fluor-488, -546, or -647 for 1 h at RT. For nuclei staining DAPI was used together with secondary antibodies at a concentration of 1:5000. Imaging was done using FV1200 Olympus inverted confocal microscope. For IHC staining, paraffin-embedded tumor tissue samples were processed in xylene and ethanol in order to remove paraffin and then rehydrated in dH2O. Following rehydration, high-temperature antigen retrieval in 1x T/E buffer was performed and samples were stained as described above. Counterstaining was performed with filtered hematoxylin for 1–2 min.

Statistical analysis

All experiments were performed at minimum in triplicate. Graphs were generated in Microsoft Excel 2015. Error bars represent the standard deviation of the mean. Statistical analysis was performed in R 3.3.3. (R Development Core team, 2013). Data were checked for normal distribution and Student's *t* test or ANOVA followed by Tukey's honest significant difference test on linear models was performed when normally distributed whereas Kruskal–Wallis chi-squared test was used when not normally distributed. The significance level was 0.05.

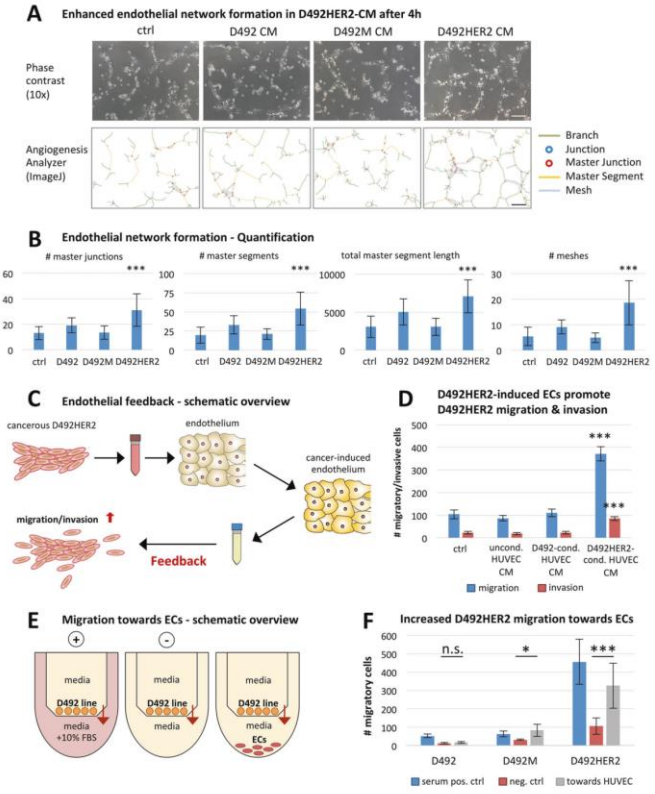
Results

CM from D492HER2 promotes endothelial network formation

Initially, we aimed to analyze the heterotypic interactions between normal and cancerous epithelial cells of the mammary gland and ECs. To address this, we used isogenic normal and cancerous cell lines derived from the D492 breast epithelial progenitor cell line [28, 30]. We collected CM from D492, D492M, and D492HER2 and analyzed the effect on endothelial network formation in vitro. ECs were seeded in CM from D492, D492M, and D492HER2 on top of rBM, and endothelial network formation was analyzed after 4, 24, and 48 h (Fig. 1a, b, Supplementary Fig. S1). Assessment of EC viability confirmed similar cell viability upon CM treatment compared with H14 ctrl medium (Supplementary Fig. S13). The established network of ECs treated with CM from D492HER2 but not from D492 and D492M showed significantly higher numbers of master junctions, master segments, meshes, and higher total master segment length after 4 h (Fig. 1a, b). This indicates that D492HER2-CM enhances early endothelial network formation in vitro. All quantified parameters were also significantly higher upon treatment with D492HER2-derived CM after 24 and 48 h indicating stability and persistence of the observed pro-angiogenic effect of D492HER2-CM over time (Supplementary Fig. S1).

D492HER2-induced ECs feedback on D492HER2 by promoting migration and invasion

After identifying D492HER2 as a cell line enhancing endothelial network formation, we collected CM from D492HER2-induced ECs and treated D492HER2 (Fig. 1c)



in order to investigate a possible feedback response through cellular cross talk. Interestingly, we observed that treatment with CM from D492HER2-induced ECs stimulated increased migration and invasion of D492HER2, in contrast to CM from untreated ECs and D492-induced ECs (Fig. 1d), suggesting a positive feedback. To identify whether migration of D492HER2 cells was specifically directed toward the endothelium rather than being general, we performed indirect co-culture of D492, D492M, and D492HER2 in a transwell assay with or without ECs present below the filter (Fig. 1e). When analyzing the number of migratory cells, D492HER2 in particular showed an increased migratory phenotype toward ECs below the filter (Fig. 1f), supporting the hypothesis that migration is increased toward the endothelium mediated by induced ECs. In line with this, we could see significantly more D492HER2 than D492 or D492M cells directly associated with ECs in 2D and 3D co-culture with ECs (Supplementary Fig. S2).

