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CELL–MATRIX INTERACTIONS: MASTER REGULATORS OF CANCER CELL FATE?

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Cell–matrix interactions: Master regulators of cancer cell fate?
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By

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"Success is not final, failure is not fatal: It is the courage to continue that counts".

Winston Churchill

Popular science summary of the thesis

The human body is estimated to consist of more than 30 trillion human cells of different types, organized into a variety of tissues and organs, all with their unique properties and specific requirements. However, tissues are not only made up of cells, there is also a non-cellular compartment that gives support and through its composition and organization, actually guides the behavior of the cells. This non-cellular compartment is called the extracellular matrix (ECM) and its properties vary substantially between tissues. One recognizable feature that differs is the stiffness of the matrix. Everyone can appreciate that our brain is softer than our bones. Cells sense and transduce the properties of the ECM via cell-matrix interactions and they will respond with changes in behavior if the property of the ECM is altered, something that is frequently seen in diseases, especially in cancer.

Cancer is not a single disease, as originally thought, but rather a group of diseases that can occur in almost any tissue and that are characterized by uncontrolled cell growth. The disease involves changes in the genome but also concomitant changes in the microenvironment and the ECM, and these alterations work together to drive normal cells progressively into malignant derivatives. ECM stiffening is an integral part of solid tumors like breast cancer; indeed, this is why a tumor in the breast is palpable. Over the last decade, it has become increasingly clear that this stiffness arises through changes in ECM organization and contributes to disease progression. However, the molecular underpinning of this phenomenon is not yet clear.

In this thesis, three out of four studies have elucidated how ECM stiffness regulates breast cancer cells to drive them into different degrees of malignancy. We observed a shift in breast cancer cell phenotype, from a pre-invasive ductal carcinoma in situ (DCIS) phenotype to an invasive ductal carcinoma (IDC) stage, depending on the stiffness of the matrix that they were cultured on. Quantification of mRNA in these two states revealed similarities to the DCIS to IDC transition in breast cancer patients, providing validity to our model and suggesting that ECM stiffness may be involved in driving this transition in the clinical setting. Further, we investigated the difference in cellular protein composition between the two stages and found that the mevalonate pathway, the target for the lipid-lowering agents statins, was important in driving the stiffness-induced transition to an invasive breast cancer phenotype. The pathway was also found to be upregulated in human breast tumors compared to normal breast tissue and to correlate with the stiffness in the tumor. Inhibition of the pathway prevented the invasive phenotype, as did inhibition of cell-matrix interaction signaling. This suggests that statins may prevent the transition from pre-invasive to invasive breast cancer, something that is also suggested by epidemiological studies. However, this needs to be further investigated. We also

performed profiling of specific signaling downstream of ECM stiffness and found yet another possible target for breast cancer therapy, the IKBKE kinase.

In addition to the specific molecular insights described above, the work presented in this thesis has also generated several large data sets. These can be further explored, by us or other scientists, to find novel targets for preventing the pre-invasive to invasive transition in breast cancer and thereby hopefully improve breast cancer survival.

Abstract

The development and homeostasis of a multicellular organism require fundamental biological processes like cell proliferation, cell differentiation, cell migration, and controlled cell death. The extracellular matrix (ECM) guides many of these functions, via cell–matrix interactions that function as mechanical and biochemical signaling hubs. Changes in the ECM composition or organization may impact cellular behavior, both in health and disease.

In this thesis, I have explored the effects of cell–extracellular matrix interactions on cellular processes, with a special focus on elucidating the molecular underpinnings of how extracellular matrix stiffening regulates breast cancer cell phenotypes.

In **study I**, we identified and characterized a new class of integrin-containing adhesion complex that we named "reticular adhesions" (RAs). They were formed by integrin αVβ5 in the absence of classical adhesion components like talin-1, vinculin, and F-actin. Unlike classical adhesions, they persisted throughout cell division during which they provided ECM anchoring necessary for efficient division and spatial memory transmission between cell generations. The characterization of reticular adhesions thus provided a solution to the long-standing question of mitotic cell-ECM attachment.

Studies II, III, and IV, all investigated the effect of ECM stiffness on breast cancer cells. The ECM stiffness increases with breast cancer progression and the stiffening is known to drive breast cancer cell proliferation and invasion. However, the molecular details of this phenomenon are not yet fully understood. In **study II**, we confirmed a stiffness-induced phenotypic switch in the high-grade breast carcinoma cell line, MCF10CA1a, with a ductal carcinoma in situ (DCIS) phenotype on a stiffness mimicking normal breast tissue stiffness, and an invasive ductal carcinoma (IDC) phenotype on a slightly higher stiffness, resembling breast tumor stiffness. Transcriptomic profiling of these two cellular states revealed only minor differences. Still, the stiffness-driven shift in mRNA resembled the changes differing IDC lesions from co-occurring DCIS lesions in patients, suggesting that stiffness may contribute to this transition and that hampering the mechanosignaling could prevent the progression of pre-invasive to invasive breast cancer. In **study III**, we used the same model as in study II, and quantitative mass spectrometry to compare the proteome of the two stiffness-dependent cellular states. The differences were much larger at the protein level, implying a previously underappreciated post-transcriptional regulation of many genes as a result of mechanical signaling. Among the stiffness-regulated genes, we found an enrichment of mevalonate pathway enzymes and confirmed the importance of this metabolic pathway for the stiffness-induced malignant phenotype. One of these enzymes, Hydroxymethylglutaryl-CoA Synthase (HMGCS1), was upregulated in human breast tumor tissue compared to normal breast tissue, and the level of expression was correlated to the collagen organization, suggesting a stiffness-dependent

regulation also in patients. Further, the synthesis rate of HMGCS1 depended on integrin and Rac1 signaling and the expression of a constitutively active Rac1 mutant could mimic matrix stiffening and promote HMGCS1 protein levels as well as a malignant phenotype on low stiffness. In **study IV**, we explored yet another level of regulation in our model when we used peptide chip arrays to profile the kinase activity in the two cellular states. The combination of the kinome profiling with a small siRNA-based screen allowed us to define the inhibitor of nuclear factor kappa-B kinase subunit epsilon, IKBKE, as a mechanosensitive kinase important for the maintenance of the stiffness-induced phenotype.

Thus, this thesis provides novel molecular insight into the regulation of cell-matrix interactions in cellular fate, especially on how mechanical properties of the ECM can induce breast cancer stage switching.

List of scientific papers

I. John G. Lock, Matthew C. Jones, Janet A. Askari, Xiaowei Gong, Anna Oddone, Helene Olofsson, **Sara Göransson**, Melike Lakadamyali, Martin J. Humphries and Staffan Strömblad (2018). Reticular adhesions are a distinct class of cell-matrix adhesions that mediate attachment during mitosis. *Nature Cell Biology* 20, 1290.
https://doi.org/10.1038/s41556-018-0220-2

II. **Sara Göransson***, Shan Chen*, Helene Olofsson, Ola Larsson and Staffan Strömblad (2023). An extracellular matrix stiffness-induced breast cancer cell transcriptome resembles the transition from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC). *Biochem Biophys Res Commun* 654, 73.
https://doi.org/10.1016/j.bbrc.2023.03.001
*Sara Göransson and Shan Chen contributed equally

III. **Sara Göransson**, Helene Olofsson, Henrik Johansson, Feifei Yan, Shuo Liang, Laia Masvidal, Inci Aksoylu, Glaucia N M Hajj, Hermano Martins Bellato, Johan Hartman, Ola Larsson, Janne Lehtiö and Staffan Strömblad. Mechanical regulation of mevalonate pathway enzyme synthesis drives a malignant breast cancer phenotype. *Manuscript*.

IV. **Sara Göransson**, Helene Olofsson, Feifei Yan and Staffan Strömblad. Matrix stiffness-induced IKBKE signaling drives a malignant breast cancer cell phenotype. *Manuscript*.

Contents

List of abbreviations

AFM	Atomic force microscopy
BM	Basement membrane
CA1a	MCF10CA1a cell line
CSC	Cancer stem cell
DCIS	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERBB2/HER2	Human epidermal growth factor 2
ERK	Extracellular–signal regulated kinase
F–actin	Filamentous actin
FAK	Focal adhesion kinase
FPP	Farnesyl diphosphate
GGPP	Geranylgeranyl diphosphate
HMGCR	3-hydroxy-3-methylglutaryl–CoA reductase
HMGCS1	Hydroxymethylglutaryl–CoA Synthase
IAC	Integrin adhesion complex
IDC	Invasive ductal carcinoma
IKBKE	Inhibitor of nuclear factor kappa–B kinase subunit epsilon
IPP	Isopentenyl pyrophosphate
IRM	Interference reflection microscopy
LINC	Linker of nucleoskeleton and cytoskeleton
LOX	Lysyl oxidases
LOXL2	Lysyl oxidase homolg 2
MET	Mesenchymal to epithelial transition
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid

MVP	Mevalonate pathway
Pa	Pascal (unit for elasticity)
PAA	Polyacrylamide–based hydrogel
PanIN	Pancreatic intraepithelial neoplasia
PDAC	pancreatic ductal adenocarcinoma
PIP2	phosphatidylinositol–4,5–bisphosphate
PIP3	phosphatidylinositol–3,4,5–triphosphate
PR	Progesterone receptor
RA	Reticular adhesion
RGD	Arginylglycylaspartic acid
SREBP	Sterol regulatory element binding protein
STORM	Stochastic optical reconstruction microscopy
TIMP	Tissue inhibitors of metalloproteinases
TIRF	Total internal reflection
TMA	Tissue microarray
TSR	Tumor stroma ratio

Preface

All cells sense, transduce, integrate, and respond to cues from their microenvironment. Cell-matrix interactions, i.e., the interaction between a cell and its non-cellular structural matrix, is crucial for virtually all biological processes that make up multicellular life. Moreover, when the balance in this interaction is disrupted, it can contribute to disease.

What you hold in your hand is my small contribution to the ever-growing body of knowledge on cell-matrix interactions and how they regulate cells in health and disease, particularly how they regulate breast cancer cell phenotypes. Once you have finished reading this thesis, the literature review as well as the description of my own work, I hope you will agree that cell-matrix interactions really are master regulators of cell fate.

If you are a biological scientist yourself, you know that only a small fraction of the experiments and hard work that we put in reach the form of a publication. If you hold a different profession, I will tell you that biological research is rarely as straightforward as it may seem when you read a nicely packaged news flash. Many hypotheses that we explore turn out to be wrong or too difficult to prove and experiments frequently fail, but we learn from every mistake and the excitement that we feel when we can add a new piece to the puzzle is priceless. As Winston Churchill once said, on a completely different and much more serious matter, "Success is not final, failure is not fatal: It is the courage to continue that counts".

