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Microenvironmental regulators of mammary gland architecture: the role of matrix metalloproteinase 3 in epithelial invasion and branching morphogenesis

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Microenvironmental regulators of mammary gland architecture: the role of matrix metalloproteinase 3 in epithelial invasion and branching morphogenesis

Tese de Candidatura ao grau de Doutor em Biologia Básica e Aplicada submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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"Don't be afraid of being the cat.

Always question the authority, and think outside the box!"

Mina J. Bissell

"Here's To The Crazy Ones. The rebels. The trouble-makers.

The ones who see things differently.

While some may see them as the crazy ones, we see genius.

Because the people who are crazy enough to think they can change the world, are the ones who DO!"

Apple Inc.

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Publications/Publicações

Ao abrigo do Artigo 17.° do Decreto-Lei n.° 216/92, fazem parte da presente dissertação os seguintes artigos científicos originais publicados ou em preparação para publicação:

1. **Correia, A.L.**, and Bissell, M.J. 2012. The tumor microenvironment is a dominant force in multidrug resistance. *Drug Resistance Updates* **15**: 39-49.
2. **Correia, A.L.**, Mori, H., Chen, E.I., Schmitt, F.C., and Bissell, M.J. 2013. The hemopexin domain of MMP3 is responsible for mammary epithelial invasion and morphogenesis through extracellular interaction with HSP90β. *Genes & Development* (accepted for publication).
3. Ghajar, C.M., **Correia, A.L.**, and Bissell, M.J. 2013. The role of the microenvironment in tumor initiation, progression and metastasis. *Molecular Basis of Cancer*, 4th Ed. chapter on the Tumor Microenvironment (in press).
4. **Correia, A.L.**, and Bissell, M.J. 2013. Coordinated expression of HSP90β and MMP3 regulates epithelial cell function during post-natal mammary gland morphogenesis (in preparation).

Em cumprimento do disposto no referido Decreto-Lei, a candidata a doutoramento declara que participou activamente na concepção e execução do trabalho experimental que esteve na origem dos resultados apresentados, bem como na sua interpretação e na redação dos respectivos manuscritos.

Abstract

Matrix metalloproteinases (MMPs) are crucial mediators in sculpting tissue architecture, and are required for many physiological and pathological processes. MMP3 has been shown to regulate branching morphogenesis in mammary gland. Ectopic expression of proteolytically active MMP3 in mouse mammary epithelia triggers supernumerary lateral branching and eventually tumors. Because the proteolytic activity of MMPs resides within the catalytic domain, it has been generally assumed that this domain is responsible for all the functions of MMP3.

Using a three-dimensional (3D) collagen-I (Col-1) gel assay that simulates epithelial invasion and branching, we found that it is the hemopexin domain of MMP3 that directs these processes. Using three different engineered constructs containing a variation on MMP3 structural domains, we confirmed the importance of the hemopexin domain not only in cultured cells but also in primary organoids of the mammary gland. A proteomic screen of MMP3 binding partners surprisingly revealed that the intracellular chaperone, HSP90β, is present extracellularly, and its interaction with the hemopexin domain of MMP3 is critical for invasion. Blocking of HSP90β with specific inhibitory antibodies added to the medium abolished invasion and branching in mammary organoids. Additionally, we observed that the levels of expression of *Hsp90ab1* and *Mmp3* are positively correlated in pubertal mammary glands, reaffirming their importance in sculpting the mammary epithelial tree during branching morphogenesis.

These findings shift the focus from the proteolytic activity of MMP3 as the central player to its hemopexin domain, and add a new dimension to HSP90β's functions by revealing a hitherto undescribed mechanism of

MMP3 regulation. Our data also may shed light on the failure of strategies to use MMP inhibitors in cancer treatment and other related disorders.

Resumo

As metaloproteinases de matriz (MMPs) são mediadores cruciais na construção da arquitectura dos tecidos, e são necessárias em muitos processos fisiológicos e patológicos. A MMP3 regula a formação de ramificações na glândula mamária. A expressão ectópica de MMP3 com atividade proteolítica no epitélio mamário de ratinhos desencadeia o desenvolvimento excessivo de ramificações laterais e, eventualmente, de tumores. O facto de a atividade proteolítica das MMPs residir no seu domínio catalítico levou os investigadores a assumirem que este domínio é responsável por todas as funções da MMP3.

Utilizando um ensaio tridimensional (3D) em colagénio tipo I (Col-1), que simula a invasão epitelial e a ramificação mamária, demonstramos que estes processos são dirigidos pelo domínio hemopexina da MMP3. Utilizando três constructos diferentes, engenhados com variações nos domínios estruturais da MMP3, confirmamos a importância do domínio hemopexina, não só numa linha celular, mas também em organóides primários da glândula mamária. Um screening proteómico para identificar proteínas que interagem com a MMP3 revelou, surpreendentemente, que a chaperona intercelular HSP90β está presente no meio extracelular, e que a sua interação com o domínio hemopexina da MMP3 é crucial para o processo de invasão. A inibição da HSP90β com anticorpos específicos adicionados ao meio de cultura aboliram a invasão e ramificação em organóides mamários. Adicionalmente, observamos que os níveis de expressão dos genes *Hsp90ab1* e *Mmp3* estavam correlacionados positivamente em glândulas mamárias em fase de puberdade, o que reafirma a sua importância na formação da árvore epitelial mamária durante o processo de ramificação.

Esta descoberta muda o foco de investigação da função da MMP3 da sua atividade proteolítica para o seu domínio hemopexina e confere uma nova dimensão às funções da HSP90β ao revelar-lhe um papel fundamental, e até agora desconhecido, no mecanismo de regulação da MMP3. Os nossos resultados podem ainda contribuir para elucidar a causa do insucesso do uso de inibidores de MMPs no tratamento do cancro e outras doenças relacionadas.

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Abbreviations

2D	Two-dimensional
293FT	Cell line suitable for lentiviral production
3D	Three-dimensional
α-SMA	Alpha Smooth Muscle Actin
ADAM	A Disintegrin and Metalloproteinase domain-containing protein
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
ANXA2	Annexin A2
APC	Adenomatous Polyposis Coli gene
AREG	Amphiregulin
AU	Arbitrary Units
bFGF	Basic Fibroblast Growth Factor
BM	Basement Membrane
BMDC	Bone Marrow-Derived Cell
BRCA	Breast Cancer Susceptibility gene
BSA	Bovine Serum Albumin
CAF	Cancer-Associated Fibroblast
CD44	CD44 antigen
cDNA	Complementary DNA
CM	Conditioned Medium
Co-IP	Co-Immunoprecipitation
Col-1	Collagen-I
copGFP	Expression cassette for the reporter gene Green Fluorescent Protein
CSF1	Colony-Stimulating Factor 1 (macrophage)
C-terminus	Carboxyl-Terminus
DAPI	4',6-Diamidino-2-Phenylindole
DDR1	Discoidin Domain Receptor 1
DMEM	Dulbecco's Modified Eagle's Medium

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dPEX-MMP3	Deleted hemopexin domain mutant MMP3
EA-MMP3	E219A mutant MMP3
ECM	Extracellular Matrix
E-cadherin	Epithelial cadherin
EF1	Elongation Factor 1α
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-Mesenchymal Transition
EpH4	Mouse mammary epithelial cell line
ERα	Estrogen Receptor Alpha
ERBB2	v-Erb-B2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
ERK	Extracellular signal-Regulated Kinase
F-12	Ham's F-12 Nutrient Mixture
F-actin	Filamentous Actin
FBS	Fetal Bovine Serum
FGFR	Fibroblast Growth Factor Receptor
FL-MMP3	Full Length MMP3
GATA3	Trans-acting T-cell-specific transcription factor GATA3
GH	Growth Hormone
GPI	Glycosylphosphatidylinositol
HGF	Hepatocyte Growth Factor
HSP	Heat Shock Protein
HSPG	Heparan Sulphate Proteoglycan
IF	Immunofluorescence
IGF1	Insulin-like Growth Factor 1
IGF1R	Insulin-like Growth Factor 1 Receptor
IGFBP	Insulin-like Growth Factor Binding Protein
IgG	Immunoglobulin G
LC MS/MS	Multidimensional Chromatography and tandem Mass Spectrometry
LEP	Luminal Epithelial Cell
lrECM	Laminin-Rich Extracellular Matrix
MAPK	Mitogen-Activated Protein Kinase
MARCKS	Myristoylated Alanine-Rich C-Kinase Substrate
MaSC	Mammary Stem Cell

MCS	Multiple Cloning Sites
MEP	Myoepithelial Cell
MMP	Matrix Metalloproteinase
MT-MMP	Membrane-type Matrix Metalloproteinase
MMTV	Murine Mammary Tumor Virus
NA	Numerical Aperture
NaOH	Sodium Hydroxide
NIH/3T3	Mouse fibroblast cell line
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PRLR	Prolactin Receptor
qPCR	Quantitative real time PCR
RAC1	Ras-related C3 botulinum toxin substrate 1
RANKL	Receptor Activator of Nuclear factor kappa-B Ligand
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
RSV	Rous Sarcoma Virus
RTK	Receptor Tyrosine Kinase
S100A4	S100 calcium-binding protein A4
SCp2	Small cuboidal functionally normal mouse mammary epithelial cells
s.d.	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
shRNA	Short Hairpin RNA
SMAD	Mad- (*Drosophila* homolog) and Sma- (*C. elegans* homolog) related protein
SPROUTY	Spry (*Drosophila* homolog) protein
STAT5A	Signal Transducer and Activator of Transcription 5A
T2A	Self-cleavable 2A peptide
TE	Tris EDTA buffer
TEB	Terminal End Bud
TIMP	Tissue Inhibitor of Metalloproteinases
TGF	Transforming Growth Factor
uPA	Urokinase-type Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor
v-src	Viral Sarcoma gene
WNT	Wingless-type MMTV integration site family
WB	Western Blotting

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Chapter 1

Introduction

1.1 Tissue Architecture

The function of a tissue or organ relies on its constituent cell types and overall organization. It is the evident uniqueness of this *architecture* that distinguishes, for example, the heart from the mammary gland, and that directs the cells within the former to pump blood and within the latter to make milk; this is so despite the fact that different tissues emerge from a single cell, and thus all share an identical genome.

Tissue and organ architecture is early specified in the embryo. Morphogens ("form producers") diffuse through embryonic tissues, setting up concentration gradients that govern pattern formation as well as the position of the several specialized cell types within a tissue (Turing 1952). All organs, with a few notable exceptions, such as the mammary gland and the brain, 'arrive' together and are complete when the organism is born. Preserving the original construction, however, is a permanent task. The ability of cells to proliferate, move, differentiate and die poses a continuous challenge to tissue homeostasis, and yet most adult tissues show architectural consistency over many decades. Loss of architecture is part of the definition of many diseases, including cancer. Indeed, this is how oncologists and pathologists diagnose tumors and determine their stage or grade (Burstein et al. 2004; Miller and Mihm 2006). Understanding the framework that maintains architectural stability is not only crucial to determine how organs re-

main functional and disease-free, but also how cells within tissues lose or overcome these controls in cancer.

1.1.1 Cell-Microenvironment Interactions are Integral Determinants of Tissue Architecture

Tissue function and homeostasis are driven by tissue architecture. But, if architecture is the product of cell arrangements, and if every cell in our body carries the same genetic information, how does each cell know how to behave within a tissue? Two-way communication has emerged as the organizing principle that enables 'dynamic and reciprocal' exchanges of information between cells and their surroundings (Bissell et al. 1982; Bissell et al. 2002). According to this model (Figure 1.1), tissues and organs are embedded in extracellular matrix (ECM)/basement membrane (BM) that provide them structural support and contextual information together with soluble factors. The model of Bissell et al. took the bidirectional cross talk between the ECM and the cell membrane (Bornstein et al. 1982), and extended it to the level of control of gene expression, by connecting ECM-ECM receptor interactions to the cytoskeleton and to the nuclear matrix and chromatin. Indeed Bissell and Hall argued that in the last analysis the organ (or indeed the organism) is the unit of function in all organisms (Bissell and Hall 1987). Cells respond then to cocktails of soluble and insoluble signaling molecules and, in turn, tune their microenvironment. It is the result of this harmonious combination that governs tissue dynamics and function.

The importance of 'tissue interaction' to formation of organs was first hypothesized by Pander in 1817 (Pander 1817). Over a century later, seminal work of early developmental biologists demonstrated that cells of distinct embryonic lineages engage in a highly organized cross talk that ensures proper cell sorting and directs tissue and organ morphogenesis and differentiation (reviewed by (Nelson and Bissell 2006)). This truism of organ development is made strikingly clear by tissue-recombination experiments, in which living pieces of tissue from various regions of the developing animal are combined and monitored. For example, combining mammary epithelium with mammary mesenchyme results in the development of a mam-

mary tree, but recombination with mesenchyme taken from the salivary gland generates structures reminiscent of the salivary epithelial tree (Kratochwil 1969; Sakakura et al. 1976). Conversely, mammary mesenchyme can induce epithelial cells from other tissues to build a lactation-competent gland (Cunha et al. 1995). The importance of reciprocal tissue interactions and the identification of the molecular mediators have now been demonstrated for several other organs, including the lung, prostate and kidney (reviewed in (Cardoso 2001; Marker et al. 2003; Yu et al. 2004)).

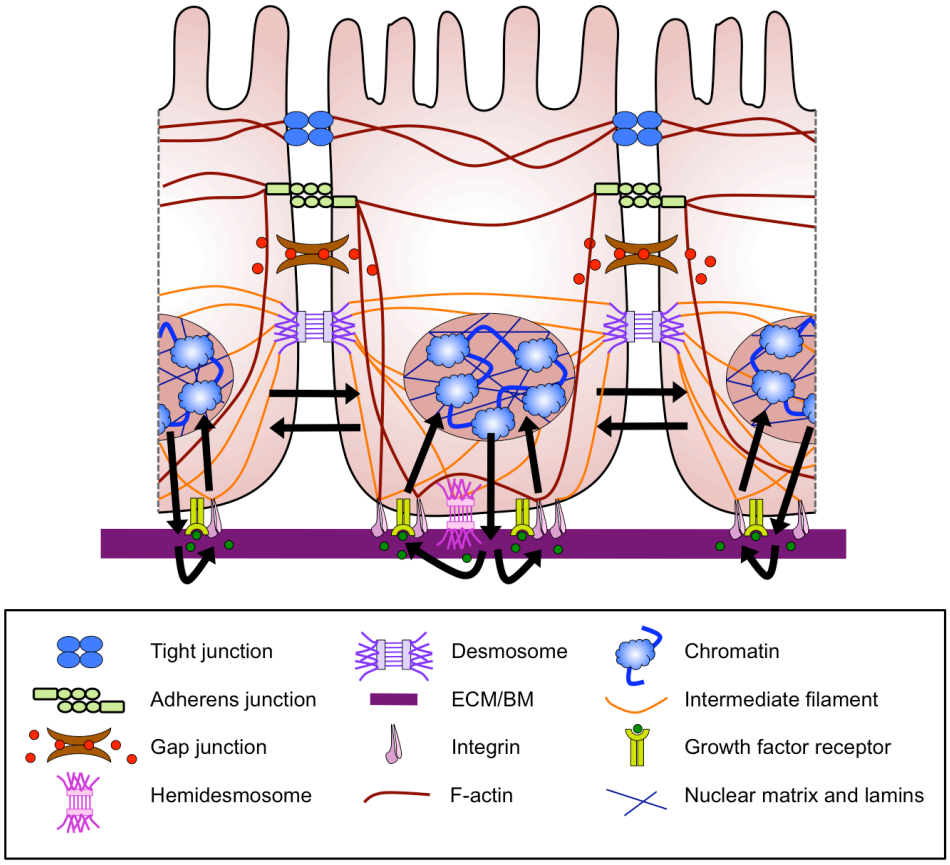


Figure 1.1 The model of 'dynamic reciprocity': the minimum required unit for tissue-specific functions.
Many mechanical and biochemical signals flow bidirectionally between the ECM and the nucleus (arrows). These signals are received at points of cell–cell or cell–ECM contact and are transduced to the nucleus, where they trigger the expression of specific gene products that are excreted back into the extracellular milieu. Integrins are connected to filamentous actin (F-actin), and sense elements of the extracellular matrix (ECM)/basement membrane (BM) to promote growth-factor activation. A combination of tight junctions, adherens junctions and gap junctions maintains the physical contact between neighboring cells. Additionally, desmosomes and hemidesmosomes serve as anchoring points for intermediate filaments and also provide signaling information.

Cell-microenvironment interactions are thus a major source of information regulating morphogenesis and differentiation. It is important to mention that phenotypic plasticity is implicit to this normal differentiation (Bissell 1981), as all living forms have the ability to adapt to changes in both the environment external to the organism and the internal microenvironment. The flexibility of differentiated cells is apparent during tissue remodeling and repair and to a remarkable degree in organisms, such as the newt, that can regenerate entire organs and limbs even in the adult animal. The fact that a differentiated cell can even react to signals that direct the development of a different tissue to express the latter's specific traits (Chiu and Blau 1984; Blau et al. 1985) should have dispelled the notion that the process of differentiation locks cells into a particular fate without recourse. There is now ample evidence that all cells retain the ability to morph from one cell type to another, and that they maintain a stable phenotype by integrating cues from the extra- and intra-cellular milieu. Dynamic reciprocity is thus scalable both in time and space and is a mechanism by which individual cells within tissues maintain the normal architecture and homeostasis in spite of an uncertain environment over the organism's lifetime.

1.1.2 Tissue Architecture and Normal Context as Overriding Tumor Suppressors

Much of the milestone research in demonstrating the importance of cell-microenvironment interactions did so by showing that *context* could override tumorigenicity; that is, tumor cells could be tricked into thinking they are normal if provided the right cues. The observation that the embryo comprises such a suppressive microenvironment is one that was first made over 100 years ago, when Askanazy showed that ovarian teratomas could form 'normal' tissues composed of the correct embryonic germinal layers when injected into embryos (Askanazy 1908). Decades later, a series of studies from different laboratories provided further evidence that the embryonic microenvironment could induce tumors to function normally in development. For example, using RSV (Rous sarcoma virus, encoding the oncogene v-*src*), Dolberg and Bissell showed that cells within injected chick

embryos expressed the virus, but that early embryos failed to form tumors (Dolberg and Bissell 1984; Howlett et al. 1988). Maintaining embryonic architecture was key, however, as dissociating the embryos and placing cells in culture resulted in rapid transformation (Stoker et al. 1990). The lasting impact of these studies is that tissue architecture is dominant to the powerful oncogene in embryos, thus overriding tumorigenicity of malignant cells. These studies offered also the clue that the malignant genotype could be suppressed if the interactions between a tumor and its microenvironment could somehow be normalized.

Evidence of the coexistence of normal and malignant cell populations within the same tissue, without resulting in a frank malignant tumor, has been reported also in human tissue specimens. Studies of large autopsy series have revealed that the majority of middle-aged and older people who die from causes other than cancer have frequent precancerous lesions throughout their bodies (Rich 1979; Harach et al. 1985; Nielsen et al. 1987; Folkman and Kalluri 2004). Analyses of 'normal' epithelial tissue adjacent to tumors have shown that similar patterns of mutations can be found in both, yet tumor growth is restrained by normal contextual cues (Deng et al. 1996; Washington et al. 2000). These and other related findings led Bissell and Hines recently to propose the microenvironment as the attenuator of both tumor onset and malignant progression, providing a rational framework to explain why the majority of people live cancer-free lives for decades, yet harboring a number of harmful mutations they accumulate over time (Bissell and Hines 2011). The phenotype is dominant over the genotype of even tumor cells; how else can one explain the occult tumors and dormancy of tumor cells that disseminate very early during tumor progression? Indeed, how else would one explain why people with familial *BRCA* or *APC* mutations have these in all their cells and yet they develop tumors only in a few of cells in specific organs?

1.1.3 Loss of Tissue Architecture and Abnormal Context as Tumor Promoters

If reciprocal communication between a normal context and ECM defines the normal tissue homeostasis, the opposite should also be true: abnormal context should lead to abnormal conversation allowing cells to disregard sorting rules and violate normal tissue boundaries, setting the stage for cancer progression. That this indeed is the case has long been obvious to pathologists, as judged by common reports of fibrotic tissue, ECM deposition, and immune and inflammatory infiltration, collectively called 'reactive' tumor stroma. As early as 1938, Orr observed that morphological changes in the microenvironment of the skin of carcinogen-treated mice appear long before neoplastic alterations in epithelial cells (Orr 1938). Subsequently, Tarin showed that complex sequential changes occur at the epithelial–mesenchymal boundary during mammary tumor progression (Tarin 1969).