◀ **Fig. 1 CM from D492HER2 enhances endothelial network and induces endothelial feedback increasing D492HER2 migration/invasion. a** Enhanced endothelial network formation in D492HER2-CM after 4 h. Top: representative phase-contrast images of HUVEC endothelial network after 4 h on top of rBM in control (unconditioned) medium and conditioned medium from D492, D492M, and D492HER2. Each condition included 4–6 wells as replicates and 1–2 images were taken per well at ×10 magnification (scale bar = 100 µm). Bottom: corresponding angiogenesis images (ImageJ, angiogenesis analyzer plugin) showing branches, junctions, master junctions, master segments, and meshes. **b** Endothelial network formation—quantification. Quantification of master junctions, master segments total master segment length and meshes for each image using angiogenesis analyzer plugin (average and standard deviation per condition). Statistical analysis performed in R, one-way ANOVA, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, <0.1. **c** Endothelial feedback—schematic overview. Schematic workflow of conditioning of ECs (see "Materials and methods"). **d** D492HER2-induced ECs promote D492HER2 migration and invasion. Transwell-migration and invasion assays of D492HER2 treated with unconditioned medium, unconditioned HUVEC-CM, D492-conditioned HUVEC-CM, and D492HER2-conditioned HUVEC-CM. Number of migratory/invasive cells is shown for the different treatments (Student's *t* test, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, <0.1). **e** Migration toward ECs—schematic overview. Schematic setup of the transwell-migration assay of D492, D492M, and D492HER2 toward HUVECs seeded below the transwell filter. Migration toward HUVECs (in H14 + EGM1) was compared with migration toward 10% FBS in H14 + EGM5 (pos. ctrl) and plain H14 + EGM5 (neg. ctrl). **f** Increased D492HER2 migration toward ECs. Migration of D492, D492M, and D492HER2 toward HUVECs. Number of migratory cells is shown for the different treatments (Student's *t* test, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, <0.1).

Enrichment of angiogenesis-regulating proteins and identification of ECM1 in D492HER2 secretome

In order to identify secreted factors from D492HER2 that are mediating the observed pro-angiogenic effect, we performed label-free mass spectrometry on concentrated CM from all three D492 cell lines. In order to determine candidates involved in tumorigenicity rather than EMT phenotype, we focused on proteins higher secreted by D492HER2 compared with both D492 and D492M. We used RT-qPCR and transcriptomic data to verify secretome candidates. After correction of the data for significance (0.05 significance level) and at least twofold difference in secretion, we received a list of a total of 77 proteins more highly secreted by D492HER2 compared with D492 and D492M (Fig. 2a and Supplementary Table S1). GO term analysis showed 6.7-fold enrichment for GO term group "regulation of angiogenesis" (GO:0045765) among D492HER2-secreted candidates (Fig. 2b), consisting of seven gene members (Fig. 2c). We then compared secretion levels of these seven candidates in our secretome dataset and identified ECM1 and chitinase 3-like-1 (CHI3L1) as the two proteins with the most prominent secretion profile (Fig. 2d). Interestingly, secretion levels of commonly recognized angiogenesis inducers such as VEGFa, FGF2, or PDGF were not significantly elevated in our cell lines, suggesting a different mechanism behind the observed pro-angiogenic effect of D492HER2 secretome (Fig. 2d). CHI3L1, also known as YKL-40, is a secreted glycoprotein, which is associated with a number of biological functions and also plays a role in chronic inflammation diseases and cancer [41–43]. Recently, it has been linked to idiopathic pulmonary fibrosis [44]. Previous studies provide evidence for a role of YKL-40 in tumor angiogenesis by mediating VEGF signaling [45, 46]. ECM1 is a secreted glycoprotein, which was first identified in the mouse osteogenic stromal cell line MN7 [47]. It was initially described as modulator of proliferation and differentiation of epidermal keratinocytes and basement membrane reconstitution in the skin [48]. However, it has also been linked to angiogenesis and malignant transformation [32–34, 37, 48–50]. Interestingly, analysis to determine correlation to different gene modules using GOBO gene set analysis database (University of Lund, Sweden) revealed a significant Spearman correlation of ECM1 to the gene module "stroma" (Fig. 2e). Kaplan–Maier survival analysis revealed for both CHI3L1 and ECM1, a significant correlation of gene expression with decreased survival (Supplementary Fig. S4). However, whereas in case of CHI3L1 this correlation is not specifically associated with a certain cancer subtype, ECM1 expression is significantly correlated with decreased distant metastasis-free survival (DMSF) when expressed in HER2+ and estrogen receptor-negative (ER−) breast tumors (Fig. 2f, g, Supplementary Figs. S3 and S4) [49]. These data put together led our focus on ECM1 as the main candidate for further investigation in the context of stromal interaction.