I hope you will enjoy reading this thesis!

1   Literature review

1.1   Multicellular organisms

The human body is estimated to consist of more than 30 trillion human cells (1) of different types, organized into a variety of tissues and organs, all with their unique properties and specific requirements. As all cells in a multicellular organism originate from a single fertilized egg cell and therefore have virtually the same DNA, this diversity is achieved through the regulation of gene expression, i.e., through switching on and off different genes. The development and homeostasis of a multicellular organism require fundamental biological processes like cell proliferation, cell differentiation, cell migration, and controlled cell death, all executed by specific patterns of gene expression, to occur in the correct space and at the right time (2–4). For this to happen correctly, cells need to integrate biochemical and biophysical cues from within the cell as well as from the outside environment (3, 4). Indeed, cells have mechanisms to sense both their neighboring cells and the extracellular matrix (ECM) in their local environment and alterations in the adhesion to both of these entities guide the cellular processes needed for proper morphogenesis and homeostasis of tissues (2, 5). The very important role of cell–cell interactions will not be further discussed here but has been nicely reviewed (6–8).

1.2   The extracellular matrix

The discovery of the extracellular matrix (ECM) preceded the discovery of cells and in the early days, before 1850, the fibers of the connective tissue were actually thought to generate spontaneously and to be the basis of life (9). When this hypothesis was disproven, the matrix was instead considered unreactive, passive, and purely structural for many years (9). However, in the last decades, numerous discoveries have overturned this view and shown how the interaction between cells and this non–cellular structure is vital for cell fate determination, differentiation, proliferation, survival, polarity, and migration of cells, i.e., for multicellular life (10).

The ECM is composed of water, proteins, and polysaccharides and is present in all tissues. The polysaccharides, i.e., glycosaminoglycans and proteoglycans, form a hydrated and porous structure in which the fibrous and adhesive proteins are embedded (11). Components of the ECM are produced and re-modeled by cells residing in the matrix and the physical, topological, and biochemical composition of the ECM varies between tissues and is even heterogenous within the same tissue (12). Further, the dynamic synthesis, modification, and degradation of ECM components alters the properties of the same tissue over time (13). There are nearly 30 different collagen types and almost as many proteoglycans that reside in the ECM, and matrix glycoproteins, like laminins and

fibronectin, also show large isoform diversity (14). The ECM components are structurally very different, and their strict organization determines the bioactivity of the ECM. Hence, even a single amino acid substitution in just one of the ECM components can result in alterations in the biochemical and physical properties of a tissue, which in turn result in changed cellular phenotypes and tissue malfunction (14). This is exemplified by congenital diseases like osteogenesis imperfecta, caused by a mutation in the collagen type I gene, or Alport syndrome, caused by mutations in the collagen type IV gene, which are associated with low bone density (15) and kidney malfunction (16), respectively. An imbalance in ECM production, degradation, and remodeling also results in disease, such as fibrosis (17), and contributes to cancer progression (18-20), as discussed in more detail below.

There are two basic forms of ECM, the interstitial ECM and basement membrane (BM) (21). The interstitial ECM is the three-dimensional lattice that surrounds the cells, as described above. BMs are specialized ECMs that line the basal side of endothelial and epithelial cells and separate them from and connect them to the interstitial ECM (22). The core structural constituents of BMs are laminins, collagen type IV, nidogens, perlecan, and agrin, and normal tissue development and function are dependent on the formation of this basement membrane (22, 23). The BM is connected to the endothelial or epithelial cell layer mainly via laminin binding to cell adhesion receptors (24).

In addition to the large and highly abundant structural components, the ECM also contains smaller secreted factors present in much lower abundance (25). For example, the ECM act as a storage site for growth factors and cytokines (26) and binding to ECM components protects these factors from degradation and helps to form gradients that direct cell migration (27). In some cases, the ECM is involved in the direct presentation of growth factors to their receptors in a way that affects activation (28, 29). Moreover, the relationship between ECM and growth factors is reciprocal and growth factors can alter ECM composition. TGF-β1 for instance, can regulate the production of multiple ECM components and further influence ECM structure by inhibiting the production of proteases (30). ECM-modifying enzymes, like lysyl oxidases (LOXs), matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs), are also localized to the ECM. These enzymes have counteracting functions and changing the balance between their respective activities can profoundly alter the properties of the ECM (13).

1.2.1 ECM elasticity

The very large structural proteins of the ECM undergo extensive post-translational modifications and assemble into higher-order molecular structures via different bonds and covalent crosslinks (25). The degree of higher-order structures results in different

physical properties, for example different elasticity (or rigidity). Rigidity, measured in pascal (Pa), is defined as a material's ability to undergo non-permanent deformation and different tissues of our body show a wide range of rigidities **[Figure 1]** (31). A soft material, like neuronal tissue, requires low stresses to deform whereas stiffer material, like bone, needs greater stress to deform. The mechanical properties of the tissue have profound effects on cellular fate and naïve mesenchymal stem cells even specify lineage depending on the stiffness of their substrate (32). How mechanical signaling regulates cell fate will be discussed in more detail in later sections.
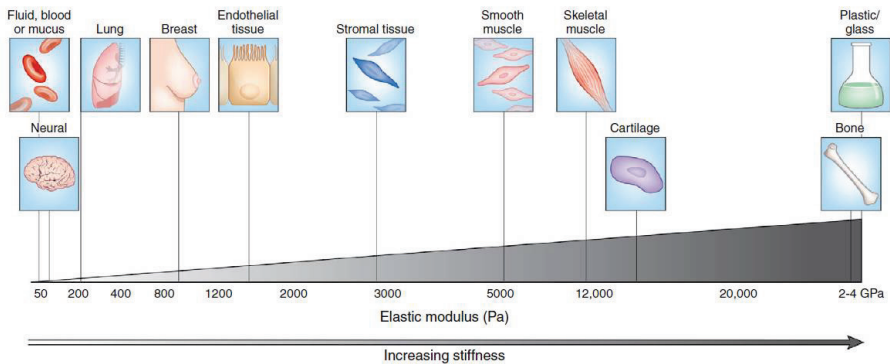

Figure 1. Stiffness variation in human tissues. The different tissues in the human body span a wide range of rigidities, from very soft neuronal tissues to stiff bone tissue.

From Cox and Erler, 2011 (31)

1.3   Cell–extracellular matrix interactions

A cell–matrix interaction is mediated via cell surface receptors, each interacting with specific ligands of the ECM. Adhesion receptors, divided into different families, are integral membrane proteins with an extracellular, a transmembrane, and a cytosolic region. In humans, integrins are the major class of ECM receptors and the family consists of 24 heterodimers formed from combinations of 18 α and 8 β subunits, each with a specific but somewhat overlapping ligand binding specificity **[Figure 2]** (33). The integrin diversity also includes a differential capacity to recruit cytoplasmic molecular interactors and to connect with the cytoskeleton (34).
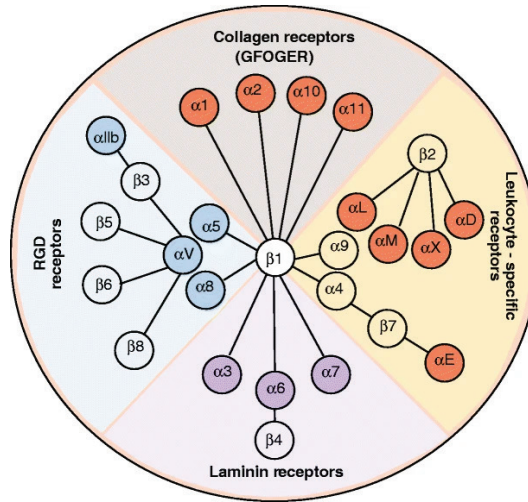
Figure 2. Representation of the integrin family of adhesion receptors. The integrin family consists of 24 heterodimers capable of binding different ECM ligands.

From Barczyk et al., 2010 (33)

The binding of a ligand to the extracellular portion of integrins results in a structural rearrangement which leads to a rapid assembly of many proteins to the intracellular part of the receptor, which then dynamically, through force generated molecular unfolding, connect the receptor to the cell's cytoskeleton and downstream signaling **[Figure 3]** (35, 36). This outside-in signaling allows the cell to sense and transmit signals from the environment to the cell interior. Conversely, the affinity and clustering of integrins can be regulated by signals from within the cell, in a process referred to as inside-out signaling (37). This activation involves the binding of talin to the cytoplasmic tail of the integrin β–subunit that induces a conformation change in the extracellular domain which results in activation **[Figure 3]**. The integrin signaling is therefore bidirectional and reciprocal in nature and the multimolecular complex, formed upon ligand binding and connected to the actin cytoskeleton, functions as a biochemical and mechanical signaling hub that detect, coordinate, transmit, adapt to, and generate signals that regulate a multitude of cellular functions (38). This is because the maturation of the multimolecular complex involves the recruitment of proteins that physically link the integrin to actin, like talin and vinculin, but also signaling molecules, like focal adhesion kinase (FAK), extracellular signal–regulated kinase (ERK), proto-oncogene tyrosine kinase Src (c-Src) and Rho family GTPases (39).
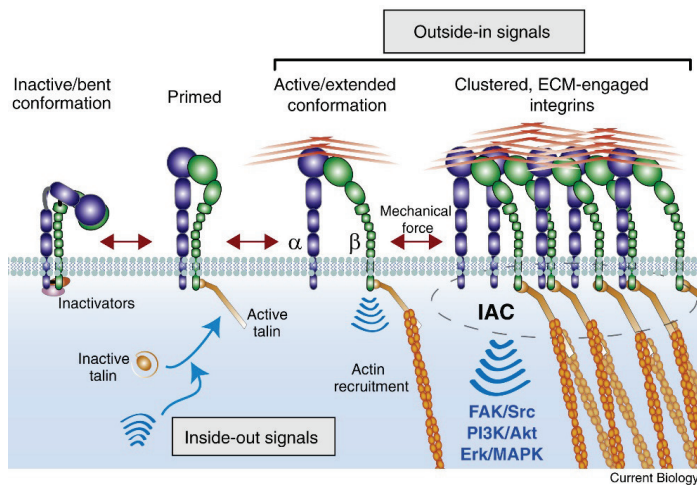
Figure 3. The steps of integrin activation. Activation of integrins involves a progressive and force-dependent conformational change that leads to the clustering and assembly of an intracellular multicomponent complex connected to the actin cytoskeleton and promoting downstream signaling.

From Chastney et al., 2021 (36). Reprinted with permission from Elsevier.