Insights into the nature of the reciprocal tumor–stromal interactions that both precede and stimulate tumorigenesis have gradually accumulated. The presence of cancer-associated fibroblasts (CAFs) has been reported in many cancer types, and bidirectional CAF–epithelial interactions were shown to precede invasion and stimulate tumor growth and progression (Picard et al. 1986; Camps et al. 1990; Hayashi et al. 1990; Skobe and Fusenig 1998; Olumi et al. 1999; Cunha et al. 2003; Bhowmick et al. 2004). Concomitantly, cancer cells overproduce proteolytic enzymes, particularly matrix metalloproteinases (MMPs) (Chambers and Matrisian 1997), which generate fragments with pro-migratory and pro-angiogenic functions (Folkman and Kalluri 2002) as well as activate cell-surface and ECM-bound growth factors (Egeblad and Werb 2002), reflecting the extensive crosstalk between the microenvironment and the malignant cells. Recently, adipocytes have been recognized as important mediators of normal context disruption as well, since they produce a host of biologically active molecules that promote the inflammatory process and angiogenesis (Iyengar et al. 2005; Motrescu and Rio 2008; Cao et al. 2010; Dirat et al. 2011). Preference for metastatic colonization is heavily influenced also by communication between circulating tumor cells and bone marrow-derived cells

(BMDCs); these cells home to the tumor and promote its progression, malignant cell escape and survival, and ultimately metastatic growth (reviewed in (Joyce and Pollard 2009)). In addition to the plethora of cell types above described, cell-intrinsic and -extrinsic forces have also a significant effect on ECM alignment (Butcher et al. 2009), contributing thus to shape the ecological landscape that is the tumor microenvironment.

1.2 The Mammary Gland as an Experimental 'Organism'

The mammary gland is an excellent example of an organ, the development and differentiation of which require dynamic and reciprocal communication between cells and their microenvironment. It is one of the very few organs that develop mainly after birth, and it undergoes multiple rounds of growth, differentiation, apoptosis, regression and remodeling during the lifetime of the organism. As such the mammary gland is a versatile experimental model for studying how structure and function unite to bring about functional differentiation.

Our understanding of the mammary gland has been enriched by the use of the mouse as an experimental system. Despite the differences between the mouse mammary gland and the human breast (Cardiff and Wellings 1999), the acinar unit of function is largely similar in the two species, making it a suitable surrogate for understanding human breast function.

1.2.1 The Structure of the Mammary Gland

The mammary gland distinguishes mammals from all other animals with its unique architecture that allows the synthesis and secretion of milk for the nourishment of the newborn. The mammary epithelium develops into an elaborate network of bilayered ducts (Figure 1.2), which begin at the nipple, are branched throughout a heterogeneous stroma, and end at mammary acini — the functional units of the gland. The mature mammary duct consists of a central layer of luminal epithelial cells (LEPs) specialized for milk production and secretion into the ducts, and an outer layer of myoepithelial cells (MEPs) that provide the coordinated contractile forces necessary to eject secreted milk through the ducts towards the nipple (Forsyth and Neville 2009). It also harbors stem and progenitor cells, which are the source of both luminal and myoepithelial cells (reviewed in (Visvader

2009)). The epithelial ductal tree is ensheathed by BM (Williams and Daniel 1983) and embedded within a complex stroma, the mammary fat pad, which contains adipocytes, fibroblasts, immune cells, blood vessels and nerves, all of which are important for normal mammary development and function.

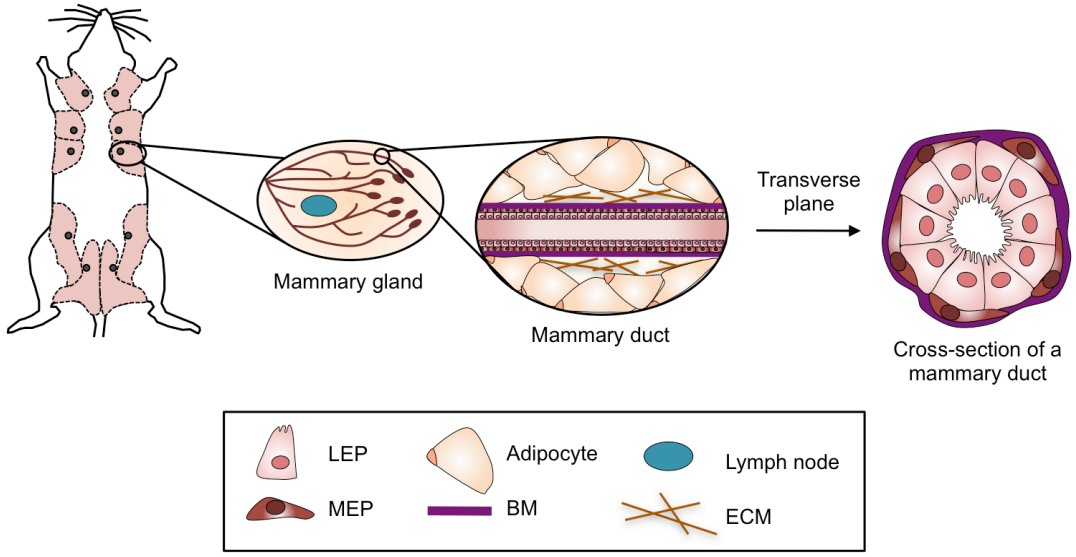


Figure 1.2 The bilayered tree-like structure of the mammary epithelium.
The mouse has five pairs of mammary glands that extend from the thoracic to the inguinal side. The mammary epithelium develops into an elaborate network of bilayered ducts (mammary gland inset), which begin at the nipple and are branched throughout a heterogeneous stroma. The mature mammary duct consists of an inner layer of milk-producing luminal epithelial cells (LEPs), surrounded by a layer of contractile myoepithelial cells (MEPs) and basement membrane (BM) (mammary duct inset and cross-section).

1.2.2 The Dynamic Journey of Mammary Morphogenesis

Mammary development occurs in three distinct and differentially regulated stages: embryonic, pubertal and adult. In mice, embryonic mammary development begins mid-gestation with the specification of two bi-lateral epidermal ridges, the milk lines, which run from forelimb to hindlimb on each side of the animal (Figure 1.3) (Cowin and Wysolmerski 2010). Five pairs of disk-shaped placodes then segregate along these lines at the site of each future nipple, and invaginate into the underlying mesenchyme to form the mammary buds, or anlagen (Watson and Khaled 2008). The buds then sprout and extend to form a rudimentary ductal structure, which in female

embryos lies quiescent until puberty, but degenerates in males due to activation of androgen receptors (Veltmaat et al. 2003).

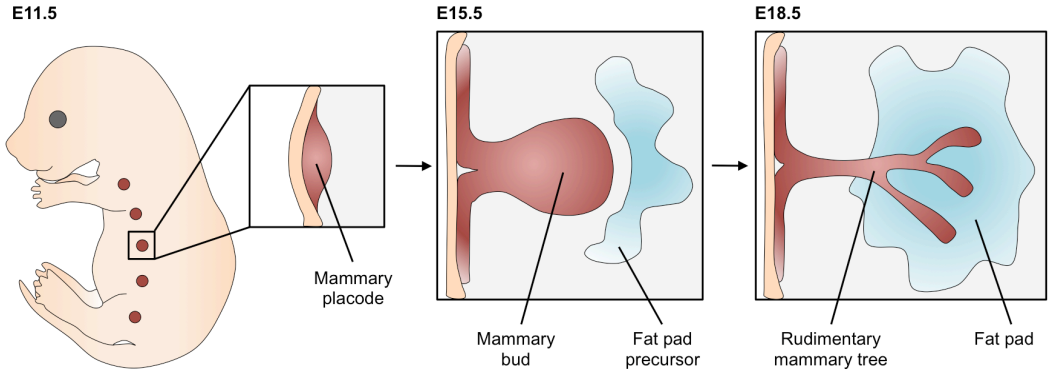


Figure 1.3 Overview of mouse embryonic mammary gland development.
In the mouse embryo, mammary development begins when five pairs of placodes form in the epithelium adjacent to the fat pad precursor (embryonic day 11.5 (E11.5)). Placodes sink into the underlying mesenchyme (grey) to become mammary buds (E15.5). These buds sprout and elongate to form a rudimentary mammary tree (E18.5), which remains morphogenetically quiescent until puberty.

Mammary gland development resumes postnatally, and the pubertal stage is perhaps the most remarkable of mammary morphogenesis (Figure 1.4). During this stage, the female mammal develops an elaborate epithelial tree through the process of branching morphogenesis. Prompted by elevated levels of hormones and growth factors, the ends of the rudimentary ducts proliferate and swell into distinct multilayered epithelial structures called terminal end buds (TEBs) (Hinck and Silberstein 2005). These ductal structures then undergo successive rounds of elongation and bifurcation to form adequately spaced primary ducts, whereas concomitant secondary side-branches sprout laterally from the trailing ducts (Wiseman et al. 2003). Presumably, distinct mechanisms control the timing of TEB branching and the periodicity of side branch eruption from pre-existing main ducts. Currently neither is understood, and it is possible that they occur stochastically. Interestingly, branching ducts never cross paths, most likely due to the secretion of inhibitory factors that act on neighboring ducts to influence their path of migration (Faulkin and Deome 1960; Silberstein and Daniel 1987). The epithelial tree ceases forward growth when it reaches the limit of the fat pad of the young adult female. Thereafter, short tertiary

side-branches form along the ducts in response to cycling ovarian hormones, further decorating the mature ductal tree.

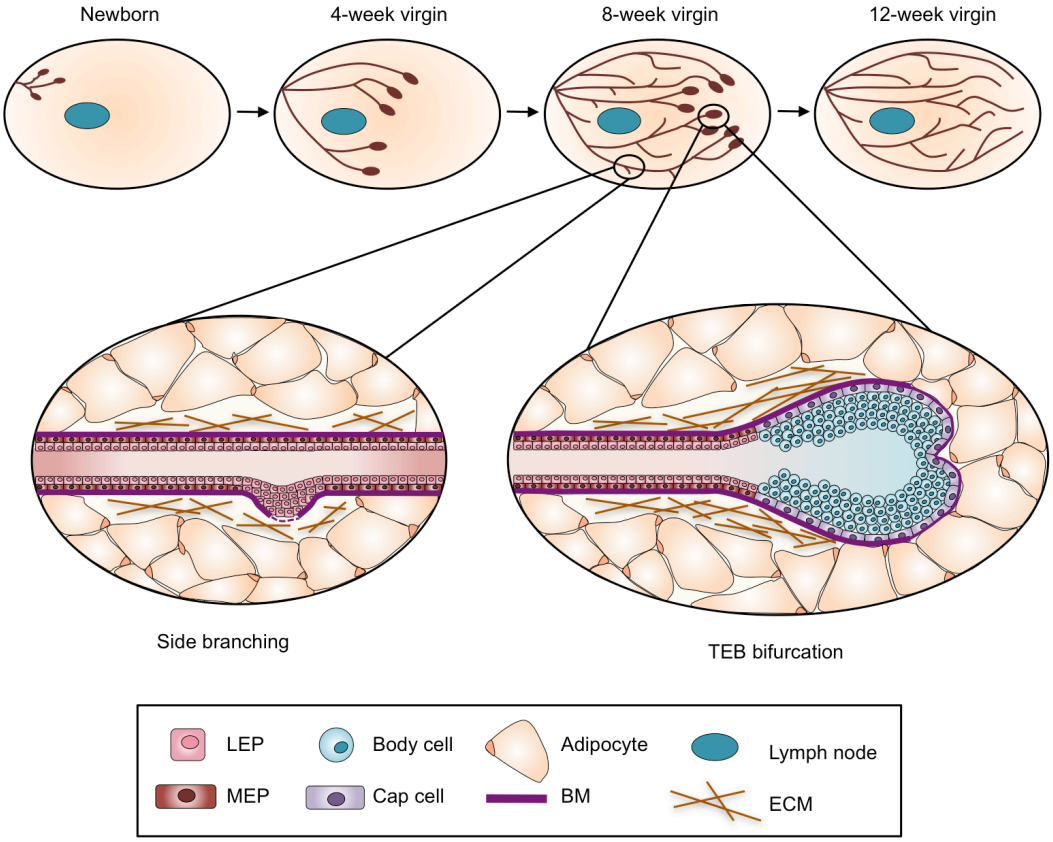


Figure 1.4 The pubertal mouse mammary gland branches through two distinct mechanisms: side branching and TEB bifurcation.
At birth, the mammary epithelium is rudimentary, consisting of only a few small ducts that grow allometrically until puberty (4 weeks in mice). With the onset of puberty, the female mammal develops an elaborate epithelial tree through two distinct processes of branching morphogenesis. Bifurcation of TEBs (lower right) occurs only from immature ducts. The branch point is formed through deposition of stroma at the cleft site, and the ducts extend directly into adipose tissue, without MEPs or stroma and with only a minimal BM at their invasive front. In contrast, in side branching (lower left), a new branch forms from a mature duct. The region where the bud is to form is first defined, and only then the emerging bud extrudes through and remodels a region containing layers of MEPs, BM and periductal stroma. The epithelial tree ceases forward growth when it reaches the limit of the fat pad of the young adult female (12 weeks in mice), and thereafter, short tertiary side branches form along the ducts in response to cycling ovarian hormones.

During pregnancy, the combined action of progesterone, prolactin and placental lactogen orchestrates remarkable changes in preparation for the important function of lactation (Figure 1.5). A massive increase in secondary and tertiary branching provides ductal arbors for alveolar development. Pro-

liferating epithelial cells give rise to alveolar buds that progressively differentiate into milk producing secretory alveoli (Brisken et al. 1999). The epithelial to adipocyte ratio increases, and each individual alveolus becomes surrounded by a basket like network of capillaries (Djonov et al. 2001). By late pregnancy, the alveoli have filled the majority of the fat pad and start showing secretory activity as pregnancy approaches term (Richert et al. 2000). The process of lactation continues for approximately three weeks until the pups are weaned.

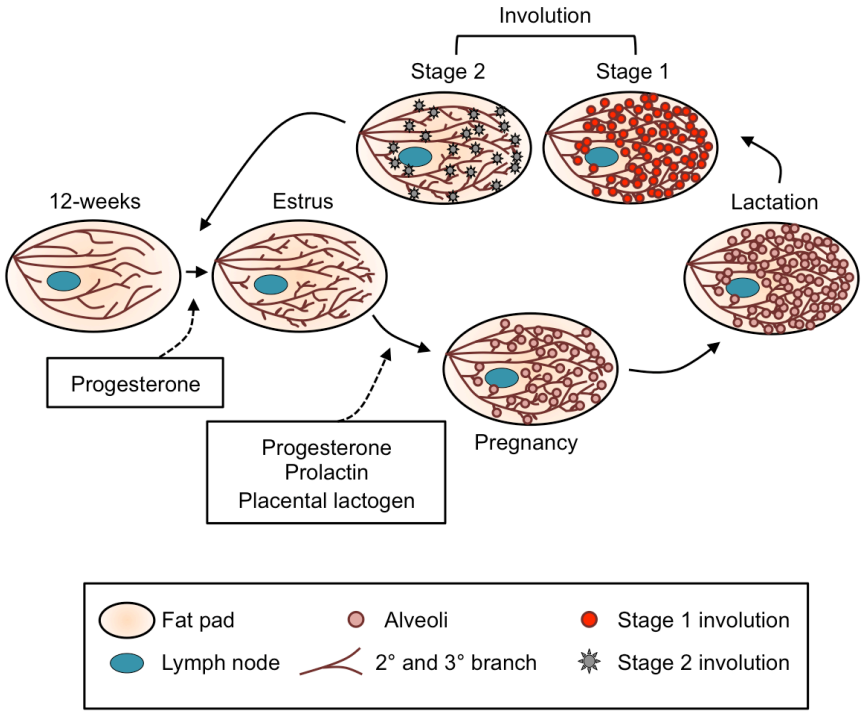


Figure 1.5 The mouse mammary gland experiences major changes to generate lactation competence during pregnancy.
During pregnancy, the combined action of progesterone, prolactin and placental lactogen orchestrates alveologenesis. Prolactin stimulation persists throughout the lactation stage, which culminates in milk production that continues until a lack of demand at weaning. The loss of suckling stimuli at weaning causes milk to stagnate in the mammary epithelium and triggers post lactational involution. This two-stage remodeling process restores a ductal structure somewhat similar to the pre gestation state.

The loss of suckling stimuli at weaning causes milk to stagnate in the mammary epithelium, initiating a remodeling program called post-lactational involution (Walker et al. 1989) (Figure 1.5). Two phases of involution have been described: the first is a potentially reversible period initiated by mechanical triggers associated with milk-stasis; and the second

is a programmed deconstruction of the alveoli and the supporting structural ECM (Lund et al. 1996). The latter phase is dominated by the involvement of extracellular proteases with concomitant reconstitution of the adipocyte compartment. The secretory alveoli collapse, eventually restoring a ductal structure somewhat similar to the pre-gestation state. The gland is then ready to initiate another cycle of pregnancy, lactation, and involution, and maintains this remarkable ability for several months throughout the female mouse lifespan.

1.2.3 The Mammary Remodeling Program

As discussed above, the mammary gland encounters constant physiological demands during the female lifespan. To maintain its unique function of lactation, the gland must repeatedly reacquire its fundamental architecture with the preservation of cell types, ratios, differentiation state, and matrix integrity. This requires a remodeling program that involves deconstruction and reassembly of multiple intricate structures throughout the gland. A plethora of molecular signals cooperate to execute the remodeling program through communication between epithelial and stromal cells. Systemic hormones induce mitogens to initiate growth, epithelial ductal branching and differentiation, and inhibitors to terminate ductal growth and balance proliferation with apoptosis. A suite of ECM modifiers, particularly MMPs, operates at the cell-microenvironment interface to tailor the structural support of the mammary tissue and rearrange cell-cell and cell-ECM adhesion. The overall tissue regenerative ability also requires concerted and reciprocal signaling between the epithelium and distinct cell types from the surrounding stroma.

1.2.3.1 Hormone-Induced Signaling

The process of mammary remodeling is set in motion by systemic hormones. The major sources of reproductive hormones in mature females include the ovaries, which secrete estrogens and progesterone, the anterior pituitary gland, which synthesizes growth hormone (GH) and prolactin, and

the adrenal glands, which release cortisol and precursors of sex steroids. Estrogen is known to play a critical role in stimulating mammary ductal elongation during mouse pubertal development. Exogenous estrogen administration can directly stimulate mammary ductal growth in female mice that have had their ovaries surgically removed (ovariectomized animals) (Daniel et al. 1987). Estrogens, however, are not sufficient, as they fail to rescue mammary branching in rodents that have been subjected to surgical removal of the pituitary gland (hypophysectomized animals) (Reece et al. 1936; Gardner and White 1941; Lewis et al. 1942). Branching is restored upon supplementation with GH (Lyons 1958; Nandi 1958) or insulin-like growth factor 1 (IGF1) (Kleinberg et al. 2000).

In contrast to estrogen, progesterone is redundant for pubertal ductal development, but instead is necessary for tertiary side branching and secretory differentiation of the alveolar compartment (Lydon et al. 1995; Brisken et al. 1998). Similarly, mice lacking either prolactin or its receptor (PRLR) show neither alveolar structures nor production of milk proteins (Ormandy et al. 1997; Brisken et al. 1999; Gallego et al. 2001). Prolactin and progesterone act partially by inducing the expression of receptor activator of nuclear factor kappa-B ligand (RANKL), and *Rankl*-null mice fail to undergo alveolar differentiation and therefore do not lactate (Fata et al. 2000).

A notable feature of the hormone-directed mammary remodeling program is the sequential action on the epithelium to orchestrate mammary gland development. During puberty, a burst of estrogen induces the expression of progesterone receptor and sets the stage for progesterone (Haslam and Shyamala 1979). Cyclic secretion of progesterone is established as the mouse attains sexual maturity, which coincides with the ducts reaching the limit of the fat pad. Progesterone and prolactin interact then in a number of ways to control alveolar development during pregnancy. This sequential action ensures that the distinct morphological steps occur in an orderly manner, so that all the ducts are established before alveoli bud, and they find adequate space to unfold and to be drained efficiently.

1.2.3.2 Epithelial Mitogens and Inhibitors

Systemic hormones stimulate a number of signaling pathways that serve specific functions in TEB proliferation, ductal elongation and side branching (Figure 1.6). For example, GR induces expression of insulin-like growth factor 1 (IGF1) in stromal cells (Gallego et al. 2001), which in turn signals to its receptor (IGF1R) in the epithelium to direct TEB formation (Kleinberg et al. 2000). Similarly, estrogen stimulates stromal cells to produce hepatocyte growth factor (HGF), which acts in a paracrine way to induce epithelial branching (Zhang et al. 2002). Estrogen also binds to estrogen receptor alpha (ERα) in the epithelium, thereby inducing the expression of STAT5A, which is required for lateral branching (Liu et al. 1997; Santos et al. 2010).