ECM1 enhances endothelial network and induces endothelial feedback

To verify that ECM1 is involved in the inducing effects of D492HER2 on endothelial network formation and positive feedback, we investigated whether rECM1 is capable of inducing network formation and feedback as well. We treated ECs with 15, 30, 60, and 120 ng/ml of rECM1 and quantified network formation as described previously. Indeed, rECM1 had significantly increased the endothelial network formed after 4 and 24 h as quantified by numbers of master junctions, meshes, master segments, and total master segment length (Fig. 3a, b). Hereby, the strongest effect was seen for treatment with 30 and 60 ng/ml rECM1 (Fig. 3b). This indicates that the system may already be saturated at these concentrations rather than rECM1 having a dose-dependent effect. To look at the endothelial feedback, we induced ECs with 60 ng/ml rECM1 and treated D492HER2 with CM of ECM1-induced ECs (Fig. 3c). CM of ECM1-induced ECs significantly increased D492HER2 migration and invasion (Fig. 3d), confirming that ECM1 does play a role in inducing the endothelial feedback induced by D492HER2.

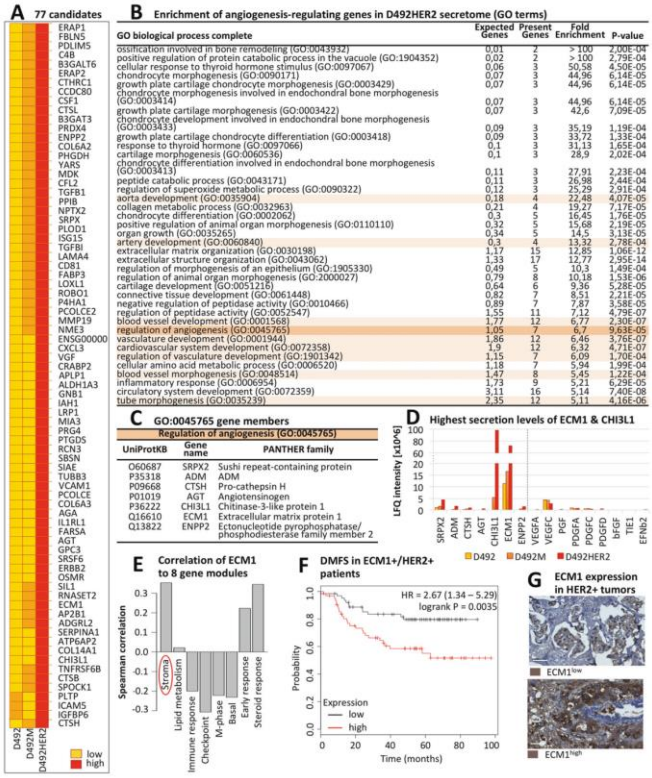


Fig. 2 Identification of ECM1 as pro-angiogenic candidate in D492HER2. a 77 candidates. Heat map of 77 proteins higher secreted by D492HER2 compared with both D492 and D492M (label-free mass spectrometry secretome data, $p < 0.05$, threshold fold change greater than or equal to twofold). **b** Enrichment of angiogenesis-regulating genes in D492HER2 secretome (GO terms). Enrichment of "Regulation of angiogenesis" GO term group GO:0045765 among D492HER2 secretome. Statistical overrepresentation test (Panther DB) was performed using "GO biological process complete" as annotation dataset with Bonferroni correction for multiple testing. **c** GO:0045765 gene members. Proteins part of GO term group GO:0045765 (Regulation of angiogenesis). **d** Highest secretion levels of ECM1 and CHI3L1. Secretion levels (label-free quantification LFQ) of the seven candidates from **c** (left of dotted line) compared WITH secretion levels of commonly known angiogenesis inducers (right of dotted line). **e** Correlation of ECM1 to eight gene modules. Spearman correlation of ECM1 expression to different gene modules in breast tumor samples (GOBO Genset analysis, Lund University). **f** DMFS in ECM1+/HER2 + patients. Kaplan–Meier plot of ECM1 in HER2+ tumors showing correlation of high expression with decreased distant metastasis-free survival (DMFS). **g** ECM1 expression in HER2+ tumors. ECM1 expression in representative HER2+ tumor samples showing low and high expression. Cells were counterstained with hematoxilyn. Scale bar = 100 µm.