Moreover, the reciprocal nature of the cell–matrix interaction allows cells to re-organize the ECM. The tension that is exerted by cells, frequently by fibroblasts, leads to the organization of collagen fibrils into sheets and cables, which influence the alignment and tensile strength of the matrix (12). Also, binding of the fibrous ECM protein, fibronectin, to specific adhesion receptors induces polymerization into fibrillar networks (40) and cellular traction forces can stretch fibronectin many times over its resting length, leading to exposure of cryptic binding sites (41).

1.3.1   Integrin adhesion complex subtypes

Integrin adhesion complexes (IACs) can be divided into different subtypes depending on morphology and composition and their special function (36). It should be noted that most of our understanding of IACs, and hence the subtype classification, come from *in vitro* studies on rigid two-dimensional (2D) substrates. Strong evidence is emerging for the existence and importance of integrin adhesions both in 3D and *in vivo*, however, they are frequently small, heterogenous, and short-lived making them difficult to visualize and characterize (42-45). The value of bringing studies on IACs into a more physiologically relevant context was recently emphasized in a study of focal adhesions during single cell migration in a zebrafish model (46). Xue et al. showed that reduced *in vivo* phosphorylation of one core adhesion protein, paxillin, resulted in increased focal adhesion disassembly rate and increased cell migration, the opposite of what has been reported *in vitro (47).*

1.3.1.1 Canonical integrin adhesion complexes

Some of the canonical IACs subtypes represent different maturation states and include focal points or nascent adhesions, focal complexes, focal adhesions, and fibrillar adhesions (34). The maturation of these canonical IACs can be observed in cells adhering and spreading on substrates and in migratory cells where adhesion complexes constantly assemble, mature, and disassemble to allow movement of the cell body (38). Both nascent and more mature IACs are actin-linked structures, and the componentry is relatively similar. A "core integrin adhesome" of around 60 proteins has been defined through the comparison of multiple proteomic studies of IACs in various cell types (48). The recruitment of components and maturation of canonical IACs is dependent on actomyosin contractility (48, 49).

Some cells exhibit more specialized integrin adhesion structures, like podosomes or invadopodia (50). These structures localize matrix-degrading activity to cell-matrix contact points to allow for the proteolytic invasion of cells (51). These IACs are also linked to the actin cytoskeleton, but they don't share the same core adhesome (48).

1.3.1.2 Atypical integrin adhesion complexes

Unlike actin and talin-dependent canonical IACs, integrins also function in a number of atypical adhesion types. These include integrin $\alpha v \beta 5$ containing clathrin plaques or reticular adhesions (RAs) (52, 53). In a study included in this thesis (study I), we identified and characterized this class of $\alpha v \beta 5$ mediated adhesions, formed independently of talin and actin and lacking almost all core adhesome components (52). The structure was shown to provide necessary ECM anchoring for efficient cell division and was present in a variety of normal cells and cancer cells in 2D cultures. An equivalent structure was characterized in a contemporaneous study in the clathrin plaque field (53). These structures formed as a result of increased substrate rigidity but independent of cell contractility, as their formation was insensitive to the Myosin II inhibitor Blebbistatin.

Hemidesmosomes is another specialized adhesion structure that facilitates the adhesion of basal epithelial cells to the basement membrane. They form specifically through $\alpha 6 \beta 4$ integrin binding to laminin, are linked to the intermediate filament system, and provide mechanical strength to epithelial monolayers (54).

1.4 Mechanotransduction

Through cell-matrix interactions, like IACs, cells are able to sense, integrate, and transmit stimuli from the extracellular environment into the cell, as discussed in previous sections.

One feature that is sensed and transmitted and subsequently elicits diverse effects on cellular processes, is the mechanical properties, e.g., the stiffness, of the matrix. This process, in which mechanical cues are converted to a biological response via the activation of signaling pathways and transcriptional regulation, is referred to as mechanotransduction (55, 56). Mechanical forces act on different scales, ranging from subcellular, to cellular and up to whole tissue level. For example, during embryogenesis, changes in membrane tension direct the transition from naïve to primed pluripotency at the cellular level but forces are also driving the larger-scale cellular rearrangements required for proper development of the embryo (57). Integrin-mediated adhesions are critical in mechanotransduction as they are intrinsically mechanosensitive and positioned to transduce forces both through the connection with the actin cytoskeleton and conversion into chemical signals via force-dependent interaction and modulation of enzymatic activity (58-60).

Other mechanochemical transducing molecules include stress-sensitive ion channels in the plasma membrane that either increase or decrease ion flux as a result of mechanical stress (61), and force-dependent receptors for biochemical ligands, like notch (62) and plexin (63). These molecules play crucial roles in the physiology of mechanotransduction but will not be further discussed here.

1.4.1 Integrin-mediated mechanotransduction

The initial interaction between integrins and ECM is independent of force (64) but from then onwards the formation of IACs and the resulting signaling is force dependent. The mechanical properties of the matrix affect integrins in multiple ways. It affects integrin ligand binding kinetics; conformation and activation; clustering and diffusion; and trafficking and subcellular localization (65). The maturation of nascent adhesions into larger focal adhesions is force-dependent (64, 66) and the necessary process of talin unfolding and subsequent vinculin binding to both talin and actin requires a rigid ECM, above a few kilopascals (67). How much force reaches talin is dependent on the integrin-ECM bond kinetics and interestingly, different integrin heterodimers are differentially regulated by force (68-71). This means that integrin-mediated mechanotransduction is influenced by integrin expression patterns as well as by the ECM composition. For example, the adaptation of breast myoepithelial cells to matrix rigidity is determined by a differential expression of α5β1 and αvβ6 where different binding kinetics of the two isoforms to fibronectin explain differences in force generation and actin flow (72). Switching between the two heterodimers allows the cells to optimize the traction force to the stiffness of healthy or malignant tissue, respectively.

The cellular shape and mechanical stability are governed by the cytoskeleton which is built up of three major filamentous components: actin microfilaments, microtubules, and intermediate filaments (73), and both extracellular and cell-generated forces are

propagated through regulation of the cytoskeletal tension (74). Alongside the force–dependent strengthening of ECM–integrin bonds and adhesion complex maturation, the cytoskeleton is also stiffening in proportion to the applied force (75, 76). The actin filament system contributes the most to cell tension, but the initial force transmission from IACs to actin is transduced among all three filament systems as they are interconnected, and all filament types can undergo remodeling as individual monomers are added or removed (75). This intricate lattice of filaments responds to applied force as a single integrated unit and in a dynamic fashion. In this integrated system, a tensile prestress is generated and maintained by actomyosin filaments and balanced by microtubules and the ECM–connected IACs (73). The cellular response to mechanical stress depends on the global structural alterations in the cell's cytoskeleton. In fact, many enzymes and substrates involved in metabolism, signal transduction, and protein synthesis are immobilized within the cytoskeleton and the physical state of this network facilitates the integration of mechanical and biochemical signals at the whole cell level (77). Further, all three filament systems converge on the nucleus where they connect to proteins in the linker of nucleoskeleton and cytoskeleton (LINC) complex (78, 79) and cytoskeletal forces determine nuclear geometry, chromatin organization, and gene expression (80, 81).

We will now take a closer look at how mechanical signaling affects the process of cell fate determination.

1.5   Cell fate determination

The generation of cellular diversity during development involves step–wise transitions to generate diverging cellular states ultimately leading to specific functional cell types (82). The specification of cell fate is determined through the interplay between extracellular signals from the local environment and intracellular, cell-autonomous signals (82, 83). The importance of mechanical forces in large–scale tissue patterning during development is well recognized and the understanding of how these forces control specific cell fate decisions during development is increasing (84, 85).

In 1957, the developmental biologist Conrad Waddington pictured the process of development and cell specification in his famous landscape metaphor, as a ball rolling down from the mountain through a landscape of watersheds and branching valleys **[Figure 4]** (86). At each watershed, the ball (the cell) must decide between two paths, leading to two different valleys (cell states).
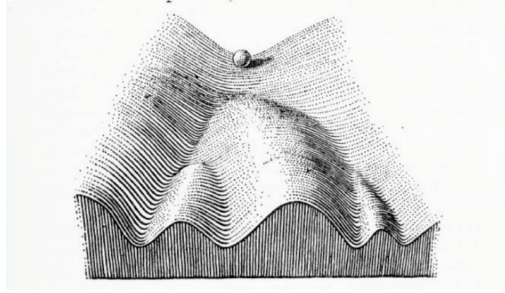
Figure 4. Waddington's landscape of cell fate determination.

From Waddington, 1957 (86)

In this view, the regulation of cell fate is based on the selection between pre-existing and more stable states. The formation of these attractor states depends on the constraints imposed by the regulatory interactions in the signaling network and to switch a phenotype would require alterations of multiple network elements at the same time, a capability inherent in the cytoskeletal structure (77). The existence of attractor states in cellular gene regulatory networks has been suggested by strongly convergent patterns of gene expression following genetic mutations and diverse chemical perturbations (87-89) Interestingly, even though the theoretical points of interaction within the actin cytoskeleton are nearly infinite, the available states of actin organization are limited, supporting phenotypic attractor states also for the cytoskeleton (90).

Force and mechanical signaling are vital for stem cell fate both *in vivo* and *in vitro*. Indeed, knockout of force-generating non-muscle myosin IIA blocks the first stages of cell differentiation in embryogenesis (91) and naïve mesenchymal stem cell differentiate into different lineages depending on the stiffness of their culture substrate (32). Mouse embryonic stem cells are very soft and when cultured on soft substrates, mimicking the intrinsic stiffness, they do not differentiate even in the absence of self-renewal promoting factor LIF (92). Interestingly, unlike mesenchymal stem cells and differentiated cells, mouse embryonic stem cells do not stiffen with increased substrate stiffness even though they increase their basal traction force, suggesting decoupling between the apical stiffness and the basal traction in these cells (93). The lower amounts of filamentous actin (F-actin) detected in these cells compared to more differentiated cells, may prevent the propagation of force to the apical surface. The application of an external force at the apical surface via integrins rescued the traction force to cell stiffness coupling, and induced cell spreading and cell differentiation (94). Further, reprogramming of somatic cells into pluripotent cells using the Yamanaka transcription factors (95) require changes in actomyosin contractility, as failure to induce such changes blocks the transition (96,

97). These examples nicely emphasize the intricate relationship between external force, cytoskeleton reorganization, and cell fate.

Force-sensitive transcription factors or transcription factor co-regulators also play a role in the mechanical regulation of cell fate in physiology and disease. For example, the YAP/TAZ transcriptional co-regulators translocate from the cytoplasm to the nucleus in response to increased matrix stiffness or cell shape changes (98, 99). TWIST1 is another example of a transcription factor regulated by mechanical cues. This factor is involved in epithelial to mesenchymal transition (100), as will be discussed below.