Several receptor tyrosine kinases have also profound effects on pubertal mammary development (Figure 1.6). Among the fibroblast growth factor receptor (FGFR) family members, FGFR2 is found to regulate proliferation of luminal epithelial cells and formation of TEBs (Lu et al. 2008; Parsa et al. 2008). Epidermal growth factor receptor (EGFR) signaling is also required for adequate branching morphogenesis, and of its seven ligands, amphiregulin (AREG) has emerged to be the most critical (Sternlicht et al. 2005). The EGFR family member ERBB2 has also been implicated in mammary morphogenesis (Jackson-Fisher et al. 2004; Andrechek et al. 2005), although its ligand and exact role remain elusive. Intriguingly, ERBB2 is required in the epithelium for normal ductal development, but the partner proteins that it normally dimerizes with, ERBB4 or EGFR, are required only in the stroma (Sebastian et al. 1998; Sternlicht et al. 2005). The compartmental localization and requirement of specific ligands and their receptors highlights the crucial importance of integrated paracrine signaling between the epithelium and stroma during pubertal development.

The involvement of many of these molecules in mammary gland remodeling has been recognized for nearly two decades. However, it has been difficult to uncouple the individual signals and receptors, given how many downstream effectors they share. For example, EGFR and FGFRs elicit at least part of their effects through mitogen-activated protein kinases (MAPKs), but they seem to yield distinct and even antagonistic phenotypic outcomes. The kinetic profile of MAPK activity may determine the final

morphogenetic response, at least of primary mammary organoids in culture; whereas sustained MAPK activation downstream of transforming growth factor alpha (TGFα) and EGFR induces branching, transient MAPK activation downstream of FGF7 and FGFR2 stimulates proliferation (Fata et al. 2007). This suggests that temporal responses may be used by the mammary epithelium to integrate and interpret distinct signals. However, the precise role of MAPKs in mammary morphogenesis *in vivo* is unclear. A combined loss of the MAPK inducers AREG, EGF and TGFα severely impairs branching morphogenesis in mice but has no discernable effect on proliferation, apoptosis or MAPK activation within the TEBs, which indicates that MAPKs may not be sufficient to promote morphogenesis (Luetteke et al. 1999).

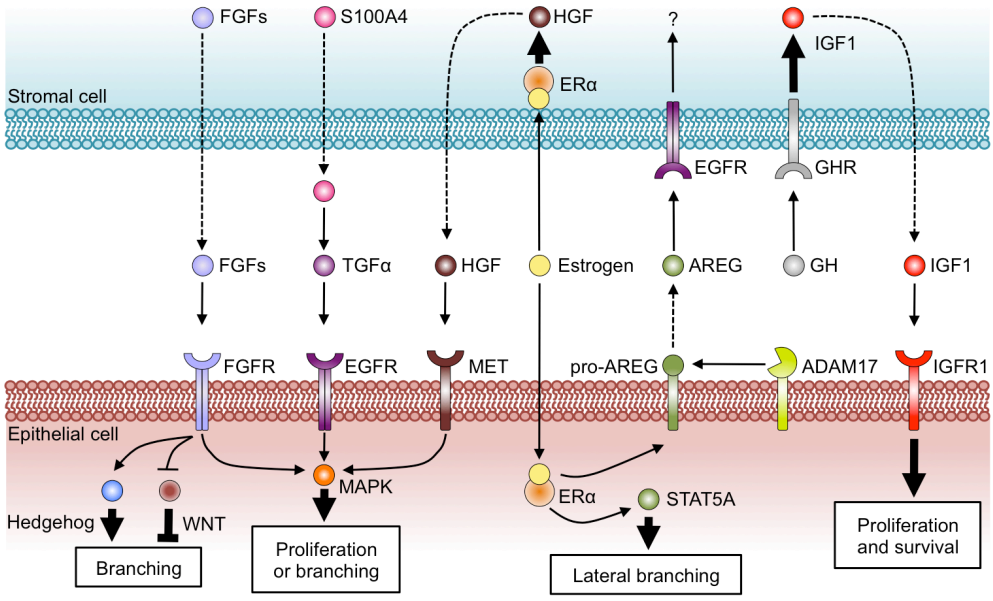


Figure 1.6 Multiple integrated signaling networks regulate mammary morphogenesis during puberty.
Global endocrine signals from the ovary (estrogen) and pituitary gland (GH) activate a plethora of paracrine signaling pathways to initiate mammary morphogenesis. Cellular crosstalk between the epithelial and stromal compartments is mediated by growth factors including IGF1, HGF, EGF and FGF, which bind to their cognate receptors to induce cell proliferation, survival and branching. Classic pathways, such as WNT and Hedgehog, are also emerging as indispensable regulators of the process.

Other classical signaling pathways have been also reported to play a role in pubertal mammary branching. Defects in branching morphogenesis found in mice genetically engineered in WNT ligands or their transcriptional tar-

gets point to the involvement of canonical WNT signaling in pubertal mammary development (reviewed in (Jarde and Dale 2012)). Factors that play important local functions in maintaining the TEB or ductal morphology include netrin-1 and its receptor neogenin (Srinivasan et al. 2003) and the cell surface morphogen epimorphin (Radisky et al. 2003). The trans-acting T-cell-specific transcription factor GATA-3 is also critical for pubertal mammary morphogenesis and maintenance of luminal differentiation in the adult mammary gland (Kouros-Mehr et al. 2006; Asselin-Labat et al. 2007).

In contrast to the above-discussed mitogens, the autocrine signaling morphogen transforming growth factor beta (TGFβ) negatively regulates mammary gland development. Studies in mice revealed that TGFβ overexpression leads to hypoplastic mammary development (Pierce et al. 1993), whereas its absence enhances ductal proliferation and accelerates lateral branching (Joseph et al. 1999; Crowley et al. 2005). Microfabrication-based culture models combined with computational approaches have also shown that the local concentration of TGFβ is determined by tissue geometry (Nelson et al. 2006). Accordingly, TGFβ gradients might specify sites of branch initiation and maintain proper ductal spacing *in vivo*, thus generating the characteristic open architecture of the gland. The precise mechanism by which TGFβ inhibits branching is still unclear. However, there is evidence that non-canonical WNT5A acts downstream of TGFβ *in vivo* (Roarty and Serra 2007) and downstream of SMADs in culture (Pavlovich et al. 2011), and *Wnt5a-null* glands phenocopy those of TGFβ-deficient animals (Roarty and Serra 2007). TGFβ and WNT5A may influence branching by modulating cell adhesion by activation of the collagen-binding protein DDR1 (discoidin domain receptor 1) (Vogel et al. 2001). TGFβ may also affect branching by inhibiting cell proliferation (Roarty and Serra 2007; Macias et al. 2011), enhancing ECM deposition (Silberstein and Daniel 1982; Daniel et al. 1996), and modulating MMP expression (Sternlicht and Werb 2001).

Besides TGFβ, other morphogens have been described as branching antagonists, including tissue inhibitors of metalloproteinases (TIMPs), heparan sulphate proteoglycans (HSPGs), IGF-binding proteins (IGFBPs) and SPROUTY family members (summarized in (Sternlicht et al. 2005)). Interest-

ingly, many of the mitogens and inhibitors here mentioned have similar functions in other branched organs, such as the lung, kidney, and salivary gland (Lu and Werb 2008).

1.2.3.3 Stromal ECM Modifiers

The mammary stroma remodels concurrently with the epithelial ductal tree. The collective activity of members of the metalloproteinase family is sufficient to degrade all proteins of the ECM network and clear paths for invasion of the growing ducts. Of the 23 MMPs (Jackson et al. 2010) and 12 proteolytically active ADAMs (Weber and Saftig 2012) that belong to this family, only a few have been studied with regard to mammary gland remodeling. Noteworthy is the finding that epithelial ADAM17 is required for mammary development and is the critical sheddase for the release of AREG (Sternlicht et al. 2005). Interestingly, the only endogenous inhibitor of ADAM17, TIMP3, is specifically downregulated in and around the TEBs (but not in trailing ducts) (Sternlicht et al. 2005). Site-specific activities of MMP2 and MMP3 contribute also to branching morphogenesis (Wiseman et al. 2003). Whereas *Mmp2*-null mice exhibit delayed mammary ductal invasion but excessive secondary branching, mice lacking MMP3 show defective side branching. On the other hand, mice overexpressing MMP3 or MMP14 yield supernumerary side branches, precocious alveologenesis, and eventually develop mammary tumors (Witty et al. 1995; Sternlicht et al. 1999; Ha et al. 2001). Although *Mmp9*-null mice have no differences in ductal length or branching (Wiseman et al. 2003), this protease may have a redundant inhibitory role in pubertal mammary development (Ucar et al. 2010). Glands lacking microRNA-212 and -132 show increased expression and accumulation of MMP9 around the ducts, and a corresponding decrease in collagen deposition within the periductal sheath and hyperactivation of latent TGFβ (Ucar et al. 2010).

The abundance and requirement of metalloproteinases is also evident at involutional stage as this involves copious restructure of the gland. Expression profiling studies have shown that MMP2 (Dickson and Warburton 1992; Talhouk et al. 1992), MMP3 (Dickson and Warburton 1992; Talhouk

et al. 1992; Clarkson et al. 2004), MMP11 (Lefebvre et al. 1992), MMP12 and ADAMTS1 (Clarkson et al. 2004) are induced at involution, with MMP3 showing the most dramatic increase (Lund et al. 1996). In addition to MMPs, there is evidence to support a role for the serine protease urokinase-type plasminogen activator (uPA) in mammary tissue remodeling, as mice lacking plasminogen show compromised lactation and involution (Lund et al. 2000; Green et al. 2006).

1.2.3.4 Stromal Cell Contribution

Sculpting of the epithelial tree requires integrated interactions among the epithelium and the cells that comprise the stroma. Recent advances in genetic manipulations have allowed the roles of individual stromal cell types to be dissected (Figure 1.7).

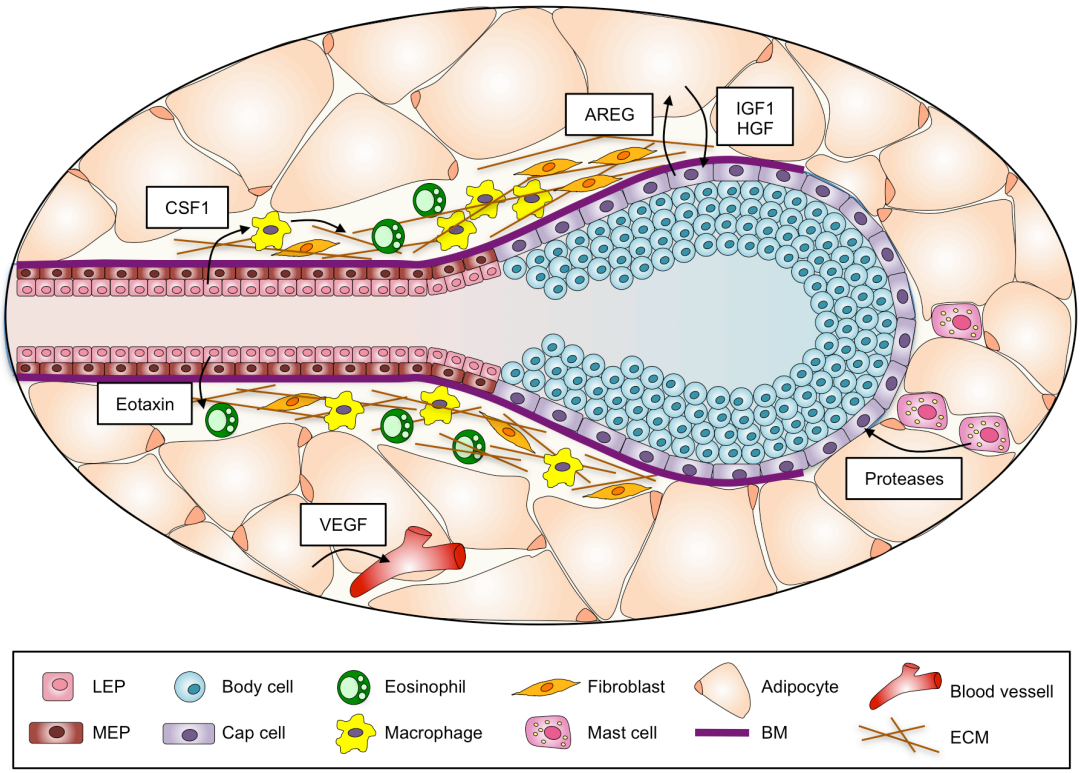


Figure 1.7 Interactions between distinct stromal cell types coordinate mammary morphogenesis.
Adipocytes form the largest component of the mammary stroma. In response to estrogen, adipocytes secrete IGF1 and HGF, which bind to their cognate receptors to induce cell proliferation, survival and branching of the epithelium; conversely, epithelial cells secrete AREG, which signals to the stromal compartment. Adipocytes also secrete adipokines and vascular

endothelial growth factor (VEGF), being the latter a potent inducer of angiogenesis. Macrophages and eosinophils are also recruited to the TEB, partly by signals released from the epithelium, such as macrophage colony-stimulating factor 1 (CSF1) and eotaxin. Additionally, macrophages enhance the formation of collagen fibers in the periductal sheath, providing a framework for ductal elongation. Mast cells localize to the stroma surrounding the invading TEB and secrete serine proteases, which are required for branching and for maintenance of the cap cell layer.

By volume, adipocytes form the largest population of cells within the fat pad. They express several critical stromal ligands and receptors, including ERα, IGF1 and HGF1, and can induce branching in culture (Pavlovich et al. 2010). Adipocytes are also required for branching *in vivo*, as their selective ablation during puberty blocks the formation and elongation of TEBs (Landskroner-Eiger et al. 2010), and mice lacking white adipose tissue show a rudimentary epithelial anlage (Couldrey et al. 2002). Moreover, pubertal branching is disrupted in obese mice (Kamikawa et al. 2009), which suggests either systemic or local effects from excess adipose tissue. In addition to signaling directly to the epithelium, adipocytes synthesize and secrete molecules that can modulate the function of other stromal cell types. For example, vascular endothelial growth factor (VEGF) is expressed by mammary adipocytes during puberty (Hovey et al. 2001). Since VEGF is a known inducer of vascular sprouting, adipocytes may therefore regulate angiogenesis during mammary branching. The presence of a robust vascular network in the fat pad becomes crucial for the transport of fluids and nutrients into milk during lactation.

Cells of the immune system have also been implicated in mammary gland remodeling. Macrophages and eosinophils are both recruited around the TEBs by macrophage colony-stimulating factor 1 (CSF1) (Van Nguyen and Pollard 2002) and eotaxin (Gouon-Evans et al. 2000) secreted locally, and the depletion of either cell type disrupts branching morphogenesis (Gouon-Evans et al. 2000). Macrophages assist the formation of long collagen fibers around the neck of the TEB (Ingman et al. 2006), providing a framework for ductal elongation. Mast cells also surround the TEBs during puberty, and induce branching by secreting serine proteases (Lilla and Werb 2010). Mice lacking mast cells or an activator of serine proteases develop hypoplastic glands (Lilla and Werb 2010), whereas accumulation of mast

cells increases collagen deposition around the growing ducts (Russell et al. 2007).

One major function of the stromal compartment is to maintain mammary stem cells (MaSCs). MaSCs are the basis of the profound renewal capacity required for branching and acinar morphogenesis, but different populations of stem cells may complete each function (Van Keymeulen et al. 2011). In mice, MaSCs located in the cap region of the TEB are responsible for the growth that drives ductal extension during branching (Shackleton et al. 2006; Stingl et al. 2006). The MaSC population is maintained by signals from its specialized local microenvironment, or niche. Protein microarrays have been used to define niche constituents, which include the BM component laminin-111, the cell-cell adhesion molecule P-cadherin and the Notch ligand jagged 1, all of which are present near MaSCs *in vivo* (LaBarge et al. 2009). Resident macrophages may be also an important constituent of the MaSC niche, as MaSCs of animals depleted of macrophages are unable to repopulate the gland (Gyorki et al. 2009). The niche is both necessary and sufficient for stem cell activity. Indeed, cells other than native MaSCs, such as neural stem cells (Booth et al. 2008) and cells from the seminiferous tubules (Boulanger et al. 2007), can function as MaSCs when placed within the niche. Further studies are required to define how the integrated signaling within the mammary gland induces maintenance and differentiation of MaSCs during pubertal branching.

1.2.4 3D Culture Models of Mammary Architecture, Function and Dysfunction

Many of the details of microenvironmental signaling in the mammary gland have been uncovered using three-dimensional (3D) culture models (reviewed in (Nelson and Bissell 2005)). The structure and function of a differentiated mammary epithelial cell can be reproduced in culture only when cells are given an appropriate microenvironment that recapitulates aspects of the above-described tissue architecture. When grown on plastic substrata, mammary epithelial cells flatten out and fail to respond to lactogenic cues. However, when grown within a compliant laminin-rich ECM (lrECM),

these same cells assemble into polarized 3D acinar structures that resemble alveoli *in vivo* (Emerman and Pitelka 1977; Lee et al. 1985; Barcellos-Hoff et al. 1989; Aggeler et al. 1991). Cells that are not in contact with BM undergo apoptosis (Boudreau et al. 1995), therefore cells in the center of 3D acinar structures die off to form hollow lumina (Blatchford et al. 1999; Debnath et al. 2002). Remarkably, when stimulated with lactogenic hormones, cultured acini start synthesizing and secreting milk proteins into the central lumina (Emerman and Pitelka 1977; Lee et al. 1985; Streuli et al. 1995).

In addition to illuminating the processes of acinus formation and milk protein secretion, 3D culture models have been highly successful in recapitulating the epithelial remodeling and invasion central to branching morphogenesis. Primary epithelial organoids or clustered mammary epithelial cells cultured within gels of collagen-I (Col-1) or lrECM can be induced to form branching structures by co-culture with fibroblasts or by exogenous addition of growth factors (Brinkmann et al. 1995; Soriano et al. 1995; Hirai et al. 1998; Simian et al. 2001) or cytokines (Lee et al. 2000; Michaelson et al. 2005).

Recreating the microenvironment in culture also allows one to distinguish between non-malignant and malignant cells on the basis of their structural integrity (Figure 1.8). Whereas non-malignant cells form polarized growth-arrested acini in 3D lrECM, primary breast tumor cells or breast cancer cell lines form highly disorganized and proliferative colonies (Petersen et al. 1992; Weaver et al. 1995). Under these conditions the balance of signaling pathways are deranged in tumor cells. Antagonizing one or more of the many signaling pathways that are deregulated in tumor cells causes them to functionally revert to a 'normal' phenotype, despite their malignant genome ((Howlett et al. 1995; Weaver et al. 1997; Wang et al. 1998; Weaver and Bissell 1999; Muschler et al. 2002; Wang et al. 2002; Kirshner et al. 2003; Liu et al. 2004; Kenny and Bissell 2007; Beliveau et al. 2010)). Interestingly, there is a reciprocal interaction between any oncogenic pathway and all the rest in 3D and the changes do not occur in 2D (Anders et al. 2003).

The same concepts were used to demonstrate that survival and sensitivity to drugs used in the clinic of human breast cells is dependent on cell and tissue polarity as well as integrin-mediated adhesion to BM, and do not correlate with the rate of growth or quiescence ((Weaver et al. 2002); reviewed in (Correia and Bissell 2012)). The 3D culture systems represent, therefore, the toolkit to more successfully translate fundamental research findings into therapies in the clinic, and may have great potential in providing answers before proceeding into costly clinical trials. So far, most 3D models available allow co-culture of epithelial cells only with one other cell type. Thus heterotypic culturing systems that more closely mimic the heterogeneity of the tumor microenvironment still need to be developed.