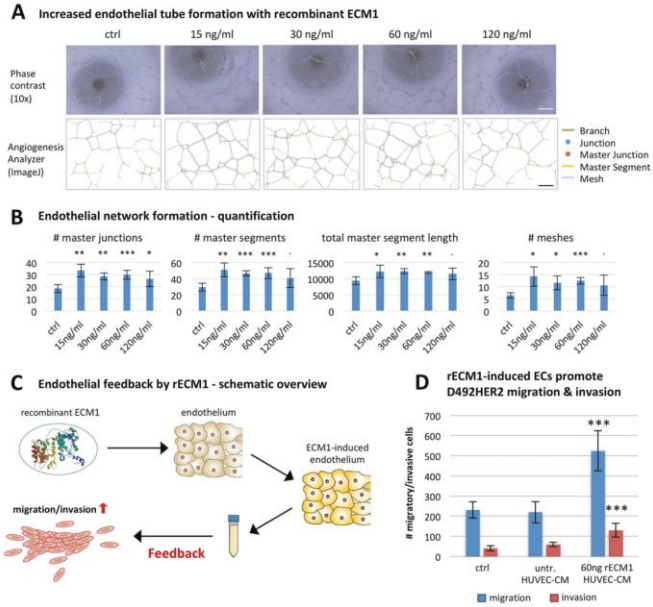


Fig. 3 Recombinant ECM1 enhances endothelial network and induces endothelial feedback increasing D492HER2 migration/invasion. a Increased endothelial tube formation with recombinant ECM1. Top: HUVEC endothelial network on top of matrigel in different concentrations of recombinant ECM1 (after 24 h). 4–6 wells as replicates per condition and 1–2 images taken per well at ×10 magnification (scale bar = 100 µm). Bottom: corresponding angiogenesis images (ImageJ angiogenesis analyzer). **b** Endothelial network formation—quantification. Quantification of master junctions, master segments, total master segment length, and meshes for each image using angiogenesis analyzer plugin. One-way ANOVA, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, <0.1. **c** Endothelial feedback by rECM1—schematic overview. Schematic workflow of conditioning of ECs using 60 ng/ml rECM1. **d** rECM1-induced ECs promote D492HER2 migration and invasion. Transwell-migration and invasion assays of D492HER2 treated with unconditioned medium, unconditioned HUVEC-CM and 60 ng rECM1-treated HUVEC-CM. Number of migratory/invasive cells shown for different treatments (Student's *t* test, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, <0.1).

To verify the activity of ECM1, we induced stable overexpression of ECM1 in D492 and D492M. CM from D492 and D492M stably overexpressing ECM1 showed pro-angiogenic properties (Fig. 4a, b). Furthermore, D492 and D492M overexpressing ECM1 show increased migration toward ECs, similar as observed for D492HER2 (Supplementary Fig. S6). Next, we generated transient KD of ECM1 in D492HER2 using siRNA (Supplementary Fig. S5) and stable KD using CRISPRi. Both transient and stable KD were successful (over 90% KD), as verified on gene as well as protein level (Supplementary Figs. S5 and S6). In accordance with the observed effects of rECM1 and ECM1 overexpression, treatment with CM from D492HER2-kdECM1 resulted in significantly lower endothelial network formation compared with control D492HER2 (Fig. 4a, b). Similar results were obtained with CM from D492HER2 with transient ECM1 KD (Supplementary Fig. S5). In addition to these results on the D492 cell lines, we investigated pro-angiogenic properties of SKBR3 (HER2+ and ECM1−) and HCC202 (HER2+ and ECM1+) breast cancer cell lines. Network formation data showed increased pro-angiogenic properties of HCC202-CM but not SKBR3-CM compared with controls, confirming the specific role of ECM1 in this context (Supplementary Fig. S10). Analysis of