1.6   Cellular plasticity

Once a multicellular organism is developed, stability of cellular identity is essential for normal tissue function and is achieved through epigenetic regulation of gene expression (101). Still, dedifferentiation (the reversion of differentiated cells to a more stem cell-like state) and trans-differentiation (the conversion from one specialized state to another) do happen both *in vitro* and *in vivo* in response to either intrinsic cellular changes or changes in the microenvironment and contribute to tissue homeostasis and regeneration (102). When trans-differentiation occurs at the level of an entire tissue, often as a result of chronic damage, the transformation is referred to as metaplasia, a phenomenon associated with a predisposition to cancer (103).

1.7   Cancer

One of the earliest descriptions of cancer dates back to an ancient Egyptian textbook on trauma surgery from around 3000 BC (104). It describes eight cases of tumors of the breast and states that the disease has no treatment (105). Thankfully, our understanding of the disease and how we can treat it have increased dramatically since those early days. Still, the cancer burden continues to grow globally with around 19 million cases and almost 10 million deaths reported worldwide in 2020 (106). Breast cancer is the most diagnosed cancer whereas lung cancer is the leading cause of cancer deaths. Huge efforts are being made to improve the prevention, detection, and treatment of cancer to reduce the burden on individuals as well as society.

Cancer is not a single disease, as originally thought, but rather a group of diseases that can occur in almost any tissue and are characterized by uncontrolled cell growth (107). The diseases involve changes in the genome in a multistep process that drives normal cells progressively into malignant derivatives with limitless replicative potential (108). In later years, we have become increasingly aware that tumors consist of more than proliferating cancer cells. In fact, tumors are complex tissues composed of many different

cell types that interact with each other and with the non-cellular ECM compartment (109). Moreover, the microenvironment plays an active role in tumor progression, both in the primary tumor and in metastasis (109, 110).

Interestingly, exome sequencing of triple negative breast cancers has revealed frequent mutations in genes coding for ECM components (laminins and collagens), integrin receptors, and proteins involved in actin cytoskeleton dynamics (111). The enrichment of cytoskeletal functions among somatic aberrations in triple negative breast cancer was also evident from the copy number and alternative splicing landscapes. Moreover, a recent network-based analysis across large cancer data sets to detect driver genes with individually sparse mutation patterns, also indicated the importance of mutations in collagen, laminin, and integrin genes (112). These studies suggest that alterations in cell-matrix signaling are very important in cancer initiation and/or progression.

1.7.1   Cancer cell plasticity

The malignant process involves the loss of cell identity and function. During cancer progression, genetic, epigenetic, and microenvironmental changes lead to molecular and phenotypic alterations that contribute to tumor heterogeneity and therapy resistance (103). Cell plasticity can be defined as the ability to transit between different cellular states or phenotypes without changing the genotype and may arise through the adoption of a more stem-like state (113). Cancer stem cells (CSCs) are a unique subpopulation of cancer cells capable of driving tumor initiation, progression, and drug resistance (114). The plasticity of this subpopulation, as well as the conversion of non-CSCs to CSC-like phenotypes, is believed to be regulated, at least in part, by the tumor microenvironment (115-117). Further, evidence for the specific importance of matrix stiffness in promoting a CSC-like phenotype is emerging in several cancer types (118-120), with an optimum stiffness depending on the tissue of origin (120).

A more specific example of cellular plasticity in cancer exists in pancreatic ductal adenocarcinoma (PDAC) where most lesions originate from pancreatic intraepithelial neoplasias (PanINs). The PanIN cells resemble ductal cells but actually originate from acinar cells that have dedifferentiated into a duct-like metaplastic state via Notch/KRAS signaling (121). Notably and as previously mentioned, Notch activation is mechanosensitive and the regulation of Notch signaling is integral to the cellular response to mechanical cues (62).

In addition, cancer cells show amazing plasticity in their invasive and metastatic cell migration, an adaptive process influenced by the surrounding ECM structure (122).

#### 1.7.1.1   *Epithelial to mesenchymal plasticity*

One of the best described examples of cell plasticity is the epithelial to mesenchymal transition (EMT). This is a dynamic process, critical during embryonic development, and

aberrantly activated during cancer progression (123). EMT allows polarized epithelial cells to gain mesenchymal cell traits like enhanced migratory and invasive capacity, apoptosis resistance, and increased production of ECM components (123). A link between EMT and stemness has also been described, as transduction of EMT transcription factors into mammary epithelial cells led, as expected, to mesenchymal morphology, but also an increase in stemness properties (124–126). The process of EMT is believed to occur across a continuum and transcriptional profiling demonstrated how cells can shift along the EMT to MET (mesenchymal to epithelial transition) spectrum (127). Further, cells with a hybrid phenotype, expressing both epithelial and mesenchymal markers appear to have increased metastatic potential (128, 129). The signaling pathways involved in EMT are numerous, including TGF-β, Wnt-β-catenin, Hedgehog, and Notch and the tumor microenvironment plays a role in inducing these signaling and EMT in cancer cells (130). Matrix stiffness has been implicated also in EMT induction, e.g., via β-catenin and YAP/TAZ signaling (131), through TWIST1–G3BP2 signaling (100), and via a combination of integrin-mediated A100A11 membrane translocation, eIF4E phosphorylation and TGF-β1 autocrine signaling (132). Mesenchymal cells can further acquire amoeboid characteristics as part of the EMT continuum (133), a cell state that can be triggered by Rac1 inhibition (134), calpain-2 mediated talin-1 cleavage (135), or via increased cortical contractility in confined environments (136). EMT, and other means of gaining plasticity, will ultimately increase a cell's ability to survive and thrive in different environments, such as the diverse conditions experienced during the metastatic journey and therapeutic interventions (113).

1.7.2   Hallmarks of cancer

Hanahan and Weinberg presented, in two conceptual landmark articles published a decade apart, a list of traits or alterations in physiology, common to most, if not all human tumors (108, 137). The first article listed six common capabilities required for tumor growth and invasion: sustaining proliferative signaling; evading growth suppressors; resisting cell death; enabling replicative immortality; inducing angiogenesis; and activating invasion and metastasis (108). Already in the first publication, the importance of the microenvironment for the development and expression of certain hallmark capabilities was appreciated and this was further elaborated in the 2011 publication when four more common traits were added. These included two emerging hallmarks: evading of immune response and interfering with cellular energetics and two enabling characteristics: genome instability and tumor-promoting inflammation (137). Enabling characteristics were features that provided means by which cancer cells could acquire the core hallmarks. In a follow-up publication in 2022, Hanahan considered the two emerging hallmarks sufficiently validated to be considered part of the core set, and further presented phenotypic plasticity, non-mutational epigenetic reprogramming, polymorphic microbiomes, and senescent cells as prospective hallmarks and enabling parameters **[Figure 5]** (138). This publication further emphasized the importance of the microenvironment and

acknowledged the importance of mechanical signaling from the ECM in regulating the phenotypic characteristics of cancer cells.
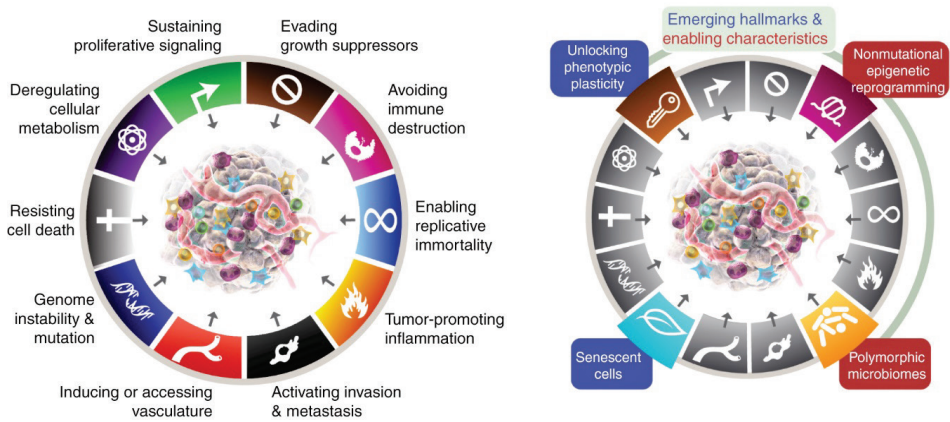
Figure 5. The hallmarks of cancer. Schematics of the common cancer hallmarks and enabling characteristics of all tumors as presented by Hanahan and Weinberg in 2011 (left), and the prospective new tumor capabilities presented by Hanahan in 2022 (right).

From Douglas Hanahan, 2022 (138). Reprinted with permission from American Association for Cancer Research.

As the core hallmarks, that at first glance appear to be inherent features of the cancer cell itself, e.g., proliferation, invasion, and apoptosis evasion, are under the influence of biophysical and biochemical cues from the extracellular matrix, a need for an intimate understanding of the reciprocal interplay between the ECM, the tumor cells, and the tumor-associated cellular stroma, is required for successful prevention and treatment (18).

1.7.3   Breast cancer

In 2020, when it surpassed lung cancer, breast cancer became the most diagnosed type of cancer worldwide with 2.3 million new cases and 685,000 deaths from this disease in that particular year (106). Just like cancer as a whole, our understanding of the biology and treatment options for breast cancer is steadily increasing. Breast cancer is a highly heterogenous disease divided into different molecular subtypes according to the expression of hormone and growth factor receptors and these diverse subtypes have different treatment options and prognoses (139–141).

1.7.3.1 Molecular subtypes of breast cancer

The original division of breast cancer into three major classes was based only on the expression of the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor 2 (ERBB2/HER2). Hormone receptor positive breast cancers are the largest group and constitute around 70% of cases. Whereas HER2 positive cancers and triple negative cancers (tumors lacking all three markers) constitute around 15% each (141). At the beginning of the 21st century, the introduction of microarray-based gene expression profiling led to a refined classification of breast cancer into five molecular subtypes: luminal A, luminal B, HER-2, triple negative/basal, and normal like, based on the expression of 50 genes (PAM50) (142, 143). An integrated copy number and gene expression analysis has suggested even more refined subgroups (144), however, these are not widely implemented. It should be noted that the identification of the five subtypes was based on gene expression profiling of primary tumors containing not only cancer cells but all other cell types present in the stroma, e.g., immune cells and fibroblasts, and the contribution of these cell types to the subtype classification is not clear (139). Yet, the molecular subtypes are linked to response to treatment and metastatic patterns and this classification is therefore useful in guiding therapy decisions (145, 146).