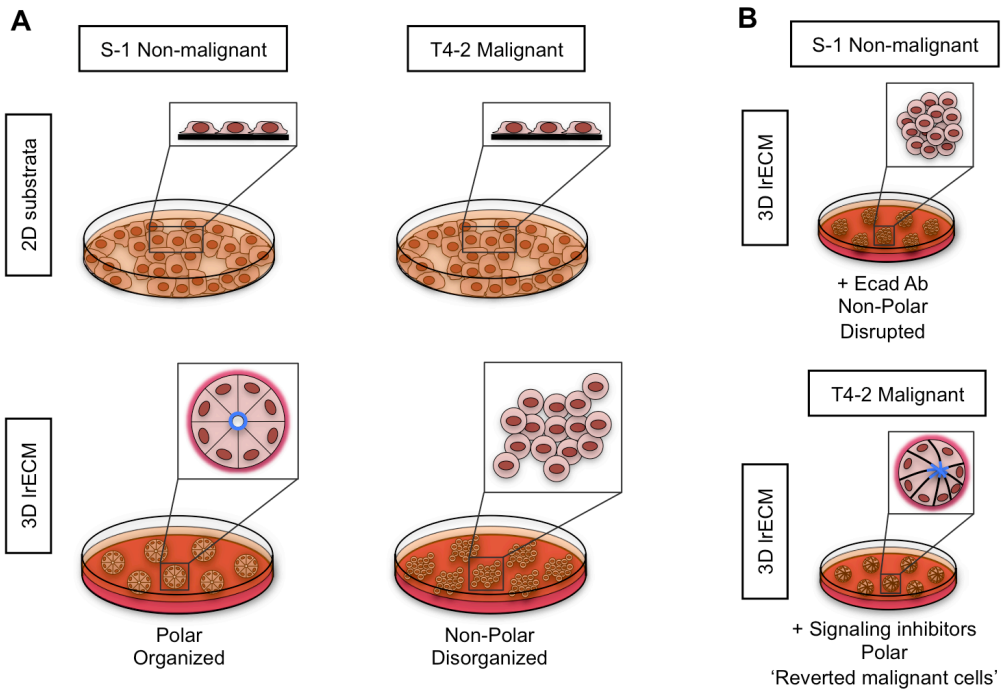


Figure 1.8 The 3D model of the normal mammary gland acinus allows the distinction between non-malignant and malignant cells.
When cultured on 2D substrata, non-malignant and malignant human breast cells are indistinguishable (**A**, upper panel). However, when placed in 3D lrECM gels, S-1 non-malignant cells form polarized growth-arrested acini (**A**, lower panel, left), whereas T4-2 malignant cells appear highly disorganized and proliferative (**A,** lower panel, right). Perturbing apical-basal polarity of S-1 acini, by treatment with E-cadherin function-blocking antibody, results in disruption of the normal organization (**B**, upper panel). Conversely, restoring cell and tissue polarity in T4-2 structures, by treatment with signaling inhibitors, induces malignant cells to functionally 'revert' (**B**, lower panel).

1.3 Matrix Metalloproteinase 3 (MMP3)

We now appreciate mammary branching morphogenesis as an ensemble performance. Epithelial cells engage in extensive crosstalk with the surrounding stroma to mobilize the necessary machinery for invasion of the growing ducts into the fat pad and formation of secondary and tertiary branches for the eventual design of the adult mammary architecture. The success of this process relies on pericellular proteolysis. MMPs are pivotal components of the cascade of locally activated proteolytic enzymes necessary for sculpting the epithelial tree.

Amongst the 23 MMP family members, MMP3 stands out as the architect of ductal side branching during mid-puberty and early pregnancy (Wiseman et al. 2003). MMP3 affects the selection of branch sites by itself, and in excess, can trigger branch formation from stem and progenitor cells that lie dormant along the ducts (Sympson et al. 1994; Thomasset et al. 1998). Remarkably, MMP3 can directly stimulate phenotypic and genotypic malignant transformation in normally functioning cells (Sternlicht et al. 1999; Radisky et al. 2005). This fact raised our interest in investigating the role of this molecule as an instructive switch for acquisition of invasive properties.

1.3.1 MMP Family Traits

The ability to degrade extracellular proteins is essential for any individual cell to interact properly with its immediate surroundings and for multicellular organisms to develop and function normally. This was obvious long before it was first shown that involuting tadpole tails contained a diffusible enzyme capable of degrading fibrillar collagen (Gross and Lapiere 1962). Since then, a family of related enzymes has been identified in species from hydra to humans and collectively called matrix metalloproteinases (MMPs).

Historically, MMPs were thought to function mainly as enzymes that degrade structural components of the ECM, creating space for cell and tissue movement. More recently, our understanding of MMP substrates and

the means by which MMPs affect cell behavior has dramatically expanded (Figure 1.9). MMPs can generate specific substrate-cleavage fragments with independent biological activity, can regulate tissue architecture through cleavage of intercellular junctions or BM, and can activate, deactivate or modify the activity of signaling molecules, thereby regulating cell behavior in several ways (Sternlicht and Werb 2001). MMPs are thus required for many physiologic and pathologic processes, including aspects of embryonic development, tissue morphogenesis, wound repair, inflammatory diseases, and cancer.

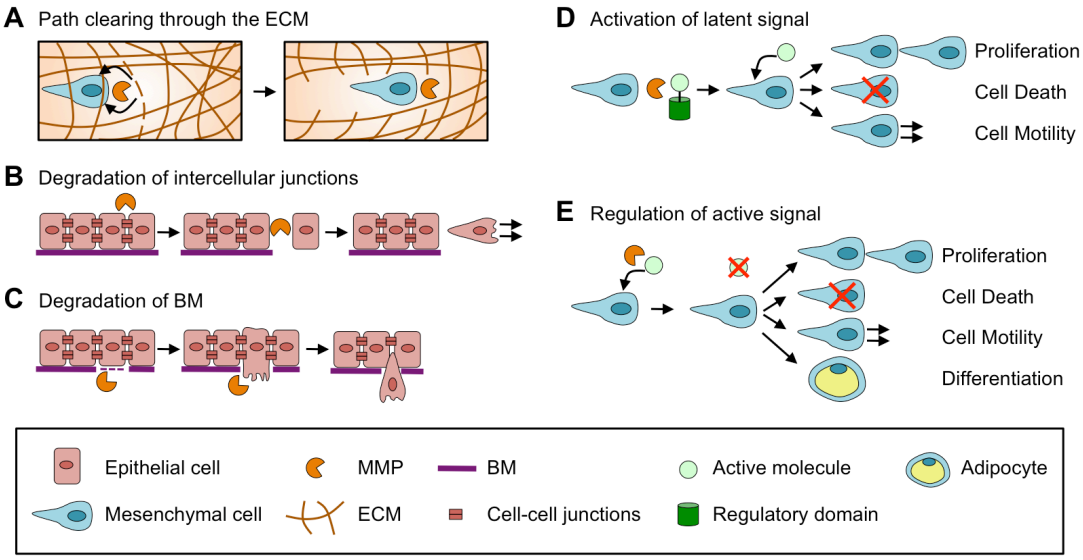


Figure 1.9 MMPs exhibit different modes of action.
MMPs can cleave ECM components, creating space for cell or tissue movement (**A**). Additionally, MMP proteolysis can generate specific cleavage products with independent biological activity. MMPs can also directly regulate epithelial tissue architecture through cleavage of intercellular junctions (**B**) or BM (**C**). MMPs can activate or modify the action of latent signaling molecules, which results in many cellular consequences (**D**). Conversely, MMPs can deactivate or modify the action of active signaling molecules, leading to changes in proliferation, cell death, differentiation or cell motility (**E**).

MMPs belong to the metzincin superfamily of proteases, which is distinguished by a highly conserved zinc-binding motif (**HE**xx**H**xx**G**xx**H**) and a conserved methionine-containing turn at the active site (Bode et al. 1993; Stocker et al. 1995). The 23 MMP family members are categorized by their modular domain structure (Figure 1.10).

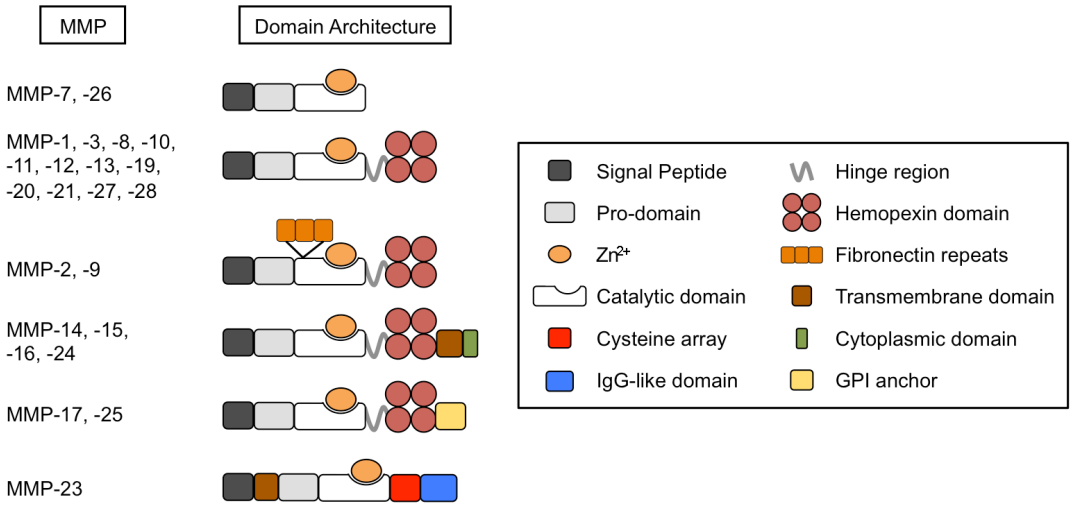


Figure 1.10 MMP domain structure.
All MMPs share a conserved minimal domain, which comprises a signal peptide, the pro-domain and the catalytic domain with a zinc-binding site. Additionally, most MMPs have a hemopexin domain and a hinge region. Besides their differential domain structure, MMPs can be principally divided into secreted (MMP-1, -2, -3, -7, -8, -9, -10, -11, -12, -13, -19, -20, -21, -22, -27, -28) and membrane-anchored proteinases (MMP-14, -15, -16, -17, -23, -24, -25), the latter of which use either a transmembrane domain with a cytoplasmic domain attached to it, a glycosylphosphatidylinositol (GPI) anchor, or an amino-terminal signal anchor, which is only the case for MMP23, as it is anchored in the plasma membrane. MMP23 contains also a unique cysteine array and an immunoglobulin-like (IgG) domain. MMP2 and MMP9 have three fibronectin type II repeats within their catalytic domains.

All MMPs share a conserved domain structure that consists of a catalytic domain featuring the zinc-binding site, and a pro-domain containing a cysteine residue that coordinates the active-site zinc to inhibit catalysis. When the pro-domain is destabilized or removed, the active site becomes available to cleave substrates (Rosenblum et al. 2007). In addition to the minimal domain, most MMPs contain a hemopexin domain, consisting of a four-bladed propeller structure that is linked to the catalytic domain via a flexible hinge region. The hemopexin domain is known to mediate protein-protein interactions, as well as contribute to proper substrate recognition and protease localization, internalization and degradation (Piccard et al. 2007). The hinge region, in turn, varies in length and composition and also influences substrate specificity (Knauper et al. 1997). Whereas MMP7 and MMP26 merely lack these extra domains, MMP23 contains a unique cysteine array and an immunoglobulin-like domain instead (Gururajan et al. 1998). MMP2 and MMP9 are further distinguished by the insertion of three

head-to-tail cysteine-rich repeats within their catalytic domain, which resemble the collagen-binding type II motif of fibronectin and are required to bind and cleave collagen and elastin (Murphy et al. 1994). Finally, the membrane-type (MT) MMPs have either a transmembrane domain with a short cytoplasmic tail attached to it (MMP-14, -15, -16 and -24) or a glycosylphosphatidylinositol (GPI) anchor (MMP17 and MMP25). These domains play a critical role in placing several important proteolytic events at specific regions of the cell surface.

1.3.2 Regulation of MMP Activity

The ability of MMPs to alter cell fate and developmental outcomes implies the need for higher levels of control. Yet, it took nearly a decade from the time collagenolytic activities were first demonstrated to realize that MMPs are synthesized as inactive zymogens that require activation (Harper et al. 1971), and even longer to demonstrate the existence of the first endogenous tissue inhibitor of metalloproteinases (TIMP) (Bauer et al. 1975). Since then, other levels of MMP regulation have been elucidated.

Because MMP substrate specificities tend to overlap, the biological function of individual MMPs is largely dictated by their differential patterns of expression. Most MMPs are tightly regulated at the level of transcription by many stimulatory and suppressive factors that influence multiple signaling pathways (reviewed in (Yan and Boyd 2007)). Importantly, different types of cells produce a specific set of MMPs and TIMPs, and their local balance defines MMP function *in vivo*.

A critical step in regulating MMP activity is the conversion of the zymogen into an active proteolytic enzyme. Although most MMPs are secreted as latent zymogens, MMP11, MMP27 and the MT-MMPs contain a furin-like enzyme recognition motif that allows them to be activated by intracellular serine proteinases before they reach the cell surface or are secreted (Pei and Weiss 1995). MMP23 also has a furin-susceptible cleavage site and is a likely target of intracellular proprotein convertases (Gururajan et al. 1998). All other MMPs lack a furin-susceptible insert and are thus activated outside

the cell by other already activated MMPs or by several serine proteinases (Sternlicht and Werb 2001).

The localization or compartmentalization of MMPs under physiological conditions often dictates their biological function. Several MMPs interact with surface receptors such as integrins or localize to specific areas of the ECM, which potentiates MMP activity by increasing their local concentration and limiting the access of TIMPs. Transmembrane and GPI-linked MT-MMPs are the most obvious mediators of proteolytic activity at the cell surface. Indeed, removal of the transmembrane domains of MMP14, MMP15 and MMP16 abolishes their ability to promote invasion (Hotary et al. 2000). Furthermore, MT-MMPs can concentrate within specific cell surface domains such as cellular protrusions named invadopodia, where active ECM degradation takes place (Nakahara et al. 1997). Another means of localizing MMPs to the cell surface is via cell surface docking receptors. For example, the binding of MMP2 to integrin-$\alpha v\beta 3$ via its hemopexin domain is crucial for endothelial and cancer cell invasive activity (Brooks et al. 1996). In summary, there are localized pericellular feedback networks that coordinate the need for a given MMP with its appropriate expression, activation and physical placement.

1.3.3 MMPs in Development and Disease

Our understanding of the role of MMPs in the regulation of tissue remodeling has been enriched by the use of genetic knockouts. MMP function can be most simply analyzed in *Drosophila*, which has only two MMP genes, *Mmp1* and *Mmp2*, each containing the conserved domain structure typical of mammalian MMPs (Llano et al. 2000; Llano et al. 2002). The fly mutants demonstrate that MMPs are dispensable, both individually and together, for embryonic fly development, but are crucial for tissue growth and tissue-ECM remodeling in the larvae and during larval development. In mammals, the 23 MMP genes seem to share redundant functions, most likely to protect against any losses of regulatory control. Analysis of single-MMP-mutant mice has identified subtle developmental phenotypes in postnatal mammary, skeletal and circulatory development, three prominent

sites of postnatal tissue and ECM remodeling (reviewed in (Page-McCaw et al. 2007)). Strikingly, MMPs are not required to build blood vessels or bones in the embryo, but rather for their postnatal development and tissue remodeling. This seeming dispensability of MMPs during embryonic development suggests that they might function specifically as regulators of post-embryonic cell motility and tissue architecture.

The role of MMPs is not limited to developmental processes. Examination of mouse mutants has revealed that MMPs are necessary to maintain homeostasis in response to environmental challenges, such as wounding, infection and inflammation. For example, MMP7 functions in intestinal mucosal defense, and *Mmp7*-null mice are more easily infected with intestinal bacteria (Wilson et al. 1999). Additionally, MMP7 seems to mediate wound-induced epithelial migration by cleaving E-cadherin and loosening cell-cell contacts (McGuire et al. 2003). MMP3 also functions in epidermal wound healing, as skin wounds of *Mmp3*-null mice heal more slowly than those of control mice, owing to a defect in 'actin purse-string' formation (Bullard et al. 1999). Both injury and infection induce inflammation, which constitutes another physiological response to environmental challenge that requires MMPs. MMPs facilitate inflammatory cell recruitment (Haro et al. 2000) and clearance of inflammatory cells (Kumagai et al. 1999; Wang et al. 1999) by cleaving inflammatory mediators, resulting in a tightly inflammatory response.

Interest in MMPs increased in the late 1960s and early 1970s following observations that MMPs are upregulated in diverse human diseases, including cancer. Significant positive correlations have been found between MMP expression and several indicators of poor prognosis in virtually all types of cancer, and in some instances, increased MMP levels represent an independent predictor of shortened disease-free and overall survival (reviewed in (Egeblad and Werb 2002)). However, there are a few cases in which increased expression of specific MMPs reflects a favorable prognosis. In colon cancer, for example, MMP12 expression by carcinoma cells is associated with increased survival (Yang et al. 2001), and expression of MMP9 by infiltrating macrophages is associated with reduced metastases (Takeha et al. 1997). Likewise, MMP12 delivered by macrophages can suppress the

growth of lung metastases (Houghton et al. 2006), and loss of the inflammation-suppressing function of MMP8 accounts for increased incidence of skin cancer in *Mmp8*-null mice (Balbin et al. 2003) and melanoma in humans (Palavalli et al. 2009). Thus, the many aspects of MMP function in cancer must be understood in the design of therapeutic agents in order to optimize their efficacy and minimize their toxicity.

1.3.4 MMP3 as an Architect of the Mammary Gland

Combined pharmacological and genetic analyses have revealed that two distinct MMPs (MMP2 and MMP3) function in the mammary epithelial microenvironment in a network of interacting pathways designed to give the gland its final branching pattern. Whereas *Mmp2*-null mice exhibit delayed TEB invasion during early puberty and increased lateral branching during late puberty, *Mmp3*-null mammary glands show normal TEB elongation, but are characterized by deficient secondary branching (Wiseman et al. 2003). MMP3 is, thus, the architect of secondary and tertiary lateral branching during mid-puberty and early pregnancy. Owing to its ability to cleave BM components, including laminin-111, nidogen and collagen-IV (Alexander et al. 1996), MMP3 clears paths for invading ducts at bud initiation sites (Wiseman et al. 2003). Additionally, MMP3 can cleave the ectodomain of the cell-cell adhesion molecule E-cadherin, thus inducing loose cell-cell contacts and epithelial invasion (Lochter et al. 1997a; Noe et al. 2001).

Paradoxically, the loss of mammary structure is dependent also on MMP3. Indeed, our laboratory showed two decades ago that during the process of involution, up regulation of MMP3 is responsible for the collapse and remodeling of the alveoli of lactating mice, indicating the intimate connection between functional differentiation and tissue structure (Talhouk et al. 1991; Talhouk et al. 1992). Conditional activation of MMP3 in functionally normal mouse mammary epithelial cells led to cleavage of E-cadherin and epithelial-to-mesenchymal transitions (EMT) (Lochter et al. 1997a). Our laboratory showed also that ectopic expression of constitutively active MMP3 in mammary epithelia enhanced lateral branching and induced precocious alveolar development in virgin mice (Sympson et al. 1994). As these

animals aged, the stroma was profoundly altered in both structure and function (Thomasset et al. 1998) and mice eventually developed mammary tumors that exhibited chromosomal aberrations (Sternlicht et al. 1999). The mechanism involved a change in cytoskeleton and cell shape through induction of RAC1B, a spliced isoform of RAC1 found in human breast tumors (Schnelzer et al. 2000). Additon of MMP3 or the expression of RAC1B also led to formation of reactive oxygen species (ROS) and genomic instability (Radisky et al. 2005).

MMP3 seems to act as an instructive switch for normal mammary epithelial invasion. Therefore, a comprehensive understanding of the role played by MMP3 in this model will shed light on how mammary gland remains functional and disease-free, but also how it may become cancerous.

Aims

It is now clear that MMPs are crucial mediators in sculpting tissue architecture, and are required for many physiological and pathological processes. Because the proteolytic activity of MMPs resides within the catalytic domain, it has been generally assumed that this domain is responsible for all the functions of MMPs. More recently some biochemical literature has indicated that the non-catalytic domains of certain MMPs, such as MMP-9, -12 and -14, may also have activities in mammalian cell lines (Mori et al. 2002; Wang et al. 2004; Dufour et al. 2008; Sakamoto and Seiki 2009). The failure of clinical trials based on inhibitors of MMP catalytic domains (Overall and Kleifeld 2006) suggested to us that the other domains of MMP3 might have crucial functions in invasion and possibly cancer.

General Aim:

The general aim of this thesis was to understand the role of MMP3 as an instructive switch for normal mammary epithelial invasion.

Specific Aims:

To achieve our goal, the following specific aims were addressed:

1. To examine activities associated with other domains of MMP3 that may be relevant for normal mammary epithelial invasion;

2. To identify novel MMP3 interacting proteins required for MMP3-induced invasion in mammary epithelial cells;

3. To validate the functional significance of distinct MMP3 domains and binding partners in a 3D culture model of mammary epithelial invasion and branching;

4. To study the expression of the identified MMP3 binding partners in different stages of normal mammary gland development.

Chapter 2

Material and Methods

2.1 Restriction Enzymes, Antibodies, Proteins and Chemical Reagents

All restriction enzymes were acquired from New England BioLabs. Bovine dermis acid-solubilized Col-1 solution (IAC-50) was purchased from Koken. Antibodies were obtained as indicated in Table 2-1. Alexa Fluor 594 Phalloidin (A12381, Molecular Probes, 1:400) was used to stain F-actin. DAPI (Sigma) was used to stain nuclei. HSP90β inhibitor CCT018159 (385920), MMP3-specific peptide-based inhibitor (444218) and recombinant HSP90β (385903) were purchased from Calbiochem/EMD Millipore.