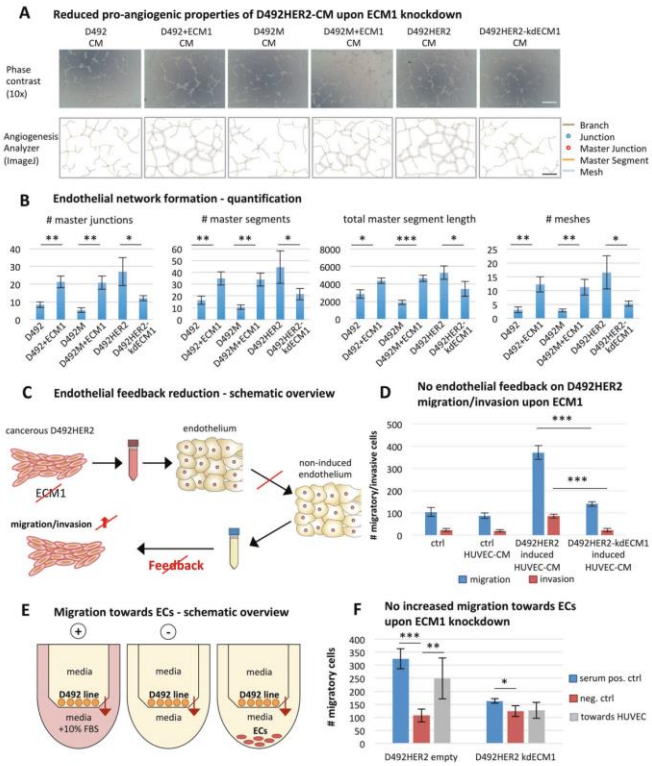


Fig. 4 ECM1 knockdown inhibits pro-angiogenic effect of D492HER2-CM and endothelial feedback on D492HER2 migration/invasion. a Reduced pro-angiogenic properties of D492HER2-CM upon ECM1 knockdown. Top: HUVEC endothelial network on top of matrigel in CM from D492 and D492M with ECM1 overexpression and D492HER2 with ECM1 knockdown compared with empty vector control (after 4 h). 4–6 wells as replicates per condition and 1–2 images taken per well at ×10 magnification (scale bar = 100 µm). Bottom: corresponding angiogenesis images (ImageJ angiogenesis analyzer). **b** Endothelial network formation—quantification. Quantification of master junctions, master segments, total master segment length, and meshes for each image using angiogenesis analyzer plugin. One-way ANOVA, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, <0.1. **c** Endothelial feedback reduction—schematic overview. Schematic workflow of conditioning of ECs using CM from D492HER2-kdECM1. **d** No endothelial feedback on D492HER2 migration upon ECM1 knockdown. Transwell-migration and invasion assays of D492HER2 treated with unconditioned medium, unconditioned HUVEC-CM, D492HER2-conditioned HUVEC-CM, and D492HER2-kdECM1-conditioned HUVEC-CM. Student's *t* test, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, <0.1. **e** Migration toward ECs—schematic overview. Schematic setup of the transwell-migration assay of D492HER2 empty ctrl. and D492HER2-kdECM1 toward HUVECs compared with pos. ctrl (10% FBS) and neg. ctrl (plain H14 + EGM5). **f** No increased migration toward ECs upon ECM1 knockdown. Migration of D492HER2 empty ctrl and D492HER2-kdECM1 toward HUVECs. Number of migratory cells for different treatments (Student's *t* test, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, <0.1).

the endothelial feedback showed that treatment of ECs with CM from D492HER2-kdECM1 did not induce an endothelial response to D492HER2 increasing cell migration and invasion (Fig. 4c, d). Also, the observed increased migration of D492HER2 toward ECs (Fig. 4c) could be depleted upon KD of ECM1, supporting the role of ECM1 in mediating the endothelial feedback and promoting migration toward the endothelium (Fig. 4e, f). These data suggest ECM1 as a specific mediator of endothelial response promoting cancer progression.

Subcutaneous injection of D492HER2 ctrl and D492HER2-kdECM1 into mice indicated a reduction in tumor volume upon ECM1 KD. However, this difference was not statistically significant (Student's t test, $p = 0.12$) (Supplementary Fig. S11). Furthermore, CD31 staining could not clearly confirm decreased angiogenesis within the tumor mass of D492HER2 with KD of ECM1 compared with D492HER2 ctrl. Interestingly though, both ctrl and kdECM1 tumors did show similar ECM1 protein expression levels although ECM1 KD had been confirmed by RT-qPCR prior injection into mice.

Triple-negative (TN) MDA-MB-231 cells also show high ECM1 expression and are capable of increasing endothelial network formation and inducing endothelial feedback

When analyzing the correlation of ECM1 and prognosis, we noted that high expression of ECM1 was not only correlated to worse survival in HER2+ breast cancer patients but also in triple-negative (TN) and generally ER− tumor patients (Supplementary Figs. S4 and S8B). In addition, correlation analysis using the Metabric discovery dataset showed a significant positive correlation between ECM1 expression and CD31 as established endothelial marker in ER− breast cancer (Supplementary Fig. S8E). Therefore, we decided to include MDA-MB-231 as TN and MCF-7 as ER+ cell line in our study. Interestingly, CM from MDA-MB-231 but not MCF-7 also increased endothelial network formation after 4, 24, and 48 h (Supplementary Figs. S8C, D, S9). Similar to D492HER2, MDA-MB-231 expresses high levels of ECM1 in contrast to MCF-7 (Supplementary Fig. S8A). Treating ECs with rECM1 also induced an endothelial feedback promoting MDA-MB-231 migration, as seen for ECM1-expressing D492HER2 (Supplementary Fig. S8F, G). Upon transient ECM1 KD in MDA-MB-231 (RT-qPCR confirmation of ECM1 KD see Supplementary Fig. S8H), its ability to increase endothelial network formation was significantly decreased (Supplementary Fig. S8I). This suggests that ECM1 might play a role in cancer progression through endothelial cross talk in a broader range of breast cancer subtypes than just HER2+.

ECM1 upregulates endothelial NOTCH1 and NOTCH3

To investigate whether treatment with D492HER2-CM upregulates commonly known angiogenesis modulators in ECs, we examined gene expression levels of endothelial VEGFa, VEGFR2 and PDGFb. However, none of these markers showed increased expression upon treatment with D492HER2-CM (Supplementary Fig. S7A).