Following surgical resection of the primary tumor, different subtypes have slightly different treatment options. The Hormone receptor (ER and PR) positive, luminal tumors, benefit from systemic anti-estrogen therapy that blocks the effect of hormones (tamoxifen) or lower the hormone levels (aromatase inhibitors) and may also benefit from chemotherapy (147). HER2 positive tumors (ER and PR negative) can be treated with targeted therapy aimed at blocking the signaling from the receptor, i.e., anti-HER2 monoclonal antibodies or similar, either alone or in combination with standard chemotherapy (147). Among the different subtypes, triple negative breast cancer has the fewest therapeutic options and is most frequently treated with standard chemotherapy, sometimes in combination with a monoclonal antibody against the vascular endothelial growth factor (VEGF) (147). Immunotherapy based on PD-1/PD-L1 immune checkpoint inhibitors is a novel therapeutic option approved for triple negative breast cancer (148).

1.7.3.2 Breast cancer stages

Breast cancer, irrespective of subtype, is divided into five different stages depending on the spread of the disease where stage 0 is a tumor confined within the ducts of the breast (ductal carcinoma in situ, DCIS) and stage IV denotes distant metastatic disease (141). As for different subtypes, the treatment options and prognosis naturally differ between these stages, and the 5-year breast cancer specific survival for stage I (local invasion only) is between 85% and 99% (depending on molecular subtype) whereas stage IV breast cancers show a median overall survival of 5 years or less (141). The introduction of mammographic screening has led to a marked increase in detected DCIS cases, a pre-invasive stage that, in some cases will progress to invasive breast cancer (149). DCIS

lesions differ in histology, progression, and molecular features (150, 151) but we cannot yet distinguish between the DCIS lesions that will progress and the indolent ones, and therefore all are treated with surgery and radiation, and sometimes hormonal therapy (152). Evidence from autopsies, missed diagnoses, and current retrospective reviews of DCIS, support a concept of DCIS as indolent in the majority of cases and hence an overtreatment of patients (152, 153).

1.7.3.3   Extracellular matrix in breast cancer

Breast ducts and lobules consist of a bilayer of inner luminal epithelial cells and outer myoepithelial cells. The ECM is made up of the basement membrane (BM) that surrounds the myoepithelial cells, and the interstitial ECM that surrounds the cells and the BM (154). During breast cancer progression, the composition and hence the mechanical and biochemical properties of the ECM are significantly altered and this contributes to disease **[Figure 6]** (155–157).
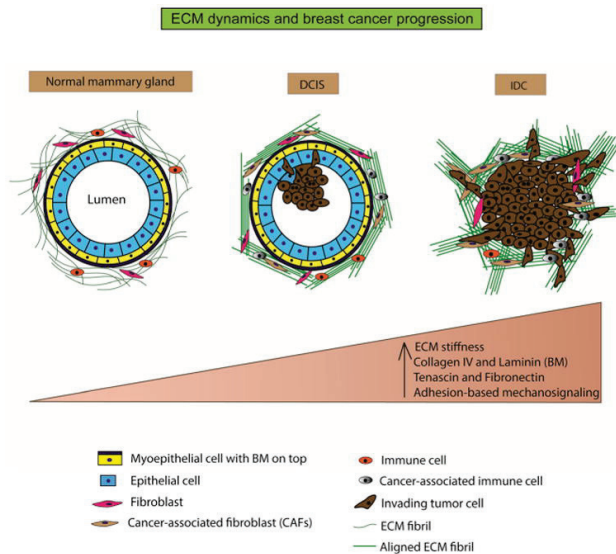
Figure 6. ECM alterations in breast cancer progression. In the normal gland, a hollow lumen is surrounded by the bilayer of inner luminal epithelial cells and outer myoepithelial cells which are encased by the basement membrane. Randomly organized fibrillar collagen makes up the majority of the interstitial ECM that also hosts fibroblasts and immune cells. During DCIS, the unregulated proliferation of the epithelial cells leads to infiltration of the central lumen and at the same time, the ECM fibrils are cross-linked and organized parallel to the tumor boundary. Stromal composition is altered, and cancer-associated fibroblasts and immune cells are present. At the invasive ductal carcinoma (IDC) stage the lumen is filled, and the ECM fibrils undergo further crosslinking and re-organization with fibers orienting perpendicular to the tumor border.

From Kaushik et al., 2017 (155). Reprinted with permission from Springer Nature

The increased organization of interstitial collagen changes the normally highly compliant ECM to a stiffer environment (158) and pre-clinical studies have shown that this stiffening drives malignancy through increased integrin-mediated mechanosignaling (159, 160). The importance of the ECM context for mammary epithelial cell behavior is further accentuated by the fact that metastatic breast cancer cells normalize and incorporate into ductal structures with proper function when subjected to normal murine mammary epithelial cells and a normal fat pad in the mouse (161).

The pre-clinical evidence for an active role of ECM in breast cancer progression is supported by clinical findings. For example, the simple tumor-stroma ratio (TSR), i.e., the proportion of tumor-related stroma within a malignancy, correlates to prognosis within different breast cancer subtypes, with inferior outcome in stroma-high tumors (162–166). More sophisticated gene expression analysis in isolated tumor stroma can also predict clinical outcome (167). Further, the prognostic value of collagen alignment has been indicated in breast carcinoma regardless of tumor grade (168) and the specific collagen organization around DCIS lesions is associated with recurrence risk (169). Interestingly, the different molecular subtypes show differences in ECM composition and rigidity with HER2 positive and triple negative subtypes showing higher collagen deposition and matrix stiffness compared to the luminal subtypes (170). In addition to the inter-tumor differences in ECM composition, there is also a significant intra-tumor heterogeneity where the organization of collagen, and hence the stiffness as well as the resulting mechanosignaling, is highest at the invasive front of tumors (170). Further, the increased stiffness correlates with infiltration of tumor promoting macrophages and with higher TGF-β signaling in the cancer cells (170).

1.8   The Mevalonate pathway

The mevalonate pathway (MVP) is a metabolic pathway that uses acetyl-CoA, NADPH, and ATP to generate many important end-products like cholesterol, isoprenoids, dolichol, vitamin D, ubiquinone, and isopenthyladenine (171). MVP is the target for the widely prescribed lipid-lowering agents, statins, which blocks cholesterol synthesis by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (172). Given the multitude of products that are synthesized from this pathway, it is not surprising that statins have pleiotropic effects that play a role in human pathologies (172, 173). Many of the metabolites are in high demand in cancer cells and the pathway, and its inhibition by statins has gained a lot of interest in the cancer research field (174–177). Cholesterol is needed for most cellular membranes and is also the precursor for steroid hormones that are involved in breast and prostate carcinoma initiation and progression (174, 178). Farnesyl-diphosphate (FPP) and geranylgeranyl-diphosphate (GGPP) are products from the MVP required for isoprenylation of proteins, a post-translational modification that is

essential for proper localization and function of small GTPases by enabling tethering to membranes (179, 180). Moreover, inhibition of MVP can induce cancer cell death, in most cases caused by loss of protein prenylation, since this inhibition can be rescued by exogenous GGPP or FPP (174, 181, 182). Dolichol is generated from the MVP product IPP and constitutes an essential component of the *N*-glycosylation of newly derived polypeptides, a process that can contribute to tumor formation, proliferation, and metastasis (183).

Activation of the MVP via ectopic expression of one flux-controlling enzyme, HMGCR, has been shown to promote the transformation of cells (184), and high mRNA expression of HMGCR or other MVP enzymes correlates with poor prognosis in breast cancer (184–186). The intracellular pools of MVP metabolites are tightly controlled by the level of enzymes (174). The transcription of MVP genes is regulated by the sterol regulatory element binding protein (SREBP) transcription factors and many oncogenic pathways converge on this transcriptional regulation. For example, PI3K–AKT pathway activation lead to increased SREBP levels (187, 188), and sterol regulatory elements are also regulated by mTORC1 (189). Gain-of-function mutants of the p53 tumor suppressor can also interact with SREBPs and increase the transcription of MVP genes (186). The MVP activation was necessary and sufficient for the tumor-promoting functionality of mutant p53 in breast epithelial cells (186). The oncogenic transcription factor MYC can also bind to promotors of MVP genes, suggesting that MYC can control the levels of MVP metabolites to ensure that these are not limiting for MYC–driven tumorigenesis (174).

The importance of many MVP metabolites for cancer cell growth and invasion suggests that this pathway can be targeted as a therapeutic strategy. Many retrospective studies have evaluated a possible effect of cancer development in statin users, with mixed results (174). However, breast cancer is one of the cancer forms where statins appear to lower the risk of recurrence (190–193). The cholesterol-lowering effect of statins is due to the inhibition of HMGCR in the liver. However, lipophilic statins, e.g., atorvastatin, simvastatin, and lovastatin, have been detected in extra-hepatic tissues but it is unclear if these statins accumulate in tumors at cytotoxic levels (174, 194).

2 Research aims

Extracellular-matrix interactions are known to guide cellular processes in normal physiology as well as in disease. The overall goal of the work described in this thesis was to provide novel molecular insight into how these interactions regulate cancer cell phenotype, with a particular focus on how matrix stiffness regulates malignant breast cancer cells.

Specific aims

Study I: To define and characterize a novel class of integrin-mediated adhesion complex that appeared in cells in long-term culture and, unlike classical focal adhesions, remained present throughout mitosis.

Study II to IV: To profile the transcriptome **(study II)**, the proteome **(study III)**, and the kinome **(study IV)** of breast cancer cells on polyacrylamide-based hydrogels of different stiffness to gain molecular insight into a stiffness-induced phenotypic switch.

3   Methodological considerations

Detailed information regarding materials and methods is found in each publication or manuscript. In this section, I will simply discuss the advantages and limitations of some of the methods that have been instrumental in this thesis, and briefly mention ethical considerations regarding the use of human biological materials.

3.1   The pre-clinical toolbox for cancer research

The tools available for pre-clinical studies of cancer biology continue to grow and *in vitro*, *ex vivo*, and animal models are getting more sophisticated and refined to closer represent the situation in a human tumor. To fully recapitulate the complexity of the human situation in a pre-clinical model is not possible but reductionistic approaches are needed to test hypotheses and gain knowledge that would not be possible in more complex settings. Also, what is found in a simple model can later be tested in more relevant situations. Each pre-clinical model has its strengths and its weaknesses, and they should be used in a complementary manner.

3.2   Cell lines

Ever since the first established cancer cell line in 1951, the HeLa cells (195), cancer-derived cell lines have continued to be established and represent a valuable resource for cancer research. They are easy and relatively inexpensive to propagate as well as to manipulate in a laboratory. The comparison of genomic data in cancer cell lines has indicated that they retain most of the genetic properties of the original cancer when appropriately cultured *in vitro (*196*)*. However, the risk for clonal selection, especially when culture conditions are not optimal, and ongoing mutational processes in genetically instable cell lines, can lead to divergence and between laboratory differences (197). To control the origin, the culture conditions, and the number of population doublings is therefore of utmost importance when using cell lines (198).