Table 2-1 Antibodies used in the immunofluorescence (IF), western bloting (WB), co-immunoprecipitation (Co-IP) and function-blocking experiments (FBlock).

Antibody	Clone, Catalogue #	Supplier	Dilution
ANXA2	AF3928, Polyclonal	R&D Systems	1:1,000 for WB
E-cadherin	13-1900, ECCD-2	Invitrogen	1:1,000 for WB
			1:200 for IF
FLAG	F1804, M2	Sigma	1:500 for WB
HSP90β	5087, Polyclonal	Cell Signaling	1:1,000 for WB

Antibody	Clone, Catalogue #	Supplier	Dilution
HSP90β	NBP1-61773, Polyclonal	Novus Biologicals	1:1,000 for WB 1:100 for IF 10 μg for Co-IP 40 μg/mL for FBlock
HSP90α	NBP1-77685, Polyclonal	Novus Biologicals	1:1,000 for WB
HSP70	610607, 7/Hsp70	BD Transduction Laboratories	1:1,000 for WB
Normal Rabbit IgG	2729, Polyclonal	Cell Signaling	40 μg/mL for FBlock
MARCKS	P0370, Polyclonal	Sigma	1:1,000 for WB
MMP3	ab18898, Polyclonal	Abcam	1:1,000 for WB 1:50 for IF
α-SMA-Cy3	C6198, 1A4	Sigma	1:300 for IF
α-Tubulin	T6074, clone B-5-1-2	Sigma	1:5,000 for WB

2.2 Construction of Expression Plasmids

All MMP3 mutants were constructed using a polymerase chain reaction (PCR)-based method. The cDNA sequence used as template was cloned from a human breast cell line and sequence confirmed by comparison with gene accession number NM_002422.3. FL contains the full-length MMP3 cDNA. EA is a catalytically inactive mutant, holding a point mutation E219A at the catalytic core. dPEX is a hemopexin domain-deleted mutant (ΔN289-C477). To generate FL and dPEX constructs, PCR fragments flanked by *EcoRI/BamHI* restriction enzyme digest sites at the 5' and 3' ends, respectively, were obtained using the same sense primer (5'-CGTTACGAATTCATGAAGAGTCTTCCAATCCTACTG-3') and different an-

tisense primers: for FL: 5'-CGAATCGGATCCCTTGTCATCGTCGTCCTTGTAG TCACAATAAGCCAG-3'; and for dPEX 5'-CCTGCAGGATCCCTTGTCATCGT CGTCCTTGTAGTCGTTGGCTGGCGTC-3'. To create the EA construct, two PCR-fragments were first generated using two different primer sets: PCR1, sense primer: 5'-CGTTACGAATTCATGAGAGTCTTCCAATCCTACTG-3'; PCR1, antisense primer: 5'-AGCAACAAGAAATAAATTGGTCCCTGTTG-3'; PCR2, sense primer: 5'-GTTGCTGCTCATGCCATTGGCCACTCCCTG-3'; and PCR2, antisense primer: 5'-CGAATCGGATCCCTTGTCATCGTCGTCCTTGTAGTCCAA TTAACCG-3'. These fragments were then linked together (using 5'-CGTTACGAATTCATGAAGAGTCTTCCAATCCTACTG-3' and 5'-CGAATCGGA TCCCTTGTCATCGTCGTCCTTGTAGTCACAATTAAGCCAG-3' as sense and antisense primers, respectively), generating the final PCR-fragment encompassing the point mutation E219A. After *EcoRI/BamHI* digestion, FL, dPEX and EA products were ligated into *EcoRI/BamHI* digested pCDH-EF1-MCS-T2A-copGFP (System Biosciences), a mammalian expression vector that was used to express the gene products. To detect MMP3 protein, the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was inserted at the C-terminus of every construct generated. All cDNA constructs were confirmed by DNA sequencing.

2.3 shRNA-Mediated Knockdowns

shRNA constructs selectively targeting HSP90β, ANXA2, MARCKS or MMP3 were purchased from MISSION shRNA library (Sigma) (sequences detailed in Table 2-2). Control cells were infected with non-targeting shRNA (SHC002, Sigma, sequences detailed in Table 2-2). Knockdown efficiency was verified by western blotting with appropriate antibodies.

Table 2-2 Detailed sequences of distinct shRNAs used in the experiments.

shRNA	Sequence (5' – 3')
HSP90β #1	CCGGCAGGAGGAGTATGGCGAATTCTCGAGAATTCGCCATACTCCTCCTG-CTTTTTG
HSP90β #2	CCGGCATGGAAGAGGTGGATTAAAGCTCGAGCTTTAATCCACCTCTTCCAT-GTTTTTG
HSP90β #3	CCGGGCTGAACAAGACAAAGCCTATCTCGAGATAGGCTTTGTCTTGTTCAG-CTTTTT
ANXA2 #1	CCGGGTATGATGCTTCGGAACTAAACTCGAGTTTAGTTCCGAAGCAT-CATACTTTTTG
ANXA2 #2	CCGGGAGCATCAAGAAAGAGGTCAACTCGAGTTGACCTCTTTCTTGATGC-TCTTTTTG
ANXA2 #3	CCGGCGAGACAAGGTCCTGATTAGACTCGAGTCTAATCAGGACCTTGTC-TCGTTTTTG
MARCKS #1	CCGGCTTCTCCTTCAAGAAGAGCAACTCGAGTTGCTCTTCTTGAAGGA-GAAGTTTTTG
MARCKS #2	CCGGGCCAAGATAATATGCCACTAACTCGAGTTAGTGGCATATTATCTTGG-CTTTTTG
MARCKS #3	CCGGCTCCTCCACGTCGTCGCCCAACTCGAGTTGGGCGACGACGTGGAG-GAGTTTTTG
MMP3 #1	CCGGCAAGATGATGTAGATGGTATTCTCGAGAATACCATCTACATCATCTT-GTTTTTG
MMP3 #2	CCGGCCCACATATTGAAGAGCAATACTCGAGTATTGCTCTTCAATATGTGG-GTTTTTG
Non-targeting	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTT-GTTTTT

2.4 Lentiviral Production and Concentration

293FT packaging cells (Invitrogen) were transfected with plasmids carrying FL, dPEX, EA, control vector or shRNA constructs using FuGENE6 (Roche), according to the manufacturer's instructions. Transfected cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/mL streptomycin, 0.1 mM MEM Non-Essential Amino Acids, 6 mM L-glutamine and 1 mM MEM Sodium for 24 h, after which the medium was replaced with fresh one. Viral supernatant was collected 48 h later, filtered with 0.45 μm filters, concentrated using Lenti-X Concentrator (Clontech), aliquoted and stored at -80 ºC until use.

2.5 Cell Culture and Transduction

SCp2 cells were cultured in DMEM/Ham's F-12 Nutrient Mixture (DMEM/F-12) supplemented with 5% FBS, 5 μg/mL insulin and 50 μg/mL gentamicin, and maintained as previously described (Desprez et al. 1993). EpH4 cells were cultured in DMEM/F-12 medium supplemented with 2% FBS, 5 μg/mL insulin and 50 μg/mL gentamicin, and maintained as previously described (Reichmann et al. 1989). For transduction, cells were seeded in 24-well plates ($1x10^5$ cells/well) and infected with lentiviral particles carrying different expression plasmids using MISSION ExpressMag Beads (Sigma), according to the manufacturer's instructions. Cells transduced with lentivirus carrying shRNA constructs were additionally selected with 2 μg/mL puromycin.

2.6 Mice

For preparation of primary mammary epithelial organoids, FVB female mice were raised until 8 weeks of age and then sacrificed. For the *in vivo* developmental studies, tissue specimens were collected from FVB female mice at different post-natal developmental stages: virgin (3-, 4-, 5-, 8-, 12-weeks),

mid-pregnant (8-12 days gestation), late-pregnant (16-19 days gestation), lactating, and involuting (1 and 3 days after weaning). Experimental animal protocols were followed in accordance with guidelines set by the Lawrence Berkeley National Laboratory's Animal Welfare and Research Committee (AWRC).

2.7 Preparation of Primary Mammary Organoids and Transduction

Primary epithelial organoids were isolated from 8-week-old, virgin FVB mice as previously described (Fata et al. 2007). Briefly, inguinal glands were removed, minced with two parallel razor blades and gently shaken for 30 min at 37 ºC in a 50 mL collagenase/trypsin mixture (0.2% trypsin, 0.2% type-IV collagenase, 5% FBS and 5 μg/mL Insulin in DMEM/F-12). After centrifugation at 80 g for 10 min, supernant was discarded and cell pellet was re-suspended in DMEM/F-12. The suspension was pelleted again, re-suspended in 4 mL DMEM/F-12 containing 80 U of DNase I (Sigma) and incubated for 5 min at room temperature with occasional shaking. After the suspension was spun at 80 g for 10 min, a series of differential centrifugations in DMEM/F-12 was implemented to separate the epithelial organoids from single cells, fibroblasts and fibrillar extracellular matrices. The final pellet was re-suspended in the desired amount of medium. For transduction, organoids were seeded in 24-well polyhema-coated plates (1,000 organoids/well) and infected with lentivirus in the presence of 8 μg/mL polybrene for 24 h.

2.8 Preparation of Cell Clusters and Transduction

EpH4 cells suspended in growth medium were plated in 6-well polyhema-coated plates ($1x10^5$ cells/well) and incubated at 37 ºC overnight, yielding rounded clusters. Single cells were removed by differential centrifugation, and the final pellet was re-suspended in the desired amount of medium.

2.9 Branching Morphogenesis Assay

Primary organoids or clustered EpH4 cells were embedded in 3D Col-1 gels as previously published (Simian et al. 2001; Mori et al. 2013). In brief, acid-solubilized Col-1 solution was mixed gently on ice with 1 volume of 10x DMEM/F-12, pH adjusted to 7.4 with 0.1M NaOH, and concentration adjusted to 3 mg/mL with DMEM/F-12. A basal layer of 80 μL Col-1 was poured into each well of an 8-well chambered coverglass (155409, Thermo Scientific) and allowed to gel for 5 min at 37 ºC. A second layer of 200 μL Col-1 containing 150 organoids or EpH4 clusters was added to each well and placed immediately at 37 ºC. After gelation, 400μL of chemically defined medium (DMEM/F-12 containing 1% insulin/transferrin/selenium and 1% penicilin/streptomycin) with 9 nM TGFα (Sigma) or 9 nM bFGF (Sigma) was added to each well (unless stated otherwise) and replaced every other day.

After 3 days of culture, gels were fixed with 4% formalin for 30 min, and stained with phalloidin and DAPI for 1h. Structures were imaged with an upright Zeiss LSM710, using a 0.8 NA 20× air objective. An organoid or cell cluster was defined as invading and branching when it had at least three independent extending processes that were at least half the diameter of the center of the organoid or cell cluster. The number of extending processes and their average length were determined using the Imaris software (Bitplane). We defined a new metric of invasion and branching, which we refer to as the 'spatial network' per organoid. This is defined as the sum of the length of all the extending processes developed from each organoid. 50 structures were counted per condition and the experiments were executed at least 3 times.

2.10 Tissue immunostaining

Tissue samples were fixed overnight in 4% paraformaldehyde/PBS, transferred to 70% ethanol, and embedded in paraffin. 5-mm-thick serial sections were then prepared. Slides were deparaffinized at 55 ºC for 10 min, and rehydrated through a series of xylene (10 min), graded ethanol (100% for 2x

1 min, 90% for 1 min, 70% for 1 min, and 50% for 5 min), and water (for 10 min). Antigen retrieval was performed with citrate buffer (pH 6.0), using two cycles of microwave boiling (5 and 9 min, respectively) followed by a period of 30 min' slow cooling at room temperature. Slides were washed in PBS, and then incubated with 20 μg/mL Proteinase K in TE buffer, for 5 min. Slides were washed again in PBS, and tissues permeabilized in 0.25% Triton X-100/PBS for 10 min, followed by incubation with blocking buffer (1% BSA, 5% Donkey Serum and 5% Goat Serum in PBS) for 1 h at room temperature, with primary antibodies diluted in blocking buffer overnight at 4 ºC, and with secondary antibodies for 1 h at room temperature.

2.11 RNA Isolation and quantitative real-time PCR (qPCR) Analysis

Total RNA was isolated from mammary tissues using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using the SuperScript II Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's instructions. Primers for MMP3, HSP90β and 18S rRNA were obtained from Qiagen (QuantiTect Primer Assays), and quantitative real-time PCR (qPCR) was performed with the LightCycler 480 SYBR Green I Master using the LightCycler 480 Multiwell Plate 384 System, following the manufacturer's instructions. Relative expression between MMP3 or HSP90β and 18S rRNA was quantified.

2.12 Caseinase Activity Assay

CM was incubated with a casein derivative quenching red-fluorescent dye (BODIPY TR-X Casein, E6639, Invitrogen). Protease-catalyzed hydrolysis released highly fluorescent BODIPY TR-X dye-labeled peptides. The accompanying increase in fluorescence is proportional to MMP3 proteolytic activity and was monitored with a microplate reader. A control without BODIPY casein was used to subtract residual fluorescence background.

2.13 Cell Scatter Assay

SCp2 cells were seeded in 6-well plates at low density ($1x10^5$ cells/well), allowed to form colonies (≈ 48 h) and serum-starved for 24 h. Epithelial cell islets were then stimulated with 9 nM EGF (Sigma) and imaged at 48 h with a Zeiss Imager Z1 microscope, using a 10x objective.

2.14 Immunofluorescence

SCp2 cells were cultured for 72 h on glass coverslips, fixed with 4% paraformaldehyde/PBS for 10 min, washed with PBS and permeabilized in 0.25% Triton X-100/PBS for 10 min. Samples were blocked with 1% BSA, 5% Donkey Serum and 5% Goat Serum/PBS for 1 h, followed by incubation with the primary antibody in blocking buffer overnight at 4 ºC and the secondary antibody for 1 h at room temperature. Images were acquired with an upright Zeiss LSM710, using a 1.4 NA 63× oil-immersion.

2.15 Morphometry Analysis

Cell edges were outlined in F-actin stained cells using an "Object Identification Module" from CellProfiler software (Carpenter et al. 2006). Cellular elliptical factors, defined as the ratio of the longest (length) to the shortest (width) axis of the cell, were calculated for 100 random cells per culture.

2.16 Invasion Assay

Cell culture inserts (8 μm, 24-well format, BD Biosciences) were evenly coated with 20 μL diluted (1:5 in DMEM/F-12 medium) Matrigel (BD Biosciences). $1x10^5$ cells in 200 μL of DMEM/F-12 medium or different CM (as indicated in each experiment) were added to the upper compartment of the chamber. The lower compartment of the chamber was filled with 300 μL medium containing 10% FBS as a chemoattractant. After 48 h of incubation at 37 ºC, the topside of the insert was cleared from non-invasive cells with a

cotton swab and washed with serum-free DMEM/F-12. The remaining (invasive) cells at the lower surface of the filter were fixed and stained with a solution of Coomassie Blue 0.125% in methanol:acetic acid:H_2O (45:10:45, v/v/v) for 15 min. Invasive cells were scored by counting 10 x20 magnification fields per filter with a Zeiss Imager Z1 microscope, using a 20x objective. Mouse embryo fibroblast NIH/3T3 cells were routinely included as a positive control. Results are expressed as mean ± s.d. from three independent experiments.

2.17 Western Blotting

Cells were lysed with a buffer containing 1% Triton X-100, 1% NP-40 and protease and phosphatase inhibitor cocktails (Calbiochem/EMD Millipore) in PBS, and the lysates were clarified by centrifugation at 16,000 g for 15 min. Protein concentration was determined using the BCA Protein Assay kit (Thermo Scientific) according to the manufacturer's instructions. Protein samples were mixed with electrophoresis sample buffer containing 5% (v/v) 2-β-mercaptoethanol and 5% (v/v) bromophenol blue and boiled for 5 min at 95 ºC. Samples were loaded in equal amounts into pre-cast 4-20% gradient polyacrylamide gels (Invitrogen) and separated by SDS-PAGE. Resolved proteins were transferred to a nitrocellulose membrane (Whatman) at 130 V for 90 min, followed by blocking of non-specific binding with 5% BSA in 0.05% Tween-20/PBS for 1 h at room temperature. The membranes were probed with primary antibodies specific to each protein overnight at 4 ºC, and then incubated with HRP-conjugated secondary antibodies (Thermo Scientific and Santa Cruz Biotechnology). Blots were visualized with an ECL detection system (Thermo Scientific) according to the manufacturer's instructions, and chemiluminescent signal was captured with a FluorChem IS-8900 (Alpha Innotech). Each western blot was done at least three times, and here we show representative experiments.

2.18 Co-Immunoprecipitation (Co-IP)

For Co-IP of FLAG-tagged MMP3 protein complexes, CM was incubated with anti-FLAG M2 antibody-conjugated agarose beads (F2426, Sigma) for 16 h at 4 ºC. The beads were then washed three times with 0.05% Tween-20/PBS and the immune complexes were directly eluted with electrophoresis sample buffer and analyzed by western blotting. For LC-MS/MS analysis, beads were washed with 0.05% Tween-20/PBS and protein complexes were eluted with a FLAG peptide (F3290, Sigma) in 0.05% Tween-20/PBS. Samples were then precipitated with trichloroacetic acid and reconstituted with a buffer (Invitrosol, MS10007, Invitrogen) suitable for mass spectrometry analysis.

For Co-IP of HSP90β protein complexes, CM was incubated with 10 μg of control rabbit-IgG or anti-HSP90β antibody for 16 h at 4 ºC. Precipitation was performed with protein G sepharose beads (17-0618-01, GE Healthcare) for 4 h at 4 ºC. The beads were then washed three times with 0.05% Tween-20/PBS and the immune complexes were directly eluted with electrophoresis sample buffer and analyzed by western blotting.

2.19 Mass Spectrometry Analysis

The detailed methodology on LC-MS/MS is given below. Scaled signal intensities were log2-transformed and analyzed by R software.

2.19.1 Trypsin Digestion of Samples for LC-MS/MS Analysis

100 μg of eluted proteins from control, FL and dPEX FLAG-immunoprecipitated samples were digested by trypsin (modified, sequencing grade, Promega) at a ratio of 1:30 enzyme/protein along with 2 mM $CaCl_2$ and for 16 h at 37 ºC. Following digestion, all reactions were acidified with 90% formic acid (2% final) to stop the proteolysis. Then, samples were centrifuged for 30 min at 14,000 rpm to remove insoluble material. The soluble peptide mixtures were collected for LC-MS/MS analysis.

2.19.2 Multidimensional Chromatography and Tandem Mass Spectrometry (LC-MS/MS)

Peptide mixtures were pressure-loaded onto a 250 μm inner diameter (i.d.) fused-silica capillary packed first with 3 cm of 5 μm strong cation exchange material (Partisphere SCX), followed by 3 cm of 10 μm C18 reverse phase (RP) particles (Aqua). Loaded and washed microcapillaries were connected via a 2 μm filtered union (UpChurch Scientific) to a 100 μm i.d. column, which had been pulled to a 5 μm i.d. tip using a P-2000 CO2 laser puller (Sutter Instruments), then packed with 13 cm of 3 μm C18 reverse phase (RP) particles (Aqua) and equilibrated in 5% acetonitrile, 0.1% formic acid (Buffer A). This split-column was then installed in-line with a NanoLC Eskigent HPLC pump. The flow rate of channel 2 was set at 300 nL/min for the organic gradient. The flow rate of channel 1 was set to 0.5 μL/min for the salt pulse. Fully automated 11-step chromatography runs were carried out. Three different elution buffers were used: 5% acetonitrile, 0.1 % formic acid (Buffer A); 98% acetonitrile, 0.1% formic acid (Buffer B); and 0.5 M ammonium acetate, 5% acetonitrile, 0.1% formic acid (Buffer C). In such sequences of chromatographic events, peptides are sequentially eluted from the SCX resin to the RP resin by increasing salt steps (increase in Buffer C concentration), followed by organic gradients (increase in Buffer B concentration). The last chromatography step consists in a high salt wash with 100% Buffer C followed by acetonitrile gradient. The application of a 2.5 kV distal voltage electrosprayed the eluting peptides directly into an LTQ-Orbitrap XL mass spectrometer equipped with a nano-LC electrospray ionization source (ThermoFinnigan). Full MS spectra were recorded on the peptides over a 400 to 2,000 m/z range by the Orbitrap, followed by five tandem mass (MS/MS) events sequentially generated by LTQ in a data-dependent manner on the first, second, third, and fourth most intense ions selected from the full MS spectrum (at 35% collision energy). Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (ThermoFinnigan).