In order to determine transcriptional changes occurring in ECs specifically in response to cancer-secreted ECM1, we performed RNA microarray analysis of ECs treated with CM from D492HER2 and D492HER2 with ECM1 KD. After p value correction and setting the fold change cutoff to twofold, analysis revealed 18 genes upregulated in ECs treated with D492HER2-CM compared with ECs treated with CM from D492HER2-kdECM1 (Supplementary Fig. S7 and Supplementary Table S2). However, the observed changes in genes expression were rather mild. One of the mildly higher expressed genes in ECs treated with D492HER2-CM was fatty acid-binding protein 4 (FABP4). FABP4 itself could not be confirmed as upregulated compared with untreated ECs, however it led our attention to the NOTCH pathway since its expression is regulated by NOTCH1 [51–53]. When investigating the expression of the NOTCH receptors 1–4 in ECs, we observed increased NOTCH1 and NOTCH3 gene and protein expression in ECs treated with D492HER2-CM and rECM1 but not with CM from D492HER2-kdECM1 (Fig. 5a).

Blocking endothelial NOTCH signaling interrupts the increase in network formation and feedback induction

Next, we wanted to investigate whether endothelial NOTCH signaling was implicated in the observed increase in endothelial network formation and the induction of an endothelial feedback promoting cancer cell migration and invasion. By blocking endothelial NOTCH using gamma-secretase inhibitor DAPT, interruption in both the increase in network formation and the feedback induction was observed. While treatment with rECM1 resulted in significantly increased numbers of master junctions, master segments, total master segment length, and number of meshes, this effect was no longer visible upon addition of DAPT (Fig. 5b, c). Compared with addition of rECM1 + DMSO, addition of rECM1 + DAPT significantly reduced numbers of master junctions ($p = 0.0004$), master segments ($p = 0.0014$), total master segment length ($p = 0.0019$), and number of meshes ($p = 0.0012$). Furthermore, CM of rECM1-induced ECs stimulated an increase in cancer cell migration and invasion whereas the addition of DAPT inhibited the induction of this feedback (Fig. 5d, e).

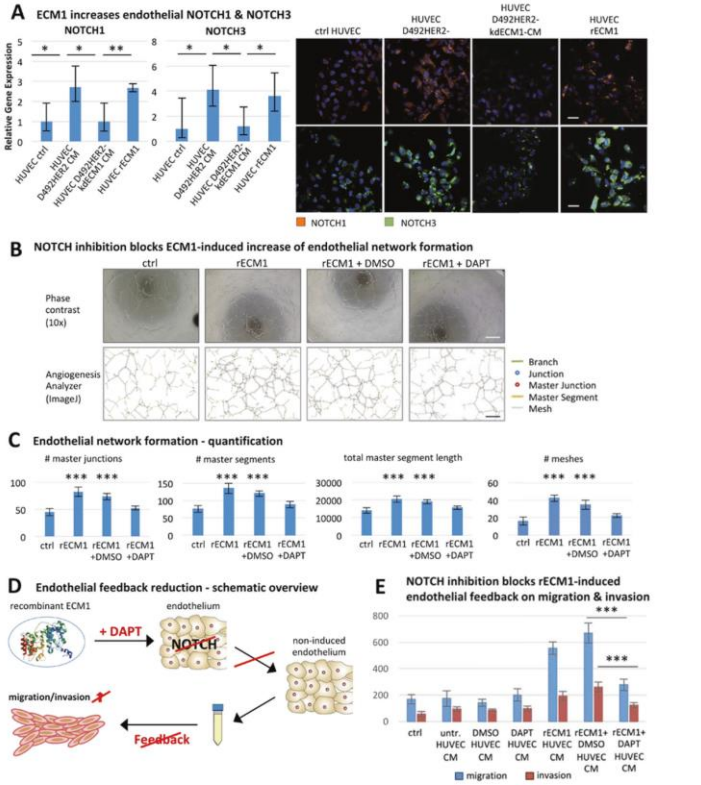


Fig. 5 Inhibition of endothelial NOTCH signaling prevents increased tube formation and feedback induction. a ECM1 increases endothelial NOTCH1 and NOTCH3. Increased NOTCH1 and NOTCH3 expression in HUVECs treated with D492HER2-CM and rECM1 but not D492HER2-kdECM1-CM on gene expression level (left) and protein expression level (IF staining, scale bar = 50 µm). Student's *t* test, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, <0.1. **b** NOTCH inhibition blocks ECM1-induced increase of endothelial network formation. Top: HUVEC endothelial network in H14 (ctrl), 60 ng/ml rECM1, 60 ng/ml rECM1 + DMSO and 60 ng/ml rECM1 + DAPT (20 µM) after 4 h. 4–6 wells as replicates per condition and 1–2 images taken per well at ×10 magnification (scale bar = 100 µm). Bottom: corresponding angiogenesis images (ImageJ angiogenesis analyzer). **c** Endothelial network formation—quantification. Quantification of master junctions, master segments, total master segment length, and meshes for each image using angiogenesis analyzer plugin. One-way ANOVA, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, <0.1. **d** Endothelial feedback reduction—schematic overview. Schematic workflow of treating ECs using 60 ng/ml rECM1 and blocking NOTCH signaling using DAPT (20 µM). **e** NOTCH inhibition blocks rECM1-induced endothelial feedback on migration and invasion. Transwell-migration and invasion assays of D492HER2 treated with unconditioned medium, unconditioned HUVEC-CM, DMSO-treated HUVEC-CM, DAPT-treated HUVEC-CM, 60 ng rECM1-treated HUVEC-CM, 60 ng rECM1 + DMSO-treated HUVEC-CM, and 60 ng rECM1 + DAPT-treated HUVEC-CM (NOTCH inhibition). Number of migratory/invasive cells for different treatments (Student's *t* test, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, <0.1).