Established cell lines have been the "work horse" in this thesis and all studies are based on this simple model. It has provided an easy and indefinite source of material in assays that require high numbers of cells or high molecular detail, where other models would have been substantially more difficult or even impossible to use with current methods. In some cases, we have confirmed key findings in multiple cell lines as well as in patient material or data sets to overcome some of the limitations.

3.3   Polyacrylamide-based hydrogels

One of the most widely used 2D systems for mechanobiology studies is polyacrylamide-based hydrogels (PAAs), first developed by Pelham and Wang in the late '90s (199). Polyacrylamide deforms in proportion to applied force over a wide range of rigidities and

the elasticity of the substrate can be reproducibly altered by changing the relative concentration of acrylamide and bisacrylamide (200). PAAs are inert and have to be functionalized by conjugation of ECM ligands via a crosslinker (200). This separates elasticity from ligand density and allows independent studies of one or the other (201).

In studies II, III, and IV we took advantage of PAAs in our mechanobiology studies. This allowed us to identify a stiffness-dependent switch in breast cancer cell phenotype. Further, the reductionistic approach was instrumental to disentangle specifically the stiffness-induced effects at a molecular level, without other confounding factors. In addition, we used PAAs in combination with rBM overlay to facilitate 3D morphogenesis to better mimic the *in vivo* setting.

One disadvantage of the PAA culture system that we used is its nearly purely elastic properties (202). Along with the elasticity, the viscous properties of tissues also vary between physiological and pathological conditions (203) and recent studies have highlighted how cells respond to changes in viscous properties (204-209). Methods to make PAAs more viscous have been developed (202) and the use of alginate polymers also allows systematic variation in viscosity (208).

3.4 Omics methods

Cancer is a complex disease involving abnormalities on many different molecular levels. The various omics methods, e.g., transcriptomics, proteomics, metabolomics, and kinomics, aim to systematically understand the disease at these different levels (210). Integration of data from different levels, acquired with different molecular profiling technologies, has the potential to improve our understanding of the disease and to provide biomarkers and targets for therapy.

In this thesis, we have used DNA microarray and bulk RNA sequencing to profile the transcriptome, quantitative mass spectrometry to quantify the proteomes, and a peptide array to elucidate the kinome, of two different cancer cell states. These methods have provided large sources of data that have been, and will be, explored to shed light on how mechanical signaling can drive breast cancer progression. As is the case with most omics data sets, our generated data has been used to form testable hypotheses for further molecular studies.

One obvious overarching shortcoming with the omics analysis in this thesis is that they are all performed in bulk, i.e., we lack single cell resolution of the changes. This likely means that we have missed important alterations on all levels and may have contributed to the small differences in mRNA between the two states in the transcriptomic profiling.

3.5 Quantification of collagen organization as a proxy for stiffness

Multiple methods are available to measure the stiffness of biological material, at the tissue scale, cellular scale, or even subcellular scale. In the laboratory setting, the use of a simple rheometer can give the elasticity of an entire tumor whereas atomic force microscopy (AFM) can give detailed information on cell and even organelle stiffness (159, 170, 211). AFM is a very specialized technique that is not readily available to most laboratories. Moreover, AFM requires fresh or fresh-frozen tissue (211), something that can be difficult to obtain from the clinic.

The stiffness of breast tissue, as measured with AFM, is correlated to the organization of the collagen, and the organization of collagen is in turn coupled to the birefringence of the material, as assessed with polarized microscopy after picrosirius red staining (170, 212, 213). This enabled us to measure "stiffness" in paraffin sections from human breast tumors in study III, and to validate the *in vitro* link between local tissue stiffness and a specific protein, HMGCS1, in the only type of patient material available to us.

3.6 Ethical considerations

The use of human tissue requires informed consent from the patient. In this thesis, we have used normal breast tissue and breast tumor tissue material originally collected at Karolinska University Hospital (ethical approval 2016/957-31) and breast tumor material collected at Camargo Cancer Centre (collection and processing approved by CEP in Brazil, decision 1844/13). All material was de-identified to us with no possibility to trace the tissue back to the patient, ensuring patient confidentiality.

4 Results and Discussion

The importance of cell–matrix interaction for cellular outcomes in physiology as well as in pathologies like cancer is undisputable. Still, due to its complex and highly interconnected biochemical and mechanical downstream signaling, there is much more to learn about the molecular details.

In **study I**, we observed a new class of integrin-mediated adhesion structures with a reticular shape and localized throughout the cell body. This adhesion structure was initially detected in U2OS cells in regular culture and subsequently discovered in a range of other cell types, both cancer cells and untransformed cells. The formation of these adhesions was dependent on integrin αvβ5, but unlike the classical αvβ5 adhesions located more peripherally, the reticular adhesions did not contain talin, vinculin, or filamentous actin (F-actin). Total internal reflection (TIRF) imaging and interference reflection microscopy (IRM) of U2OS cells co-expressing fluorescently tagged β5 and vinculin confirmed that the vinculin negative β5 structures resided in close range of the substrate and were indeed cell–matrix adhesion complexes. The complexes were named reticular adhesion (RA).

Further characterization of these integrin αvβ5-mediated adhesion structures showed that they formed as puncta and grew by net peripheral integrin recruitment into ring-like or reticular structures and surprisingly, there was no correlation between RA size and β5 clustering density. Stochastic optical reconstruction microscopy (STORM) revealed no difference in integrin β5 nanoscale organization in RAs compared to classical focal adhesions (FAs), however, the dynamic behavior was very different. An isotropic growth and relative immobility of RAs suggested an absence of directed mechanical cues, which was corroborated by the lack of F-actin and supported by a locally disordered motion of adhesion trajectories. The overall lifetime was increased compared to FAs but the β5 turnover rate was faster and more extensive.

Disrupting actin polymerization or knock down of talin-2 in talin-1 null mouse embryonic stem cells did not affect the formation of RAs. Moreover, the remaining cell attachment to the substrate in the absence of F-actin was dependent on αvβ5, as competitive inhibition of αvβ5 to vitronectin binding using cyclic RGD peptides blocked this adhesion. A fusion protein of the extracellular domain of β5 and the integrin β3 intracellular tail domain also localized to RAs, identifying the extracellular domain of β5 as the facilitator of αvβ5 clustering in RAs.

The use of mass spectrometry to define adhesion compositions in U2OS cells, with or without F-actin disruption, revealed an almost complete lack of consensus adhesome components (48) in RAs, with only tensin-3 and talin-2 being present. In contrast, several endocytic adaptors were identified and validated by immunofluorescence. Out of the 53

detected RA proteins, 41 formed a highly connected protein–protein interaction network with many components reported to bind phosphatidylinositol–4,5–bisphosphate (PIP_2). Experiments using RNAi–based knockdown of PIP regulators predicted to shift the balance between PIP_2 and phosphatidylinositol–3,4,5–triphosphate (PIP_3) in different ways, also shifted the relative RA to FA ratio in a manner that suggested that PIP_2 is promoting RAs and PIP_3 is promoting FAs.

Unlike FAs that completely disassembled during mitosis, RAs persisted in a virtually unchanged fashion, and membrane dye–labeled retraction fibers were shown to angle down and attach precisely at sites of RAs during cell division. Mitotic retraction fibers contained dense actin filaments, in line with what has been reported (214), and we detected weak F–actin staining in RAs at the tips of these retraction fibers. This indicates that RAs do have limited coupling to F–actin at the time of mitotic cell rounding. The link between actin and the plasma membrane could potentially occur via ezrin or moesin, two ERM domain proteins that were identified in the RA adhesome and that are known to link the plasma membrane to cortical actin in other situations (215, 216). siRNA–mediated knockdown of integrin β5 interfered with spatial memory, as indicated by a random orientation of the mitotic axes relative to the pre-mitotic major axis. β5 depletion also reduced cell proliferation and caused a range of mitotic defects, including delayed mitosis, repeated cell rounding and re-spreading without division, and failure of cytokinesis resulting in binucleated daughter cells.

The identification and characterization of RAs in study I provide a mechanism for how adhesion is maintained during mitosis, how previously recognized mitotic retraction fibers (214) are tethered to the substrate, and how spreading is guided afterward. Given that cells can also divide on other ECM ligands, not involving αvβ5 binding, they must use alternative adhesion receptors for mitotic anchorage under these circumstances. Investigation into the role of other integrins for possible mitotic adhesion is therefore warranted. Interestingly, both β5 knockout (217) and overexpression (218) in mice cause deficiencies in osteoblast/osteoclast function indicating a specialized role for αvβ5 in cells on rigid RGD-rich substrates like bone, an environment that is quite similar to the culture conditions used in this study.

The enrichment of PIP_2 binding proteins in RAs is interesting and includes adaptors involved in clathrin-mediated endocytosis. Indeed, RAs appear to be identical to αvβ5 containing clathrin plaques (53, 215). As mentioned earlier, RA/Plaque formation is sensitive to mechanical signaling but insensitive to myosin II–dependent actomyosin contractility, suggesting that their formation allows the cell to sense and distribute forces differently. Interestingly, other myosin isoforms, like myosin Ic, were detected in RAs in our study. Myosin I isoforms can bind PIP_2 and prefer Arp2/3–nucleated over tropomyosin-coated actin filaments and hence localize to membranes where it is involved in the generation of resting cortical tension (219, 220). As cortical tension is crucial in

pluripotency regulation (221) it is tempting to speculate that RAs may be involved in cell fate determination. In addition to mechanosensing, RA/Plaques appear to be involved in chemical signaling (215). For example, recruitment of the epidermal growth factor receptor (EGFR) to clathrin plaques is necessary for optimal signal transduction (222) and the full activation of ERK requires localization to clathrin plaques (53).

In conclusion, RA/Plaques are distinct cell-matrix adhesion structures that control numerous important cellular processes, including adhesion, cell division, mechanosensing, receptor-mediated signaling, and endocytosis (215). Whether RAs/clathrin plaques play a role *in vivo* during physiology or disease remains to be determined.

Studies II, III, and **IV** constitute consecutive studies, where we explored the effect of ECM stiffness on breast cancer cells. Using polyacrylamide-based hydrogels to culture the high-grade breast carcinoma cell line, MCF10CA1a (CA1a), on ECM stiffness mimicking normal breast and breast tumor, respectively, we observed a stiffness-dependent shift in phenotype. Higher matrix stiffness led to a phenotype resembling invasive carcinoma whereas the normal breast tissue stiffness reverted this aggressive behavior into a more DCIS-like phenotype, with integrin β4 binding to an intact basement membrane at the outer rim of the cell clusters. The phenotype on higher stiffness coincided with increased mechanosignaling from FAK and ERK and inhibiting FAK with a small molecule inhibitor, severely affected the phenotype. These stiffness-dependent behaviors are in line with what has previously been reported regarding mechanical signaling in breast cancer (159, 160, 223, 224).