2.19.3 Database Search and Interpretation of MS/MS Datasets

Tandem mass spectra were extracted from raw files, and a binary classifier — previously trained on a manually validated data set — was used to remove the low quality MS/MS spectra. The remaining spectra were searched against a mouse protein database containing 56,871 protein sequences downloaded as FASTA-formatted sequences from EBI-IPI (database version 3.75, released on July, 20, 2010) (Kersey et al. 2004), and 124 common contaminant proteins, for a total of 56,871 target database sequences. To calculate confidence levels and false positive rates, we used a decoy database containing the reverse sequences of 56,871 proteins appended to the target database (Elias and Gygi 2007), and the SEQUEST algorithm (Eng et al. 1994; Yates et al. 1995) to find the best matching sequences from the combined database.

SEQUEST searches were done using the Integrated Proteomics Pipeline (IP2, Integrated Proteomics Inc.) on Intel Xeon X5450 X/3.0 PROC processor clusters running under the Linux operating system. The peptide mass search tolerance was set to 50 ppm. No differential modifications were considered. No enzymatic cleavage conditions were imposed on the database search, so the search space included all candidate peptides whose theoretical mass fell within the 50 ppm mass tolerance window, despite their tryptic status.

The validity of peptide/spectrum matches was assessed in DTASelect2 (**Tabb** et al. 2002) using SEQUEST-defined parameters, the cross-correlation score (XCorr) and normalized difference in cross-correlation scores (DeltaCN). The search results were grouped by charge state (+1, +2, and +3) and tryptic status (fully tryptic, half-tryptic, and non-tryptic), resulting in 9 distinct sub-groups. In each one of the sub-groups, the distribution of XCorr and DeltaCN values for (a) direct and (b) decoy database hits was obtained, and the two subsets were separated by quadratic discriminant analysis. Outlier points in the two distributions (for example, matches with very low Xcorr but very high DeltaCN were discarded. Full separation of the direct and decoy subsets is not generally possible; therefore, the discrimi-

nant score was set such that a false positive rate of 1% was determined based on the number of accepted decoy database peptides. This procedure was independently performed on each data subset, resulting in a false positive rate independent of tryptic status or charge state.

In addition, a minimum sequence length of 7 amino acid residues was required, and each protein on the final list was supported by at least two independent peptide identifications unless specified. These additional requirements — especially the latter — resulted in the elimination of most decoy database and false positive hits, as these tended to be overwhelmingly present as proteins identified by single peptide matches. After this last filtering step, the false identification rate was reduced to below 1%.

2.20 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0 software. Student's *t*-test (unpaired with Welch's correction, two-tailed, 95% confidence interval) was used to determine statistical significance. Statistical analyses were always performed in relation to vector control cells (unless stated otherwise).

Chapter 3

Results

3.1 The Hemopexin Domain of MMP3 is Required for a Change in Cell Shape in 2D Substrata and Invasion in Boyden Chambers

3.1.1 Overexpression of Distinct MMP3 Constructs in Functionally Normal Mouse Mammary Epithelial Cells (SCp2)

To investigate the function of different domains of MMP3, we engineered three FLAG-tagged constructs containing different domains of the MMP3 molecule: a wild type (FL) MMP3, a mutant lacking the hemopexin domain (dPEX) and a construct containing a point mutation E219A (EA) at the catalytic core (Figure 3.1A). We overexpressed the distinct MMP3 constructs in SCp2 (Figure 3.1B), a mammary cell line previously shown to undergo EMT upon expression of MMP3 (Lochter et al. 1997a; Radisky et al. 2005). SCp2 cells have a low level of endogenous MMP3 activity that resembles that found in vivo in mammary epithelia; we chose to maintain this activity advisedly to have a positive control for the overexpression of the human homologues in murine cells. This was additionally useful because we observed that the concurrent knockdown of endogenous MMP3 and the intro-

duction of the exogenous levels of the human constructs would lead to aberrant cell behavior. To compare the cultures transduced with different constructs to each other and to the control, we ensured that the endogenous as well as the exogenous levels of MMP3 were comparable in all engineered cell lines (Figure 3.1C,D). Overexpression of the exogenous constructs in SCp2 showed that the proteolytic activity (measured by casein-quenched degradation) in dPEX was similar to FL and they both were higher than EA-SCp2 or control cells (Figure 3.1E).

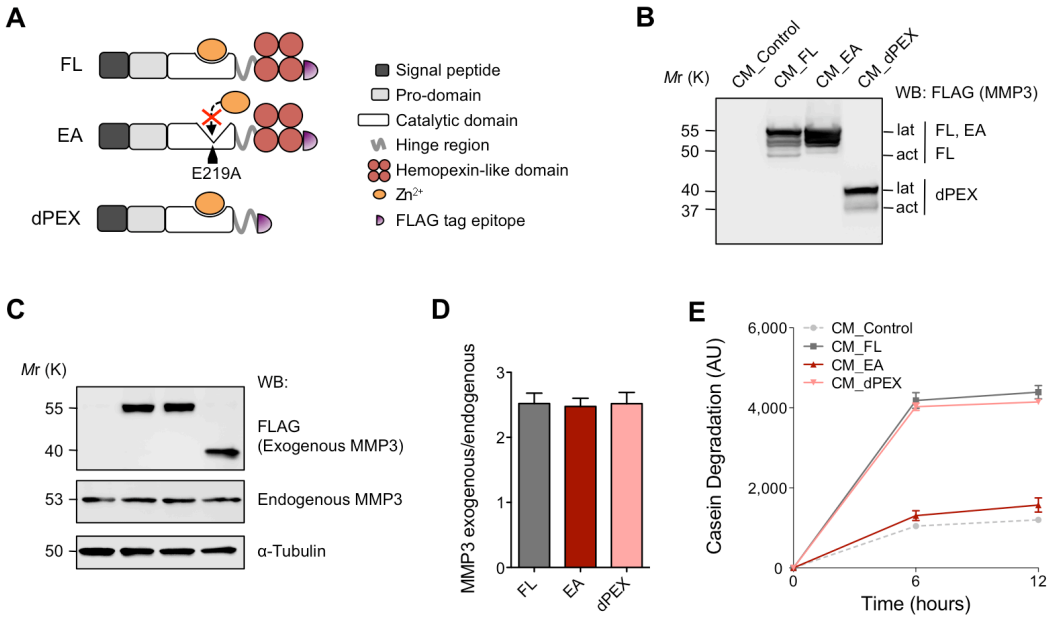


Figure 3.1 Overexpression of distinct MMP3 constructs in functionally normal mouse mammary epithelial cells (SCp2).

(**A**) Schematic representation of engineered MMP3 constructs. (**B**) Overexpression of MMP3 and its mutants in SCp2 cells assessed by western blotting (WB). Conditioned-medium (CM) was isolated from cells transduced with each of the MMP3 constructs and the control vector. FLAG epitope tag was detected with anti-FLAG antibody. Both latent (lat) and activated (act) forms of MMP3 were recognized. (**C**) Expression of endogenous and exogenous MMP3 in SCp2 cells assessed by WB. Whole cell lysates were isolated from each engineered cell line. Exogenous and endogenous MMP3 were detected with anti-FLAG antibody and an antibody specific for murine MMP3, respectively. α-Tubulin was used as loading control. (**D**) Quantification of the ratio between exogenous and endogenous MMP3 in each culture. Results are indicated as mean ± s.d. from three independent experiments. (**E**) MMP3 proteolytic activity of SCp2 cells overexpressing each construct assayed by casein degradation. CM was incubated with a dye-quenching casein substrate (BODIPY TR-X casein). MMP3-mediated degradation of casein generated fluorescent dye-labeled peptides that were monitored over time. Fluorescence intensity is indicated as arbitrary units (AU).

3.1.2 MMP3 Hemopexin Domain Induces Scattering, Loose Adherens Junctions and Elongated Morphology in both SCp2 and EpH4 Cells

Cell scattering is a functional consequence of EMT (Vincent-Salomon and Thiery 2003); overexpression of FL-MMP3 induced scattering in 2D cultures (Figure 3.2A, first and second rows). The EA mutant also stimulated a spindle-shaped morphology and scattered phenotype, albeit to a lower extent (Figure 3.2A, third row). In contrast, dPEX-SCp2 did not scatter and resembled the control cultures (Figure 3.2A, fourth row). Others and we have shown that E-cadherin is a substrate for MMP3 and its loss is associated with scattering (Lochter et al. 1997a; Noe et al. 2001). Consistent with these observations, we found that FL and dPEX-MMP3 both reduced the expression of E-cadherin (Figure 3.2B, second and fourth rows) by shedding its extracellular domain (Figure 3.2C,D). Surprisingly, however, EA-SCp2 cells (which lack the proteolytic activity) exhibited a stretched phenotype even in the presence of E-cadherin levels similar to control cultures (Figure 3.2B, third row), suggesting that the ability of MMP3 to disrupt epithelial morphology was due to activities residing in its other domains.

Using changes in cell morphology and reorganization of filamentous actin (F-actin) as additional endpoints, we observed that in dPEX-SCp2 and control cultures, F-actin was predominantly organized in cortical bundles and cells had a classical epithelial morphology in 2D (Figure 3.3A, first and last rows). In sharp contrast, actin filaments were extended in FL and EA-SCp2 cultures and cells were elongated (Figure 3.3A, second and third rows). We quantified these morphological changes by calculating the ratio of the longest (length) to the shortest (width) axis of the cell that we refer to as cellular elliptical factor (Figure 3.3B). Whereas FL and EA-SCp2 displayed elliptical factor >2, cells expressing control vector or dPEX had elliptical factors close to 1. These observations show a critical role for MMP3 hemopexin domain in altering epithelial cell shape.

Despite the small amount of proteolytic activity of SCp2 cells, these exhibit little invasive behavior (Lochter et al. 1997b); the same is true in SCp2 cells transduced with control vector (Figure 3.3C, control). SCp2

transduced with FL-MMP3 had the highest invasive rate, followed by EA and dPEX-SCp2, respectively (Figure 3.3C). These data indicate that despite the background proteolytic activity, MMP3 requires the hemopexin domain to induce invasion in SCp2 cells. A similar trend was obtained with EpH4, another mouse mammary epithelial cell line (D-F).

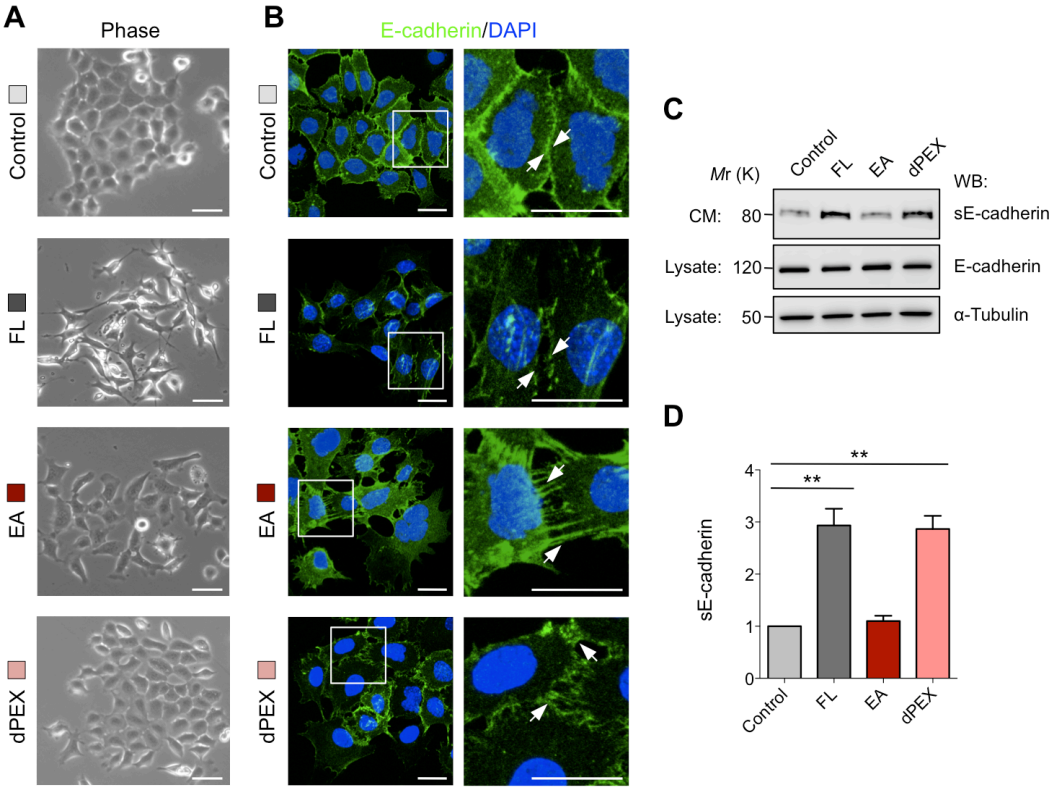


Figure 3.2 Overexpression of MMP3 containing the hemopexin domain induces scattering and loose adherens junctions in SCp2 cells.

(**A**) The hemopexin domain of MMP3 is required to induce scattering in SCp2 cells. Scattering ability was evaluated in cells transduced with each construct upon stimulation with EGF. Scale bars: 20μm. (**B**) MMP3 hemopexin domain disrupts adherens junctions. Immunofluorescence images show E-cadherin distribution (green) in cells expressing each construct. Arrows depict areas of cell-cell contact. Nuclei stained with DAPI (blue). Scale bars: 10μm. (**C**) E-cadherin is a substrate for MMP3. WB showing soluble fragments of E-cadherin (sE-cadherin) detected in CM from SCp2 cells overexpressing distinct MMP3 constructs and control vector. Total E-cadherin and α-tubulin from whole cell lysates were used as loading controls. (**D**) Quantification of sE-cadherin levels in each culture. Results are indicated as mean ± s.d. from three independent experiments (** $P < 0.001$ by Student's *t*-test).

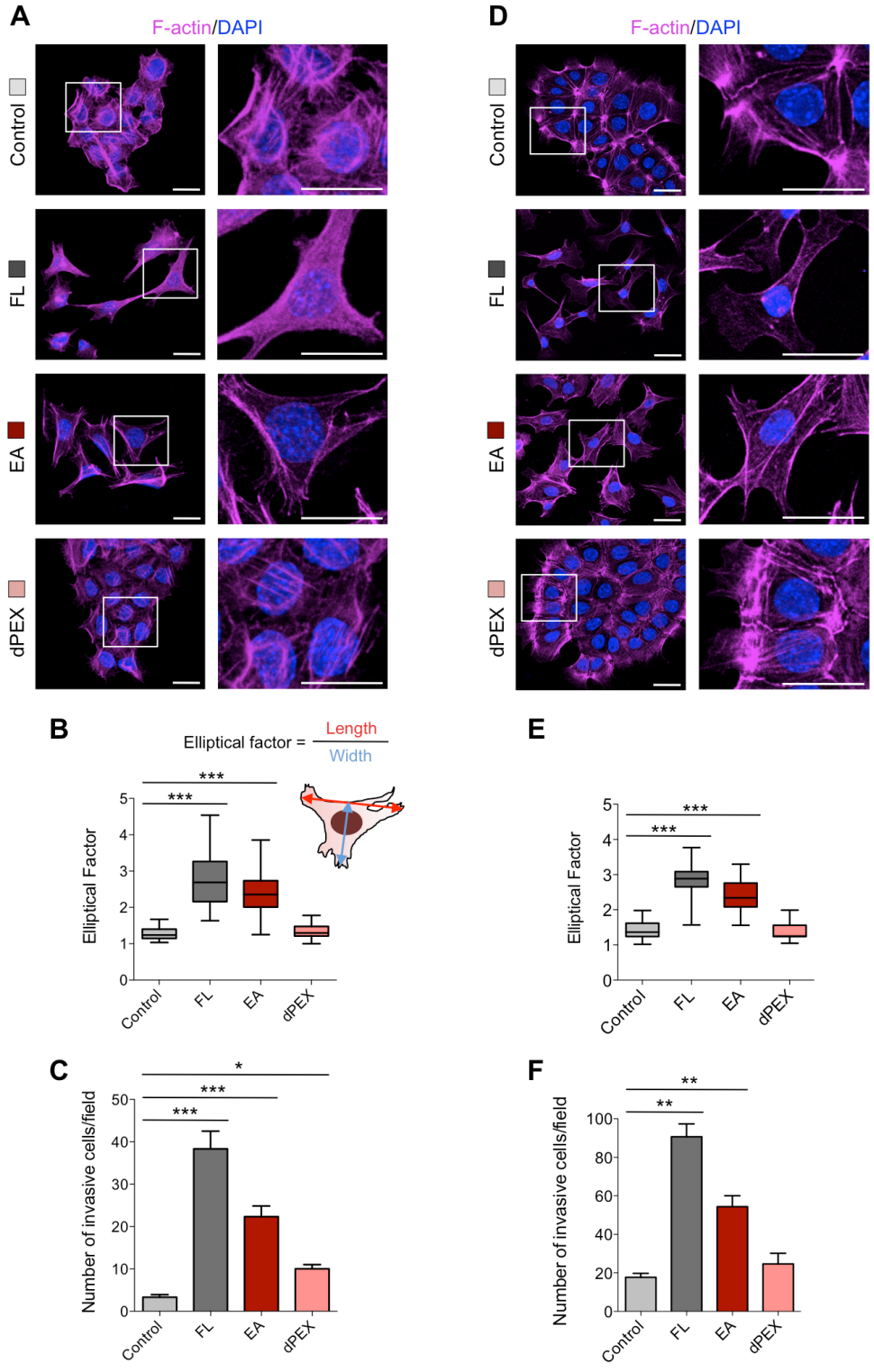


Figure 3.3 MMP3 hemopexin domain induces elongated morphology and invasion in both SCp2 and EpH4 cells.

Panels (**A-C**) and (**D-F**) refer to SCp2 and EpH4 cells, respectively. (**A**,**D**) MMP3 hemopexin domain induces reorganization of F-actin. Images show F-actin (magenta) and nuclei (DAPI; blue) in each culture. Scale bars: 10μm. (**B**,**E**) The hemopexin domain of MMP3 stimulates elongated cell shape. Quantification of morphological changes in each culture by calculation

of the cellular elliptical factor. The box plot shows the median and the interquartile range, and the whiskers show the extreme values (n = 100 cells for each stable cell line). (**C**,**F**) The hemopexin domain of MMP3 directs signaling for invasion. Invasiveness in each condition was assayed in Boyden chambers. Results are indicated as mean ± s.d. from three independent experiments (10 bright field images in x20 magnification were counted). *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$ by Student's t-test.

3.1.3 Secreted MMP3 is Required to Induce Morphological and Functional Changes in Parental SCp2 Cells

Because MMP3 is a secreted protein, we asked whether the secreted form of this enzyme and its mutants were required to induce the morphological and functional changes observed (Figure 3.4).

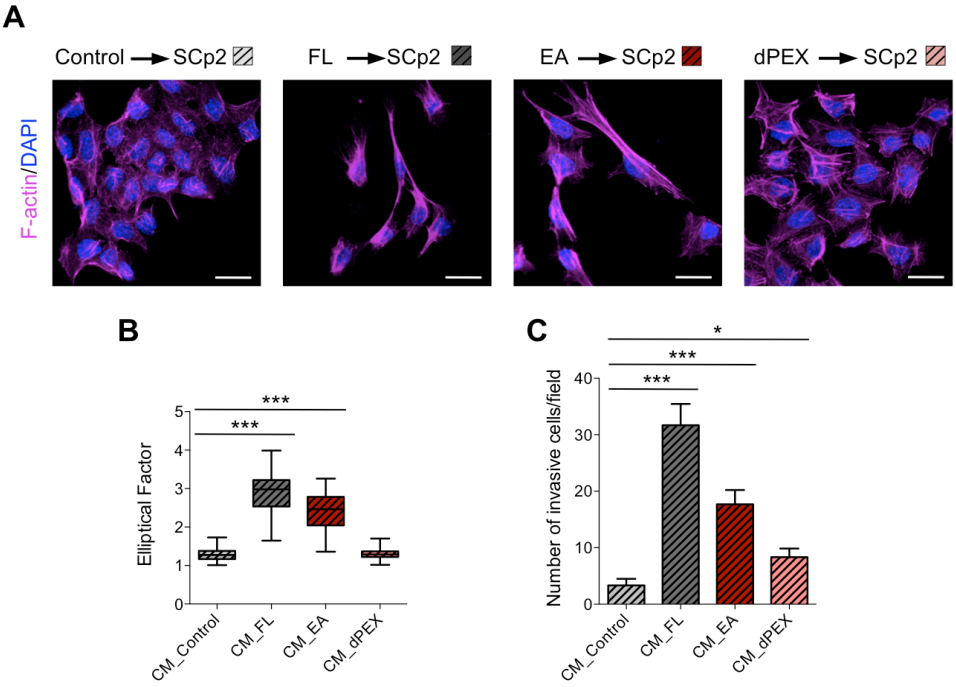


Figure 3.4 Conditioned medium from cells overexpressing each of the MMP3 constructs is sufficient to induce morphological and functional changes in parental SCp2 cells.
(**A**) Images showing F-actin organization (magenta) and nuclei (DAPI; blue) in each culture. Scale bars: 10 μm. (**B**) Plot of the cellular elliptical factors of each culture. The box shows the median and the interquartile range, and the whiskers show the extreme values (n = 100 cells per culture). (**C**) Invasiveness in each condition assayed in Boyden chambers. Results are expressed as mean ± s.d. from three independent experiments (10 bright field images in 20x magnification were counted). *** $P < 0.0001$, * $P < 0.05$ by Student's t-test.