Blocking endothelial NOTCH signaling therefore appears to interrupt both cancer-increased network formation as well as feedback induction. Interestingly, addition of DAPT alone to HUVEC did not affect the endothelial network (Supplementary Fig. S12) and feedback on migration and invasion (Fig. 5e). This suggests that blocking endothelial NOTCH by DAPT reduces network formation and feedback only upon elevated NOTCH signaling conditions.

Discussion

In this study, we have demonstrated that the HER2-overexpressing breast cancer cell line, D492HER2, stimulates endothelial network formation in vitro and is capable of inducing an endothelial feedback promoting cancer cell migration toward the endothelium and invasion. We have further identified ECM1 as a secreted factor in D492HER2 that plays a role in mediating both the pro-angiogenic effect of D492HER2 and the feedback induction. rECM1 increases endothelial network formation and induces the feedback while KD of ECM1 interrupts both. Furthermore, D492HER2 no longer show an increased migrative phenotype toward ECs upon ECM1 KD. However, the fact that we observed only a marginal reduction of tumor volume upon ECM1 KD indicates that it may be necessary to knockout ECM1 for in vivo studies and further to evaluate angiogenesis of the tumor-surrounding stroma. This is supported by the study of Wu and colleagues that have performed in vivo studies with ECM1 knockout rather than KD cells [54]. Interestingly, ECM1 protein expression levels were found to be similar in both ctrl and kdECM1 tumors although ECM1 KD had been confirmed by RT-qPCR prior injection into mice. Rather than suggesting no difference in tumor formation between ctrl and kdECM1 cells, these results are a possible indication that a positive selection of ECM1+ cells might have occurred in mice injected with kdECM1 cells. Therefore, our results do not necessarily stand in conflict to previously published data on ECM1 promoting tumor and metastasis formation in vivo [36–38, 54, 55]. Indeed, new studies could show that injection of equal amounts of different clones of the same breast cancer cell line MDA-MB-231 leads to formation of tumors with very few cells of one but many cells from another clone [56]. Tracing ECM1+ and ECM1− clones in vivo would offer a great approach to further investigate the occurrence of positive selection of ECM1+ clones and clarify the role of ECM1 during tumor formation.

ECM1 is a secreted glycoprotein and was first identified in the mouse osteogenic stromal cell line MN7 [47]. It was initially described as modulator of proliferation and differentiation of epidermal keratinocytes and basement membrane reconstitution in the skin [48]. However, it has also been linked to angiogenesis and malignant transformation [32–34, 37, 38, 48–50]. Han et al. demonstrated that rECM1 stimulated proliferation and blood vessel formation in the chorioallantoic membrane of chicken embryos [32] and Wang et al. found it to be expressed particularly in highly malignant breast epithelial tumors [34]. Recently, Wu et al. linked ECM1 to breast cancer progression by suggesting a role of ECM1 in facilitating metastasis through increased invasion and adhesion capacity of ECM1-expressing cancer cells [54]. These data are in line with our findings in the current study. Ferraro et al. recently showed that ECs are capable of increasing migration and invasion of SKBR3 breast cancer cells [57]. However, a direct involvement of cancer-secreted ECM1 in cancer progression through induction of an endothelial feedback supporting cancer migration and invasion has not been reported yet. Here, we show that the increased migration is directed toward the endothelium, suggesting that ECM1 may play a mediating role in inducing ECs to promote cancer cell migration from the primary tumor toward the vasculature and therefore support metastasis formation and tumor progression.

Survival analysis shows that expression of ECM1 in HER2+ breast tumors is associated with significantly reduced distant metastasis-free survival (DMFS). Therefore, targeting ECM1 in tumors overexpressing HER2 could lead to better patient prognosis. This is supported by a study of Lee et al., which showed that ECM1 regulates resistance to trastuzumab, a recombinant antibody targeting HER2 and the most common drug used in treatment of HER2+ breast cancer [49]. Interestingly, the TN breast cancer cell line MDA-MB-231 also expresses high levels of ECM1 and is capable of inducing endothelial network formation in the present study and previous studies [18, 19, 54]. KD of ECM1 in MDA-MB-231 inhibits these pro-angiogenic effects, supporting the mediating role of ECM1 in increasing the endothelial network. We also report that rECM1 is capable of inducing a positive endothelial feedback on MDA-MB-231 migration. Since expression of ECM1 also correlates with worse outcome in TN and generally ER− breast cancer, its molecular involvement in breast cancer progression might be more general rather than only limited to HER2+ cancer. Silencing of ECM1 in MDA-MB-231 led to a decrease in cancer cell migration and invasion capacity, linking its expression to increased tumor progression aggressiveness [54, 55]. Therefore, ECM1 might represent a possible therapeutic target for a broader range of breast cancer patients than just HER2+ individuals. However, the specific relationship between HER2 overexpression and ECM1 still needs to be elucidated. ECM1 expression is upregulated in several HER2+ breast cancer cell lines including ZR7530, SUM44PE, and HCC202. However, other HER2+ cell lines such as SKBR3 or BT474 show no ECM1 upregulation. However, among the breast cancer cell lines with strong ECM1 upregulation according to the GOBO database there