We used an omics approach to elucidate molecular mechanisms behind the stiffness-driven effects and profiled the transcriptome, the proteome, and the kinome in the two observed cellular states. Transcriptional changes are an inherent feature of mechanotransduction in both normal cells and cancer cells (80, 225-227). We were therefore surprised to find only minor changes at the mRNA level in CA1a cells on high stiffness compared to low in **study II**. The range of expression fold changes was moderate and the number of differentially expressed genes (DEGs) was small. Our initial DNA microarray results were confirmed with RNA sequencing, affirming that our finding was not a result of technical limitations. Analysis using gene sets of the 200 most up- and down-regulated genes from each technique revealed an overlap in cellular functions for these genes. The enrichment of lipid metabolism and immune response genes, two processes linked to matrix stiffness and breast cancer (228, 229), suggest that ECM stiffness induces small, coordinated changes in mRNAs involved in cellular processes relevant to breast cancer progression. Until experimentally tested, we also cannot rule out the importance of the upregulation of individual genes. Noteworthy, one of the DEGs upregulated on high stiffness was fibronectin (FN). This suggests that ECM stiffness influences the cancer cell's ability to generate their own matrix and subsequently the integrin signaling that, as we know, has profound effects on cellular processes.

Previous studies displayed similarly limited changes in mRNA levels when comparing the epithelial compartment in DCIS and IDC in patients (230, 231). We generated gene sets from three different studies comparing the transcriptome of co-occurring DCIS and IDC lesions (230, 232, 233) and compared those to our stiffness data set. Interestingly, in all three cases, the genes upregulated in IDC relative to DCIS, correlated to our high stiffness signature, supporting a role for ECM stiffness in the pre-invasive to invasive transition in humans. We therefore speculate that increased matrix stiffness may facilitate the invasion of cells with a specific molecular make-up, however, whether the small transcriptional changes that we detected really are important in this process or if larger post-transcriptional alterations are required, is still an open question.

How do we reconcile our finding with the well-established role of mechanotransduction in transcriptional regulation (80, 225–227)? A small sample size (n = 3) may of course affect our ability to detect differentially expressed genes in a noisy system. However, if the stiffness-dependent switch in phenotype is regulated exclusively at a transcriptional level in our system, we envisage that the changes in mRNA would be large enough in magnitude for us to pick them up as differentially expressed. Two previous studies may be able to shed some light on our findings. First, the number of stiffness-regulated genes in mouse mesenchymal stem cells were shown to drop dramatically if 3 kPa and 18 kPa hydrogels were used as compared to if 3 kPa and 30 kPa were used in the experiment (234). We used a different cell type and a different range of stiffness (0.4–0.5 kPa and 5–8 kPa) making it difficult to directly compare the two studies but we can conclude that the difference in stiffness between our two conditions is smaller than in many other gene expression studies in cancer cells (235–237). Second, the type of ECM ligand used may also play a role as exemplified by a study showing differential regulation of gene expression following EGF treatment in HEK293 cells attached to fibronectin or laminin respectively, with laminin giving a smaller response (238). Many studies on the mechanical regulation of gene expression have used collagen I or fibronectin-functionalized hydrogels as opposed to the rBM-conjugated hydrogels used in our study.

The idea that stiffness can regulate cellular phenotypes post-transcriptionally is supported by the fact that transcript levels are insufficient to predict protein levels in many biological scenarios, especially when cells are adjusting to changes in environmental conditions (239). Further, transcriptomic analysis of primary breast carcinomas and their corresponding lymph node metastasis revealed that in roughly half of the cases, the paired samples were closely matched whereas in the other half, the difference at mRNA level was more pronounced (240). This shows that some breast cancer cells change to a metastatic phenotype without significantly changing their transcriptomes.

In **study III**, we looked beyond the well-established transcriptional response to mechanical signaling. Using quantitative mass spectrometry, we profiled the proteomes of the low and high stiffness CA1a phenotypes to investigate a possible post-

transcriptional regulation of the stiffness-induced phenotypic switch. In contrast to the limited changes on the transcriptional level, the proteomes showed significant differences. Out of the 6513 proteins quantified in both conditions, nearly 10% were significantly altered by an increase in ECM stiffness (176 upregulated and 417 downregulated, log2 FC = 0.3 and q-value<0.01). This shows that mechanical cues from the ECM can generate sustained alterations in the proteome without concomitant mRNA changes and highlights the need for further post-transcriptional studies in the field of mechanobiology. Indeed, over-representation analysis of the differentially expressed proteins (DEPs) indicated a stiffness-dependent upregulation of ribosomal biogenesis and protein translation machinery, e.g., ribosomal proteins (RPs), initiation factors, elongation factors, and tRNA synthetases. The stiffness-dependent regulation of specific ribosomal subunits is particularly interesting as heterogeneity of ribosomal composition is known to regulate distinct sub-pools of mRNAs (241) and ribosomes lacking specific RPs show a preference for other classes of transcripts (242). We can speculate that the differential regulation of RPs in our model changes the translational landscape to increase the level of certain proteins that in turn drive the phenotypic switch. Indeed, other studies have pointed to the importance of specific ribosomal proteins and ribosomal biogenesis in breast cancer cell plasticity and metastasis (243, 244).

The comparison of the stiffness-regulated proteins to proteomic signatures representing different molecular subtypes of breast cancer indicated that the cells on high stiffness showed a significant correlation to the basal subtype whereas cells on low stiffness more resembled the normal-like or luminal A subtype. This suggests that mechanical signaling may drive cells into different subtypes, at least on a proteomic level. Because the stiffness of a breast tumor is heterogenous (170) this could generate intratumor molecular subtype heterogeneity and potentially affect responses to treatment. Future studies should evaluate how this may be achieved at a single cell level, i.e., investigate if mechanical signaling drives CA1a cell state transitions involving EMT or stemness properties.

As proof of the generalizability and importance of the generated proteomic data set, we decided to follow up on the regulation of a specific pathway. Over-representation analysis indicated a specific upregulation of the mevalonate pathway. Many of the enzymes of this important metabolic pathway showed increased expression on high stiffness and we confirmed the stiffness-dependent upregulation of one of these enzymes, Hydroxymethylglutaryl-CoA Synthase (HMGCS1), in several breast cancer cell lines as well as in non-transformed breast epithelial cells. In most cell lines, the increase in protein was not concordant with HMGCS1 mRNA levels, suggesting that mechanical cues from the microenvironment can lead to the biosynthesis of sterols and isoprenoids without SREBP-dependent transcriptional activation of the pathway. Moreover, the increase in these metabolites is crucial for the stiffness-induced phenotypic switch as inhibition of the

pathway with RNAi or the inhibitor simvastatin reverted the IDC phenotype and inhibited proliferation.

We also found increased HMGCS1 expression in human breast tumors versus normal breast tissue. Importantly, the level of HMGCS1 in tumors correlated with local collagen organization, i.e., ECM stiffness, both in a tissue microarray (TMA) of 87 invasive breast cancers and in a smaller cohort of whole tumor sections, picked for their variability in HMGCS1 labeling. This indicates that HMGCS1 expression may be regulated by mechanical signaling also in human breast tumors. In addition, polysome profiling in breast tumor tissue from 161 patients, revealed a poor correlation between total levels of HMGCS1 mRNA and polysome-bound, i.e., actively translating, HMGCS1 mRNA, suggesting regulation at the level of mRNA-translation in patients. Interestingly, translational regulation of HMGCS1, although not previously experimentally demonstrated, has been suggested by 5´-UTR sequence homology to the reductase of the pathway, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (245). HMGCR, the most studied rate-limiting enzyme in the mevalonate pathway, is known to be regulated at multiple levels (171). Translational regulation of HMGCR was indeed apparent in our patient polysome profiling, whereas other downstream enzymes of the pathway showed a good correlation of total mRNA and polysome-bound mRNA, indicating regulation primarily at a transcriptional level. Together, these patient data support our *in vitro* findings for a role of tissue stiffness in post-transcriptional regulation of the Mevalonate pathway *in vivo* and underscores the relevance of our proteomics dataset.

To investigate the role of integrin signaling in the mechanical regulation of HMGCS1 levels, we cultured the CA1a cells on different concentrations of ECM ligands. Indeed, higher ligand concentrations led to higher HMGCS1 expression, irrespective of ECM type, arguing for a general integrin regulation from different heterodimers. Inhibition of integrin β1 signaling using a monoclonal antibody (AIIB2) attenuated HMGCS1 protein levels without a concomitant decrease in HMGCS1 mRNA, supporting a role for integrins in post-transcriptional regulation of HMGCS1. AIIB2 treatment also induced a reversion of the stiffness phenotype, in line with what has been reported previously on a role of integrin β1 in breast cancer cells (246, 247). Further, the activity of the small GTPase Rac1, a well-known effector of integrin signaling and important in mammary branching morphogenesis (248, 249), normal secretory function (250), and breast cancer progression (251), was increased by ECM stiffness in CA1a cells. The Rac1 activity was necessary for maintaining HMGCS1 levels on high stiffness as the levels were decreased when cells were treated with the Rac1 inhibitor NSC23766 or transfected with efficient Rac1 siRNAs. Just like stiffness and integrin signaling, Rac1 appeared to act post-transcriptionally, since the effect of Rac1 inhibition on HMGCS1 protein levels was not reflected by changes in mRNA.

In addition, using metabolic labeling to detect nascent proteins, we showed that the synthesis rate of HMGCS1 was significantly higher on breast tumor stiffness compared to

normal breast tissue stiffness. In a 4 h window, around 2.4 times more HMGCS1 was synthesized on high stiffness compared to low. Importantly, Rac1 inhibition by NSC23766 treatment or Rac1 RNAi decreased the synthesis rate of HMGCS1 dramatically. Interestingly, the transforming activity of Rac1 is dependent on geranylgeranylation (252), i.e., the addition of a mevalonate pathway metabolite. Also, Rac1 was recently shown to control local GTP availability and subsequently its own activity and cancer cell invasion, through the interaction with inosine monophosphate dehydrogenase 2 (IMPDH2) (253). Together with the data presented here, this indicates that Rac1 can control the availability of two different metabolites needed for its full activation. Moreover, our finding that the prenylation-dependent small GTPase Rac1 regulates HMGCS1 synthesis post-transcriptionally, supports an earlier theory that suggested translational regulation of the mevalonate pathway via an unknown prenylated protein (171).