Conditioned-medium (CM) from FL-SCp2 was sufficient to induce scattering, elongated shape and a substantial increase in invasion in parental SCp2 cells. Whereas dPEX-SCp2 CM did not trigger scattering or enhance the el-

liptical factor, there was a small but significant increase in invasion. However when the proteolytic activity of the MMP3 construct was ablated (CM from EA-SCp2) there was still a considerable increase in invasion and cells were elongated. This finding additionally supports the fact that the hemopexin domain is required for invasion in SCp2 cells.

3.2 Proteomic Screen Identifies HSP90β as Interacting with MMP3 Hemopexin Domain in the Extracellular Milieu

3.2.1 HSP90β, ANXA2 and MARCKS are Present Extracellularly and Interact with MMP3 via Hemopexin Domain

The fact that CM from cells overexpressing each of the MMP3 constructs was sufficient to induce morphological and functional changes in parental SCp2 cells raised the question of whether MMP3 functions alone or depends on other factors being present in CM. The hemopexin domain of MMPs is known to interact with other proteins. MMP14 hemopexin domain was reported to be required for invasion through Col-1 (Tam et al. 2002; Wang et al. 2004) and for binding to the adhesion receptor CD44 and integrin-β1 (Mori et al. 2002; Mori et al. 2013).

To explore what other factors may be required for the functional activities of MMP3, we isolated FLAG-tagged-FL or -dPEX protein complexes from CM and performed a proteomic analysis to identify proteins that interact with MMP3 hemopexin domain (Figure 3.5A). Based on spectra counts, we selected proteins with abundances greater than 1.5-fold change in FL compared to dPEX (Figure 3.5B, left; Figure 3.6). Amongst the 75 proteins that passed the selection criteria, we selected myristoylated alanine-rich C-kinase substrate (MARCKS) and annexin A2 (ANXA2), which were previously implicated in regulation of cell shape, motility and invasion in *Xenopus* embryos and canine kidney cells (Iioka et al. 2004; de Graauw et al. 2008). Additionally we selected heat shock protein 90 beta (HSP90β) detected in both FL and dPEX but much higher in FL (Figure 3.5B, right). We validated the interaction of the hemopexin domain of MMP3 with these three proteins by co-immunoprecipitation (Co-IP) (Figure 3.5C).

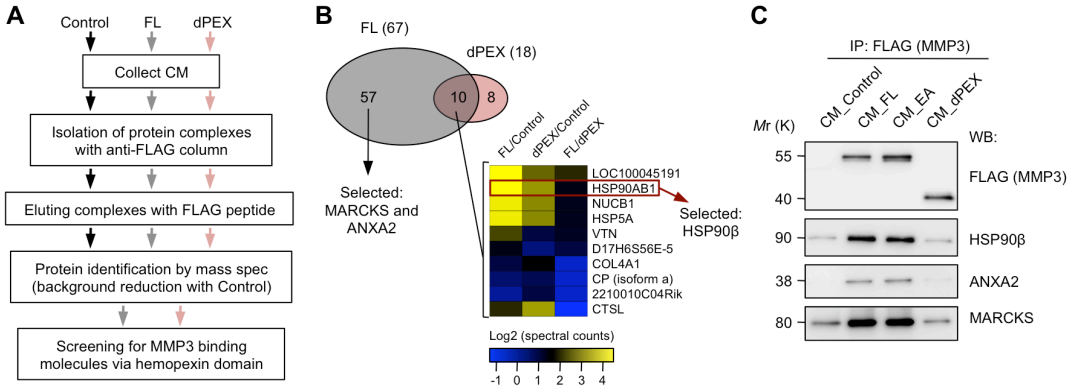
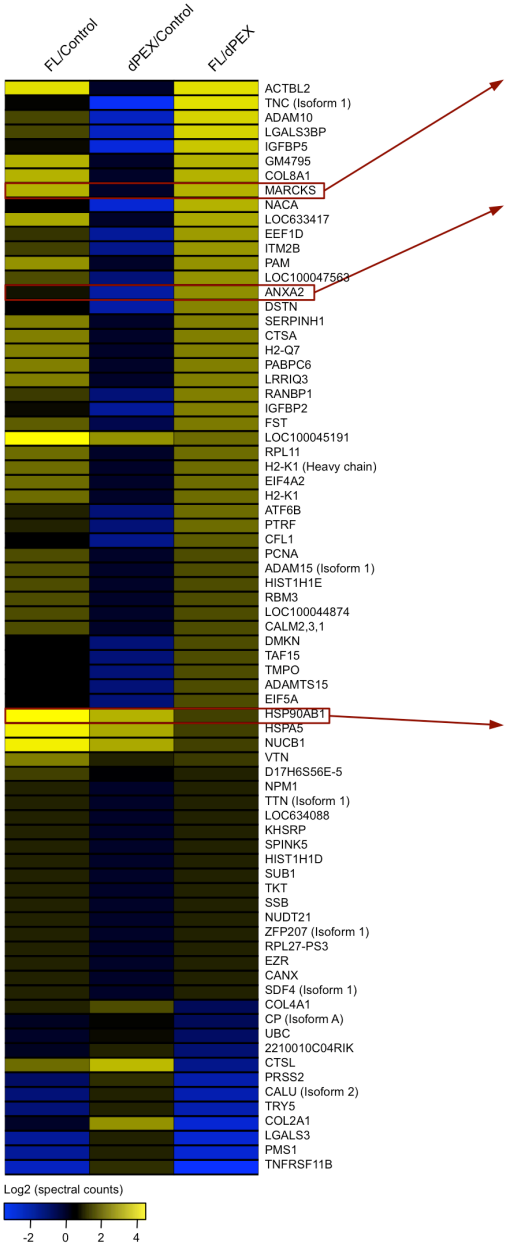


Figure 3.5 Proteomic screen of MMP3 binding partners reveals the extracellular interaction with HSP90β, ANXA2 and MARCKS via the hemopexin domain.
(**A**) Strategy for screening MMP3 binding partners through the hemopexin domain. (**B**) Selection of MARCKS, ANXA2 and HSP90β from proteomic analysis. Left: Venn diagram showing the spectrum of proteins detected in FL and/or dPEX FLAG-immunoprecipitated samples. Right: Heat map illustrating the relative difference in abundance of proteins detected both in FL and dPEX but much higher in FL. Proteins were sorted by the highest ratio between FL and dPEX. (**C**) Co-IP of each mutant shows the association between MMP3 and the selected targets via hemopexin domain. FLAG-tagged MMP3 FL, EA and dPEX were immunoprecipitated from CM with an anti-FLAG antibody, and blotted with antibodies for its binding partners.

3.2.2 Silencing of HSP90β, ANXA2 and MARCKS Reduces MMP3-Driven Invasion in SCp2 Cells when the Hemopexin Domain of MMP3 is Present

We then asked whether this interaction is functionally significant and required for MMP3-induced invasion. We generated SCp2 cell lines co-expressing each of the MMP3 constructs and either non-targeting short hairpin RNA (shRNA; negative control) or shRNA selectively targeting each of the three proteins (Figure 3.7A-C,G-I,M-O, three distinct shRNAs for each of the targets). We treated parental SCp2 with CM from each engineered cell line and screened for invasion using Boyden chambers (Figure 3.7D-F,J-L,P-R).



Protein	log2 FL/Control	log2 dPEX/Control	log2 FL/dPEX
ACTBL2	4.0875	0.0000	4.0875
TNC (Isoform 1)	0.7059	-3.3458	4.0517
ADAM10	1.4854	-2.3219	3.8074
LGALS3BP	1.4854	-2.3219	3.8074
IGFBP5	0.7776	-2.8074	3.5850
GM4795	3.1699	0.0000	3.1699
COL8A1	3.1699	0.0000	3.1699
MARCKS	3.1699	0.0000	3.1699
NACA	0.5850	-2.5850	3.1699
LOC633417	3.0000	0.0000	3.0000
EEF1D	1.2224	-1.5850	2.8074
ITM2B	1.3785	-1.3219	2.7004
PAM	2.5850	0.0000	2.5850
LOC100047563	1.5850	-1.0000	2.5850
ANXA2	0.8231	-1.7004	2.5236
DSTN	0.5850	-1.8074	2.3923
SERPINH1	2.3219	0.0000	2.3219
CTSA	2.3219	0.0000	2.3219
H2-Q7	2.3219	0.0000	2.3219
PABPC6	2.3219	0.0000	2.3219
LRRIQ3	2.3219	0.0000	2.3219
RANBP1	1.3219	-1.0000	2.3219
IGFBP2	0.7370	-1.5850	2.3219
FST	1.8074	-0.4150	2.2224
LOC100045191	4.5850	2.5850	2.0000
RPL11	2.0000	0.0000	2.0000
H2-K1 (heavy chain)	2.0000	0.0000	2.0000
EIF4A2	2.0000	0.0000	2.0000
H2-K1	2.0000	0.0000	2.0000
ATF6B	1.0000	-1.0000	2.0000
PTRF	1.0000	-1.0000	2.0000
CFL1	0.5406	-1.2895	1.8301
PCNA	1.5850	0.0000	1.5850
ADAM15 (Isoform 1)	1.5850	0.0000	1.5850
HIST1H1E	1.5850	0.0000	1.5850
RBM3	1.5850	0.0000	1.5850
LOC100044874	1.5850	0.0000	1.5850
CALM2,3,1	1.5850	0.0000	1.5850
DMKN	0.5850	-1.0000	1.5850
TAF15	0.5850	-1.0000	1.5850
TMPO	0.5850	-1.0000	1.5850
ADAMTS15	0.5850	-1.0000	1.5850
EIF5A	0.5850	-1.0000	1.5850
HSP90AB1	4.5850	3.1699	1.4150
HSPA5	4.3923	3.0000	1.3923
NUCB1	4.3923	3.0000	1.3923
VTN	2.3219	1.0000	1.3219
D17H6S56E-5	1.4150	0.4150	1.0000
NPM1	1.0000	0.0000	1.0000
TTN (Isoform 1)	1.0000	0.0000	1.0000
LOC634088	1.0000	0.0000	1.0000
KHSRP	1.0000	0.0000	1.0000
SPINK5	1.0000	0.0000	1.0000
HIST1H1D	1.0000	0.0000	1.0000
SUB1	1.0000	0.0000	1.0000
TKT	1.0000	0.0000	1.0000
SSB	1.0000	0.0000	1.0000
NUDT21	1.0000	0.0000	1.0000
ZFP207 (Isoform 1)	1.0000	0.0000	1.0000
RPL27-PS3	1.0000	0.0000	1.0000
EZR	1.0000	0.0000	1.0000
CANX	1.0000	0.0000	1.0000
SDF4 (Isoform 1)	1.0000	0.0000	1.0000
COL4A1	1.0000	1.5850	-0.5850
CP (Isoform A)	0.1375	0.7225	-0.5850
UBC	0.0000	0.7370	-0.7370
2210010C04RIK	0.0902	1.0000	-0.9098
CTSL	2.0000	3.3219	-1.3219
PRSS2	-0.7370	1.1375	-1.8745
CALU (Isoform 2)	-1.0000	1.0000	-2.0000
TRY5	-1.0000	1.0000	-2.0000
COL2A1	0.0000	2.5850	-2.5850
LGALS3	-1.5850	1.0000	-2.5850
PMS1	-1.5850	1.0000	-2.5850
TNFRSF11B	-2.3219	1.1375	-3.4594

Figure 3.6 Proteins identified as interacting with MMP3 in the extracellular milieu.
Left: Heat map showing the relative difference in protein abundance in FL vs. control, dPEX vs. control and FL vs. dPEX FLAG-immunoprecipitated samples. Proteins are sorted by the ratio of FL and dPEX. Right: Complete list of the identified proteins. Targets selected for validation and further studies are highlighted.

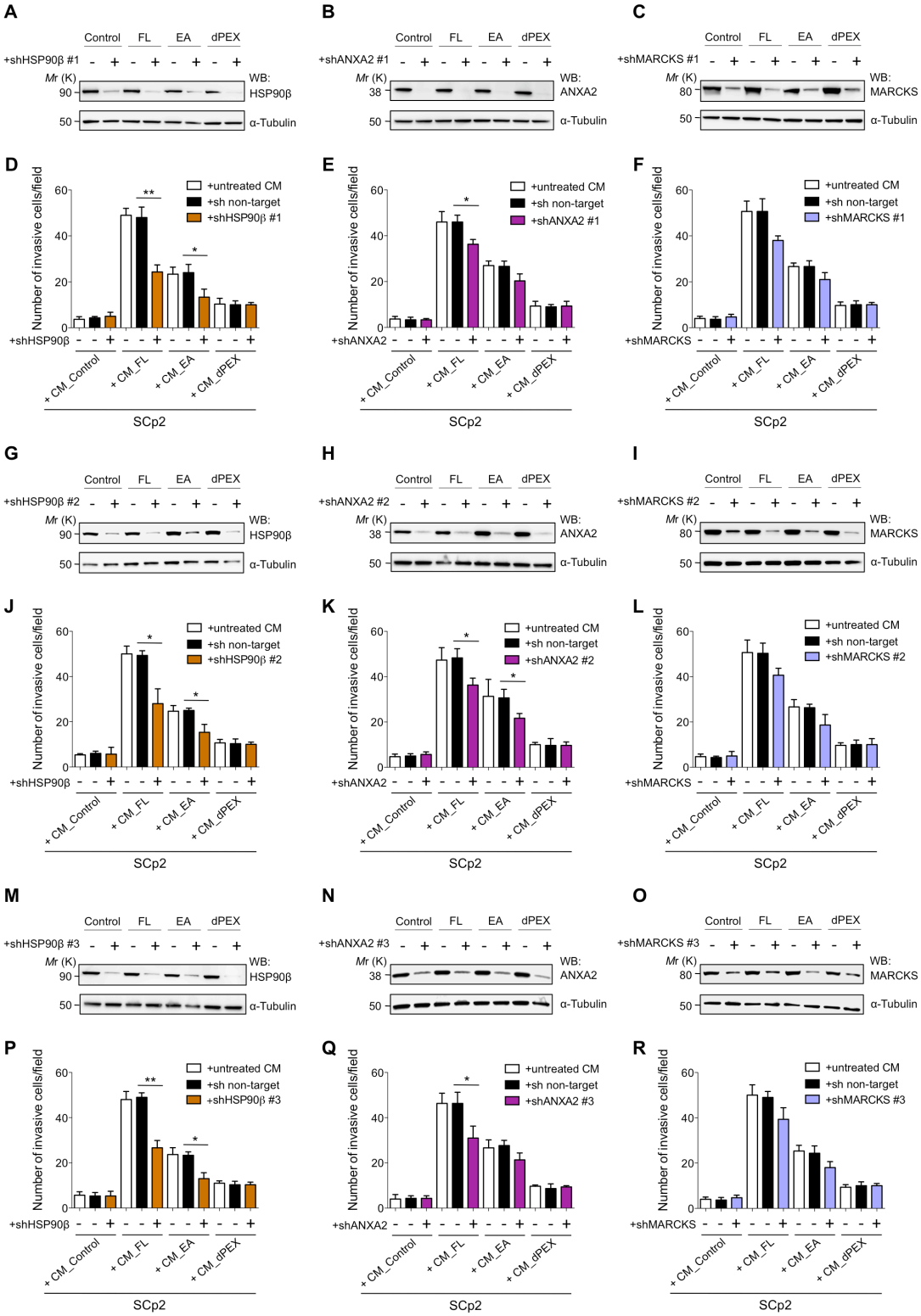


Figure 3.7 Silencing of HSP90β, ANXA2 and MARCKS reduces MMP3-driven invasion in SCp2 cells when the hemopexin domain of MMP3 is present.

(**A**-**C**), (**G**-**I**) and (**M**-**O**) Blots showing shRNA-mediated silencing of HSP90β (**A**,**G**,**M**), ANXA2 (**B**,**H**,**N**) and MARCKS (**C**,**I**,**O**) in SCp2 cells overexpressing each of the MMP3 constructs and the control vector. Non-targeting shRNA was used as negative control. Knockdowns were

verified by WB of whole cell lysates with antibodies specific for each target protein. α-Tubulin was used as loading control. (**D**-**F**), (**J**-**L**) and (**P**-**R**) Silencing of HSP90β, ANXA2 and MARCKS reduces MMP3-driven invasion in SCp2 cells when the hemopexin domain of MMP3 is present. SCp2 cells were co-transduced with each of the MMP3 constructs and either non-targeting shRNA or shRNAs selectively targeting HSP90β (**D**,**J**,**P**), ANXA2 (**E**,**K**,**Q**) or MARCKS (**F**,**L**,**R**). SCp2 parental cells were treated with CM from each engineered cell line and assayed for invasiveness in Boyden chambers. Parental cells treated with CM from SCp2 cells expressing each of the MMP3 constructs and the control vector (untreated CM) were used as control. Results are expressed as mean ± s.d. from three independent experiments (10 bright field images in x20 magnification were counted in each experiment). ** $P < 0.001$, * $P < 0.05$ by Student's *t*-test.

The knockdown did not affect invasion of cells treated with CM from control or dPEX-SCp2, but it significantly reduced invasiveness of cells treated with FL or EA-SCp2 CM. These results indicate that binding of each one of these three proteins to the hemopexin domain of MMP3 has functional significance, but the inhibition was much more dramatic when HSP90β was inhibited (Figure 3.7D,J,P). The nature of the complexes containing MMP3 and HSP90β was clarified further by reverse Co-IP of HSP90β protein complexes from CM of control SCp2 cells (Figure 3.8).

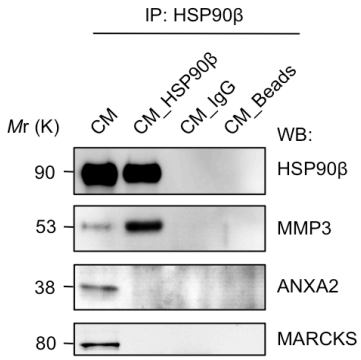


Figure 3.8 Co-IP of HSP90β protein complexes confirms the extracellular association of MMP3 and HSP90β in reverse.
HSP90β protein complexes were immunoprecipitated from CM from control SCp2 cells with a rabbit anti-HSP90β antibody, and blotted with antibodies for HSP90β, MMP3, ANXA2 and MARCKS. Control rabbit-IgG and plain protein G sepharose beads were used as controls.

Whereas the association of MMP3 and HSP90β was confirmed in reverse, ANXA2 and MARCKS could not be recovered in the immunoprecipitated fraction under these conditions. This suggests that either these proteins do not exist in a single complex at a given time, but may instead represent a network of proteins interacting with one another at different times for dif-

ferent purposes, or the interaction of the other two proteins is weak and thus cannot be detected easily by the reverse Co-IP. These data also justify the importance of HSP90β as the major player in regulation of MMP3 function.

3.2.3 The Levels of Extracellular HSP90β Determine MMP3-Induced Invasion

Given the significance of HSP90β in cellular and tissue function, we concentrated on understanding the role of this molecule in regulating MMP3. The levels of HSP90β in each engineered cell line were tuned by adding either a recombinant protein or a specific inhibitor (CCT018159; (Sharp et al. 2007)) (Figure 3.9).