are numerous ER− cell lines (e.g., HCC1143, HBL100, SUM1315MO2, MDA-MB-435, and MDA-MB-231). Lee et al. report that high ECM1 expression in HER2+ breast cancer is correlated with poor prognosis and trastuzumab resistance [49]. They suggest that ECM1 may interact with EGFR family receptors that interact with HER2, attenuating therapeutic action by trastuzumab. However, the mechanisms of ECM1 expression regulation itself are not very well understood in HER2+ breast cancer. Smits et al. identified conserved potential binding sites for transcription factors of the Ap1, Ets, and Sp1 family in the human ECM1 gene in MN7 cells [58]. Ye et al. provide evidence that ECM1 is a target of miR-486-3p in cervical cancer. Overexpression of miR-486-3p thereby inhibited cell growth and metastasis by targeting ECM1 [59]. However, we could not detect differential expression of miR-486-3p between D492, D492M, and D492HER2, suggesting that ECM1 might be regulated through a different mechanism in our HER2+ breast cancer system. Generally, investigating the involvement of epigenetic regulation in modulating ECM1 expression and downstream signaling could be of great interest though as it could potentially give novel insights also in the context of cellular cross talk.

In our model, ECM1 was identified as cancer-secreted protein with pro-angiogenic properties that is capable of inducing an endothelial feedback promoting cancer cell migration toward ECs and invasion (Fig. 6). However, the molecular mechanism through which ECM1 mediates this feedback of the vascular niche on cancer progression has not been described previously. Here, we identified an upregulation of NOTCH1 and NOTCH3 receptor expression in ECs upon treatment with D492HER2-CM and rECM1 but not CM from D492HER2 with ECM1 KD. However, the initial microarray candidate, FABP4, could not be confirmed in further experiments, indicating that the subsequent downstream signaling may not involve FABP4. This would coincide with the findings that in addition to NOTCH1, also VEGFa, bFGF, and FOXO1 play potential roles in regulation of FABP4 expression [60]. So far, ECM1 has been associated with EGF signaling [49], and ITGB4/FAK/SOX2/HIF1a [36] and WNT1/ß-catenin [37] pathways, but a mediating role in NOTCH signaling has not been described. NOTCH signaling is very complex and plays a role in a large number of biological processes [61, 62]. Nevertheless, recent studies suggest NOTCH1 as a key player in inducing tumor angiogenesis and therefore promoting tumor progression in cutaneous melanoma and myeloma [25, 26]. Kalucka et al. have shown that NOTCH1 is involved in endothelial barrier function and angiogenesis by controlling fatty acid metabolism [53]. These data would support a possible involvement of endothelial NOTCH in mediating the increase in endothelial network and possibly the induction of the pro-cancerous feedback in breast cancer. Indeed, upon inhibition of endothelial NOTCH signaling, the enhancing effect of ECM1 on network formation and feedback was no longer observed, indicating the existence of a ECM1/NOTCH axis involved in mediating the cross talk between cancer cells and endothelium in terms of promoting cancer progression (Fig. 6).

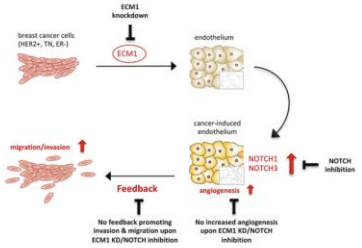


Fig. 6 Schematic summary of ECM1 inducing an endothelial feedback promoting cancer cell migration/invasion. ECM1 secreted by HER2+, TN, or ER− breast cancer cells increases endothelial network formation and induces endothelial feedback promoting cancer cell migration and invasion through upregulation of NOTCH1 and NOTCH3 expression. Knockdown of ECM1 in cancer cells and NOTCH inhibition in ECs inhibits the network formation increase and the feedback induction.

In conclusion, we have identified ECM1 as a cancer-secreted pro-angiogenic factor involved in inducing the endothelial niche to support cancer progression through increased cancer cell migration and invasion. Moreover, our data indicate a novel, mediating role of ECM1 in endothelial NOTCH signaling, which appears to be involved in mediating the induction of the cancer-promoting endothelial feedback (Fig. 6). Taken together, our findings might open up new possibilities for cancer treatment by understanding how an activated vascular niche provides positive feedback to the cancer and targeting this interaction between cancer cells and their microenvironment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. The funding body had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

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