Finally, the importance of Rac1 signaling and HMGCS1 expression for the stiffness-induced phenotypic switch was shown via lentiviral expression of a naturally occurring cancer mutant, Rac1[P29S]. The expression of this constitutively active Rac1[P29S] promoted HMGCS1 protein expression and induced an IDC phenotype in CA1a cells on low stiffness. The phenotypic switch required an increase in mevalonate pathway metabolites as it was inhibited by simvastatin. Further, the Rac1 protein levels were significantly positively correlated to HMGCS1 protein levels, but not HMGCS1 mRNA levels, in the CPTAC breast cancer cohort (254). Other related GTPase proteins, RhoA and Cdc42, displayed no significant correlation with HMGCS1 protein levels. Overexpression of Rac1 correlates to advanced tumor stage and poor prognosis in breast cancer patients (255). Our data support a role for Rac1 in bypassing the need for ECM stiffness that could have implications in the metastatic colonization of soft tissue, like lung or brain, and suggest that mevalonate pathway inhibitors, i.e., statins may inhibit metastatic outgrowth. Intriguingly, a recent study observed a significant association between statin use and improved breast cancer-specific survival in triple negative breast cancer (256), the subtype possessing the stiffest stroma (170) and where Rac1 signaling plays important roles in metastasis formation (257-259).

To complement our studies on the ECM stiffness-induced changes in the transcriptome and proteome of CA1a cells, we set out to investigate the role of kinase signaling for the stiffness phenotype in **study IV**. In this study, we included integrin β1 inhibition on high stiffness as a third condition to determine the contribution of β1-mediated signaling to the overall mechanical signaling. The use of commercially available tyrosine (PTK) and serine/threonine (STK) peptide arrays (PamGene) revealed significant stiffness-dependent alterations in phosphorylation patterns. Out of the 79 PTK peptides that passed the quality control, 59 were significantly more phosphorylated when incubated with lysates coming from CA1a cells cultured on high stiffness. For the STK array, 67 out of 109 were more phosphorylated. Principal component analysis (PCA) based on all peptides

passing the quality control (QC) successfully separated low and high stiffness samples in the first component. Integrin β1 inhibition affected the stiffness-induced phosphorylation pattern to some degree with 4 PTK peptides and 11 STK peptides significantly altered compared to high stiffness control. The relatively small effect of AIIB2 treatment on peptide phosphorylation suggests that other integrin isoforms or alternative mechanosensitive pathways play a role in CA1a cells. Interestingly, an EphA2 peptide on the PTK array is the second most differentially phosphorylated in the stiffness comparison. Ligand-independent, non-canonical EphA2 signaling is known to be mechanosensitive (260), involved in EMT (260) and cancer cell invasion (261, 262) as well as in stem cell regulation (262). To what extent EphA2 signaling plays a role in our system remains to be investigated.

Next, the differentially phosphorylated peptides were used to predict kinase activity, using a proprietary software, Bioconductor (PamGene). Among the kinases predicted to be activated by stiffness are well-characterized mechanosensitive signaling components such as ERK1 and Src (263) and also kinases not previously linked to mechanosignaling, including IKBKE and ZAP70. Treatment with AIIB2 attenuated ERK, JNK, and p38 serine/threonine kinases, known integrin signaling mediators, providing validity to the peptide array as a tool for kinase activity profiling.

To specifically interrogate the importance of kinase activity downstream of integrin β1 for the stiffness-induced phenotype, we compared the list of kinases whose activity was predicted to be upregulated by stiffness to the list of kinases inhibited by AIIB2 treatment. This revealed an overlap of 16 kinases in total. A small image-based RNAi screen, in which these kinases were knocked down using siRNA pools on high stiffness and evaluated for their ability to revert the stiffness phenotype, indicated that at least 4 out of 16 were important. We did not control for knockdown efficiency at the time of phenotype assessment meaning that we may have false negative results. The inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKBKE), a non-canonical I-kappa-B kinase, was one of the hits in the screen.

IKBKE has been identified as a breast cancer oncogene, amplified and overexpressed in more than 30% of breast carcinomas (264). Increased expression of IKBKE in cancer has been detected both with and without corresponding copy number gain (264, 265) and several recent studies implicate this kinase in breast cancer progression and metastasis (264-267). Our results suggest that IKBKE is activated as a result of augmented mechanical signaling, adding yet another layer of regulation of this kinase in breast cancer cells. The use of two individual siRNAs with confirmed knockdown efficiency at the mRNA level validated our screen finding and suggested that IKBKE was indeed required for the stiffness phenotype. Further, CA1a cells transfected with either of these IKBKE siRNAs showed impaired proliferation on high stiffness compared to control. Pharmacological inhibitors of IKBKE have been developed (268) and one of them, a dual IKBKE/TBK1 inhibitor

named Amlexanox, is in clinical use for the treatment of recurrent aphthous ulcers (269). This inhibitor has been reported to repress cancer cell proliferation and invasion (270–272) and has shown efficacy in different experimental tumor models (273). In line with these studies, the treatment of CA1a cells on high stiffness with Amlexanox led to impaired growth and a reversion to a DCIS-like phenotype. Together, these results indicate that IKBKE signaling is involved in driving the stiffness-induced malignant breast cancer cell phenotype and warrant further investigations into the possible use of IKBKE inhibitors in preventing DCIS to IDC transition.

5 Conclusions

The work presented in this thesis contributes to the constantly growing body of evidence of the importance of cell–matrix interactions in guiding cellular processes.

We identified and characterized a novel and distinct class of integrin–mediated adhesion complexes, the reticular adhesions, which persist throughout mitosis and therefore provide an answer to the long-standing question of ECM attachment during cell division. Moreover, the specific componentry of RAs suggests a possible role in the regulation of cortical tension, which in turn has implications for cell fate determination.

In addition, we add to the knowledge of cell-matrix interactions in breast cancer progression. We show that breast cancer cells switch between a DCIS–like state and an IDC–like state depending on the ECM stiffness and that this *in vitro* switch resembles the DCIS to IDC transition in breast cancer patients, in phenotype as well as in transcriptomic alterations. This shows that mechanical forces can drive larger-scale collective growth patterns of cancer cells into different states, resembling different breast cancer stages. Further, we provide molecular insight into this switch and show that the mevalonate pathway as well as IKBKE signaling support the stiffness-induced IDC phenotype and hence may represent targets for therapy. The transcriptomic, proteomic, and kinase activity profiling also offer a source for future hypothesis generation both in the field of mechanotransduction and in the field of breast cancer research.

In conclusion, the novel data in this thesis together with the vast amount of available literature on the subject, of which I have presented a small selection in the literature review, link cell-matrix interactions to cancer cell fate regulation both at single cell level and at larger tissue scale. Hence, I believe that this thesis provides strong evidence for cell-matrix interactions as master regulators of cancer cell fate.
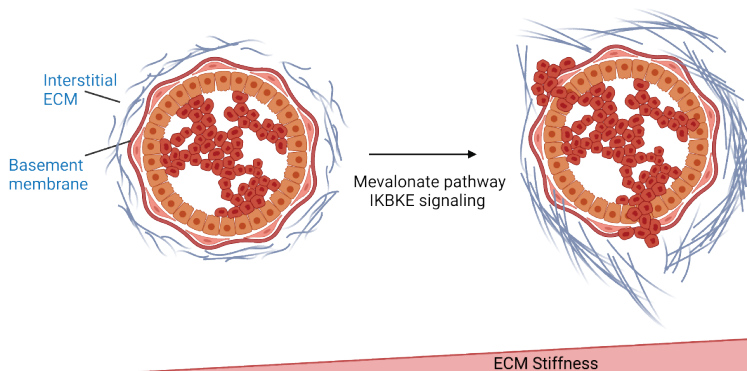
Figure 7. Schematic summary of studies II, III, and IV. Created with in Biorender.com

6 Points of perspective

A general point of perspective

Studies on cell–matrix interactions have evolved from being heavily biologically oriented to more occurring at the crossroad of biology and physics, as our awareness of the importance of physical properties on cellular behavior is increasing. I believe that multidisciplinary efforts are key to continuing to bring this field forward and creating environments where biologists, material physicists, and bioinformaticians can work together and learn from each other is most desirable.

Future research and clinical implications

The work presented here gives hints at molecular details underlying the effect of cell–matrix interaction on cancer cell fate at different scales. However, all findings need to be verified in additional and more relevant models. One obvious next step would be to bring the mechanotransduction studies into a proper 3D context. Technical developments have made it possible to generate 3D environments with different stiffness without altering other properties, like ligand density and pore size, something that has not been possible when using natural matrices like rBM or collagen type I. There are also interesting mouse models available, like the Col^{tmJae} transgenic mouse that present with high mammary collagen density due to a mutation in collagen type I near the matrix metalloproteinase cleavage site (274). Combining this model with the Mouse-INtraDuctal (MIND) model of DCIS (275) could generate valuable insight into the role of ECM stiffness and cell-matrix interactions in DCIS to IDC transition. Studying DCIS to IDC transition in the clinical setting is limited by the standard surgical removal of lesions at the DCIS stage. Looking at molecular differences between co-occurring DCIS and IDC lesions is a start but is unlikely to fully recapitulate the molecular mechanisms driving the transition. Correlating molecular markers in resected DCIS lesions to recurrence is also not an accurate way to establish markers for the DCIS to IDC transition. Clinical trials, like the COMET trials in the U.S. or the LORD trial in the Netherlands, are evaluating active monitoring as an option to standard surgical and radiation treatment for low-risk DCIS cases (276). Biopsies from DCIS lesions that are later monitored for potential progression will be an important source of material for molecular characterization to differentiate aggressive and indolent DCISs.

In addition, technical advances in spatial omics and multiplexed imaging that allow us to overlay the molecular information with the cellular location within the tumor microenvironment will likely provide a more comprehensive understanding of the interplay between cancer cells and their surroundings. For example, overlaying measurements of stiffness or collagen organization with spatial transcriptomics or

multiplexed protein detection would likely be much more informative than the bulk measurements that we have performed in this thesis.

With increased knowledge of how alterations in the ECM and/or in the components that transduce these changes contribute to cancer progression, the idea of targeting this signaling has manifested. Therapies targeting the ECM organization, such as LOX and LOXL2 inhibitors, or therapies targeting the signaling, e.g., inhibitors of integrins, FAK, or YAP/TAZ, have shown promising results in preclinical and sometimes in early phase clinical studies. However, none of these have yet reached clinical use. The role of the ECM in cancer biology is complex and in some contexts, the ECM acts to restrain the cancer, as was the case in pancreatic cancer where nonselective depletion of stroma led to acceleration of disease and interrupted clinical trials (277). Continued pre-clinical and clinical efforts to increase our understanding will likely improve our chances to curb cell-matrix signaling in a way that will be useful in the treatment of cancer.

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