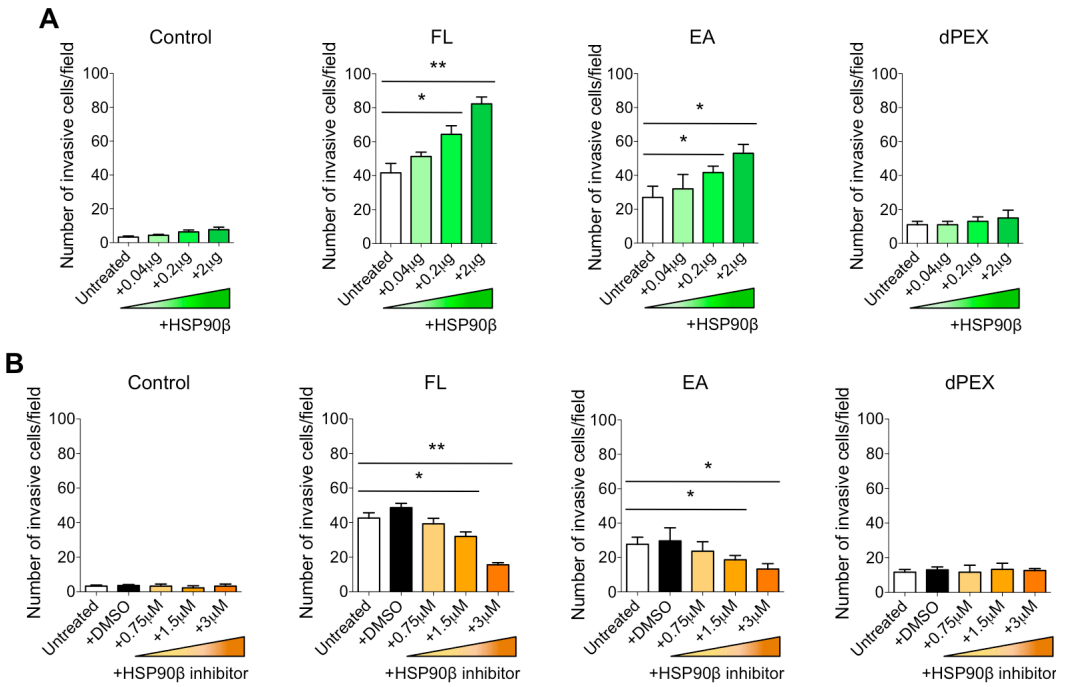


Figure 3.9 HSP90β regulates MMP3-driven invasion in a dose-dependent manner when the hemopexin domain is present.
(**A**) Addition of purified HSP90β enhances the invasiveness of SCp2 cells overexpressing MMP3 constructs containing the hemopexin domain. Cell invasiveness of FL, EA, dPEX-overexpressing SCp2 and control vector cells cultured in the presence of increasing doses of recombinant HSP90β. Untreated cells were used as a control. (**B**) Inhibition of HSP90β reduces invasiveness of SCp2 cells overexpressing MMP3 constructs containing the hemopexin domain. Cell invasiveness of FL, EA, dPEX-overexpressing SCp2 and control vector cells cultured in the presence of increasing doses of a cell-permeable HSP90β inhibitor. Cells cultured with vehicle DMSO and untreated cells were used as controls. Invasiveness

from experiments in (**A**,**B**) was assayed in Boyden chambers. Results are expressed as the mean ± s.d. from three independent experiments (10 bright field images in 20x magnification were counted). ** $P < 0.001$, * $P < 0.05$ by Student's *t*-test.

Increasing HSP90β levels enhanced invasion significantly in FL and EA-SCp2 (Figure 3.9A, second and third panels), but did not raise the invasive potential of dPEX-SCp2 or control cells significantly (Figure 3.9A, first and last panels). Conversely, inhibition of HSP90β reduced invasion in FL and EA-SCp2 (Figure 3.9B, second and third panels) and had no significant effect on dPEX-SCp2 or control cells (Figure 3.9B, first and last panels).

The above pattern was reproduced when we used a function-blocking antibody against HSP90β and demonstrated that inhibition of extracellular HSP90β was sufficient to reduce invasiveness of FL and EA-SCp2 (Figure 3.10). These data show that MMP3 is unable to perform much of its invasive functions without interacting with HSP90β in the extracellular milieu.

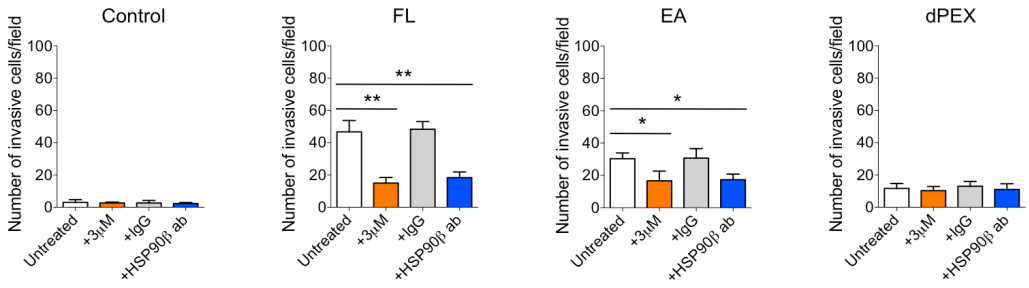


Figure 3.10 Blocking of extracellular HSP90β is sufficient to reduce invasiveness of SCp2 cells transduced with MMP3 constructs containing the hemopexin domain.
Cell invasiveness of FL, EA, dPEX-overexpressing SCp2 and control vector cells cultured in the presence of a cell-permeable HSP90β inhibitor or a function-blocking antibody against HSP90β. Untreated cells and rabbit IgG were used as controls. Invasiveness was assayed in Boyden chambers. Results are expressed as the mean ± s.d. from three independent experiments (10 bright field images in 20x magnification were counted). ** $P < 0.001$, * $P < 0.05$ by Student's *t*-test.

3.3 The Hemopexin Domain is Required for the Invasive Function of MMP3 During Branching Morphogenesis

The finding of the critical role of the hemopexin domain in the invasion function of MMP3 in cell lines needed to be confirmed in a more physiological context. We used two culture models that simulate the normal processes of mammary invasion and branching: cell clusters of a functionally normal mouse mammary epithelial cell line (EpH4) (Figure 3.11A; (Hirai et al. 1998; Mori et al. 2013)) and primary mammary organoids (Figure 3.12A; (Simian et al. 2001)), embedded in Col-1 gels. The physiological relevance of this model is illustrated by the presence of copious amounts of Col-1 in the stroma surrounding epithelial ducts in the murine mammary gland (Williams and Daniel 1983).

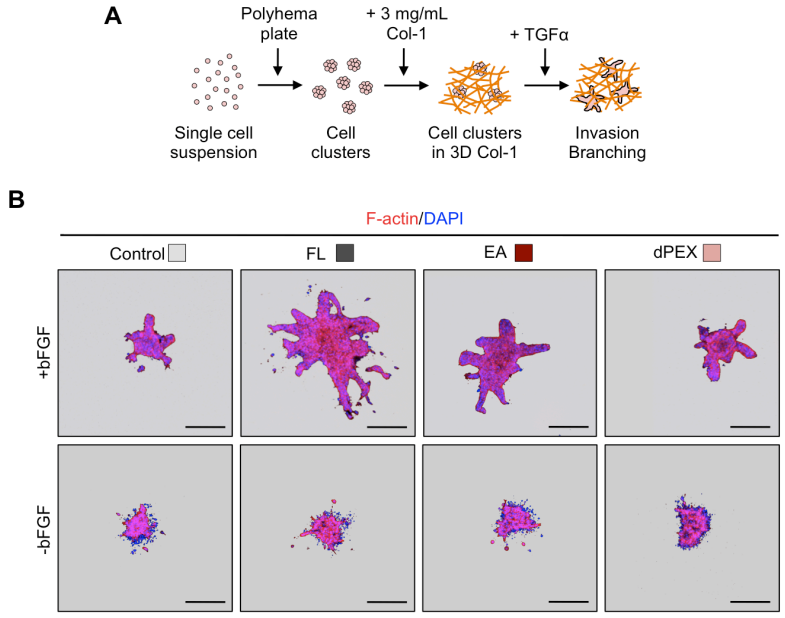


Figure 3.11 The hemopexin domain of MMP3 directs invasion and branching of clustered EpH4 cells in 3D Col-1 gels even without the proteolytic activity.
(**A**) Schematic representation of cell cluster preparation and culture in 3D Col-1 gels. (**B**) Images of maximum-intensity projection of EpH4 cell clusters transduced with each of the MMP3 constructs as well as the control vector, and cultured in 3 mg/mL Col-1 for 4 days. EpH4 clusters invaded and branched only in the presence of the growth factor (bFGF). Structures were stained for F-actin (red) and nuclei (DAPI; blue). Image background was pseudo-colored in grey. Scale bars: 100μm.

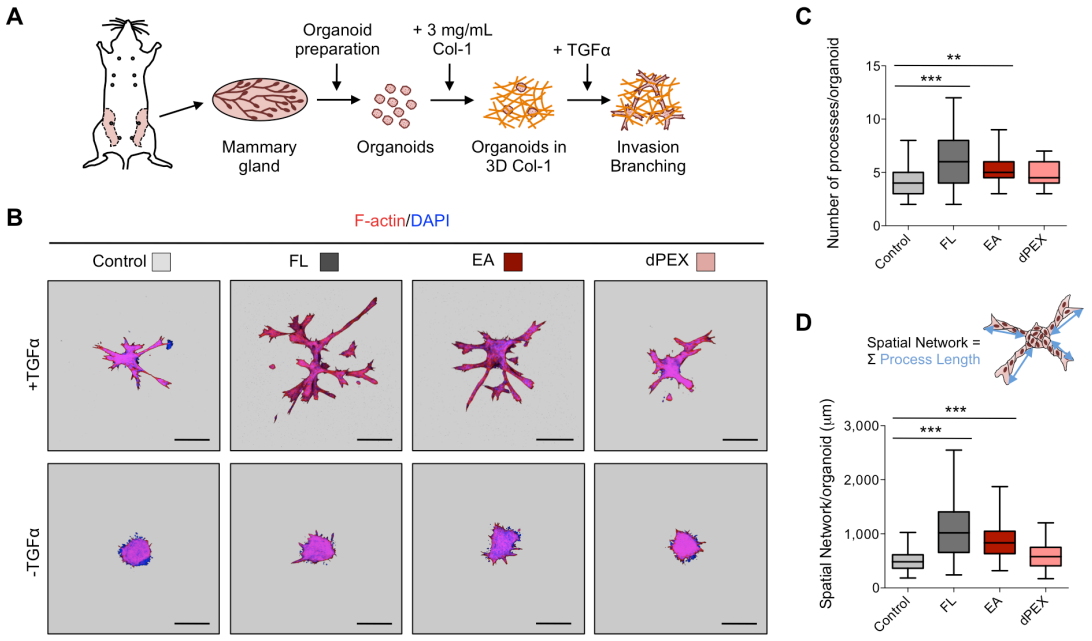


Figure 3.12 MMP3 hemopexin domain signals for epithelial invasion and branching of mammary organoids in 3D Col-1 gels even without the proteolytic activity.
(**A**) Schematic representation of primary mammary organoid preparation and culture in 3D Col-1 gels. (**B**) Overexpression of MMP3 containing the hemopexin domain enhances invasion of mammary organoids in Col-1. Images of maximum-intensity projection of mammary organoids transduced with each of the MMP3 constructs as well as the control vector, and cultured in 3 mg/mL Col-1 gels for 3 days. Organoids invaded and branched only in the presence of the growth factor (TGFα). Structures were stained for F-actin (red) and nuclei (DAPI; blue). Image background was pseudo-colored in grey. Scale bars: 100μm. (**C**) The presence of the hemopexin domain of MMP3 increases the number of extending processes developed from each organoid invading through Col-1 (150 organoids were counted per culture). (**D**) The size of the 'spatial network' per organoid is increased by overexpression of MMP3 containing the hemopexin domain. The 'spatial network' per organoid is defined as the sum of the length of all the extending processes of an organoid (50 organoids were counted per culture. *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$ by Student's *t*-test.

There are a number of advantages of using the versatile assay employing organoids from the mammary gland. Cell-cell and cell-matrix interactions remain intact, and the architecture of the tissue is not disrupted. Additionally, we can prepare enough mammary organoids from a single mouse (≈1200) and infect with the four distinct constructs. Even inbred mice are known to change biochemical and morphological characteristics at different stages of estrogen cycle as well as in response to handling and context. In this way we could control for all variations and avoid the excessive use of animals, but also achieve statistical significance. Lastly we could mark

them: the presence of the GFP in the constructs indicated that more than 80% of the cells were infected. These cultures allow us not only to create a physiological condition where the organoids regenerate an epithelial tree-like structure, but also to observe and control extracellular events much more robustly.

The functional significance of the hemopexin domain was reproduced in our 3D assays with clustered EpH4 cells (Figure 3.11B) and most importantly with primary organoids (Figure 3.12B). We used two different criteria to quantify invasion and branching of organoids: the number of extended sprouts and processes developed from each structure (Figure 3.12C), and the 'spatial network' per organoid (Figure 3.12D). As expected, organoids overexpressing FL-MMP3 had the highest number of extending processes and longest spatial network, indicating that the proteolytic activity would be still necessary if the path is obstructed.

For the purpose of the current experiments, we did not distinguish between branches that were more than one cell layer thick and demonstrated basal and apical polarity, and strands that grew as a single file. However, there were very few of the latter in dPEX-overexpressing and control cultures. As mentioned above we advisedly decided against inhibiting the endogenous MMP3 activity using multiple genetic manipulations because both cells and organoids were sensitive to more than one set of viral infections. We therefore used a peptide that has been shown to inhibit MMP3 proteolytic activity effectively and specifically (Fotouhi et al. 1994; Farina et al. 2002). Inhibition of both endogenous as well as exogenous MMP3 proteolytic activity decreased branches in a dose-dependent manner in all organoids (Figure 3.13A,B). Nevertheless, there still was invasion of cells individually or in a single file, with less branching than untreated cultures (Figure 3.13A). These data indicate that the hemopexin domain of MMP3 allows epithelial invasion, but that in the presence of proteolytic activity there are more multilayered branches. Additionally, when we knocked down MMP3 in control organoids there was a significant decrease in invasion and branching (Figure 3.14). This reaffirms the requirement for MMP3 for mammary branching morphogenesis, and provides additional reason for our choice of preserving the endogenous MMP3 intact.

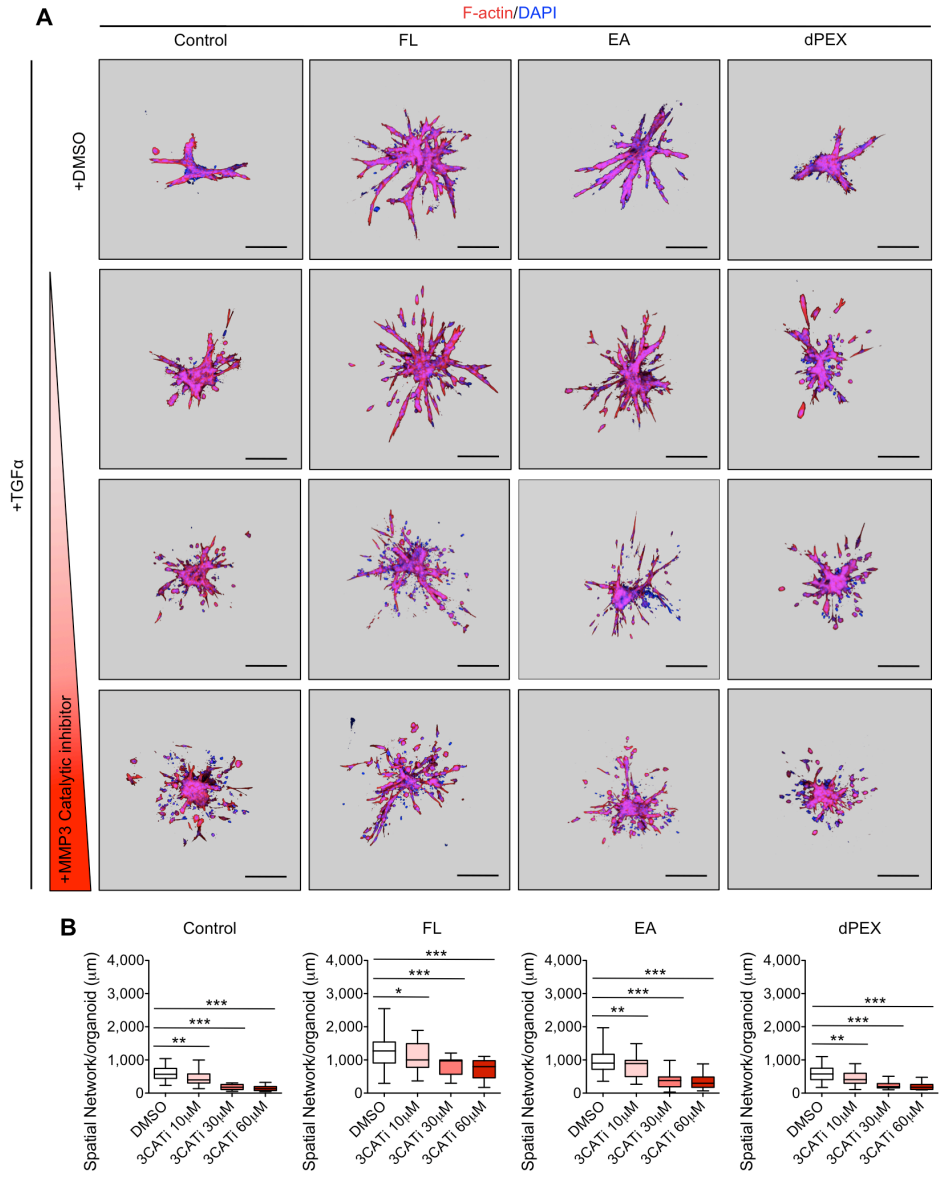


Figure 3.13 The hemopexin domain of MMP3 is sufficient for epithelial invasion, but the proteolytic activity is still required for the formation of branches.

(**A**) Images of maximum-intensity projection of mammary organoids transduced with each of the MMP3 constructs as well as the control vector, and cultured in 3 mg/mL Col-1 gels for 3 days. Organoids were cultured in the presence of the growth factor (TGFα) and increasing doses of a peptide that was shown previously to inhibit MMP3 proteolytic activity specifically and significantly. Structures were stained for F-actin (red) and nuclei (DAPI; blue). Image background was pseudo-colored in grey. Scale bars: 100 μm. (**B**) Quantification of invasion and branching by measuring the 'spatial network' per organoid (50 organoids were counted per culture). *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$ by Student's *t*-test.

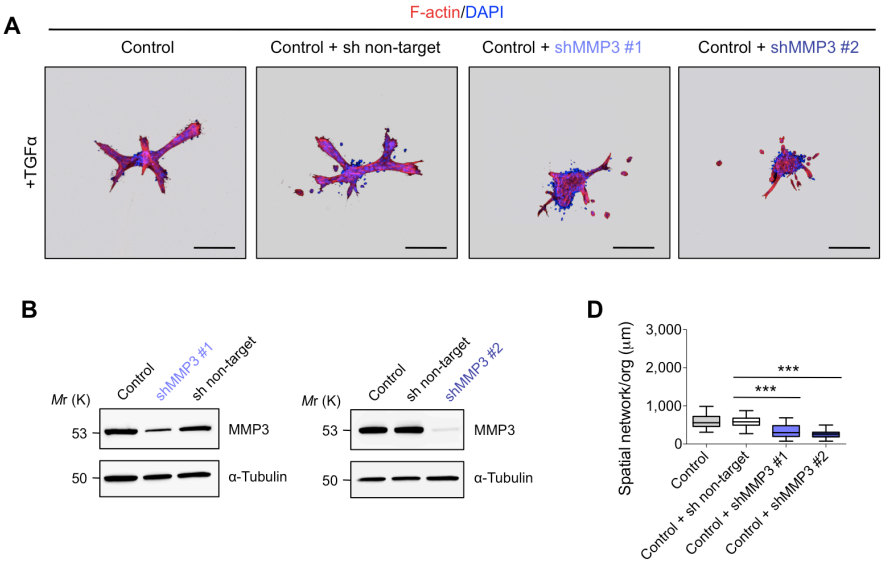


Figure 3.14 shRNA-mediated knockdown of MMP3 reduces invasion and branching significantly in control organoids.

(**A**) Images of maximum-intensity projection of control mammary organoids infected with either non-targeting shRNA or shRNA selectively targeting MMP3, and cultured in 3 mg/mL Col-1 gels for 3 days. Organoids were cultured in the presence of the growth factor (TGFα). Structures were stained for F-actin (red) and nuclei (DAPI; blue). Image background was pseudo-colored in grey. Scale bars: 100μm. (**B**) Blots showing silencing of MMP3 using two distinct shRNAs. Knockdown was verified by western blotting of whole cell lysates with an antibody specific for murine MMP3. α-Tubulin was used as loading control. (**C**) Silencing MMP3 decreases the size of the 'spatial network' per organoid (50 organoids were counted per culture). *** $P < 0.0001$ by Student's *t*-test.

3.4 Extracellular Interaction of MMP3 with HSP90β is Required for Invasion and Branching, with Binding Occurring via Hemopexin Domain

Having shown the relevance of the distinct domains for invasion and branching also in organoids, we examined the requirement of HSP90β in organoids transduced with different constructs receiving either recombinant protein (Figure 3.15A) or a function-blocking antibody against HSP90β (Figure 3.15B).

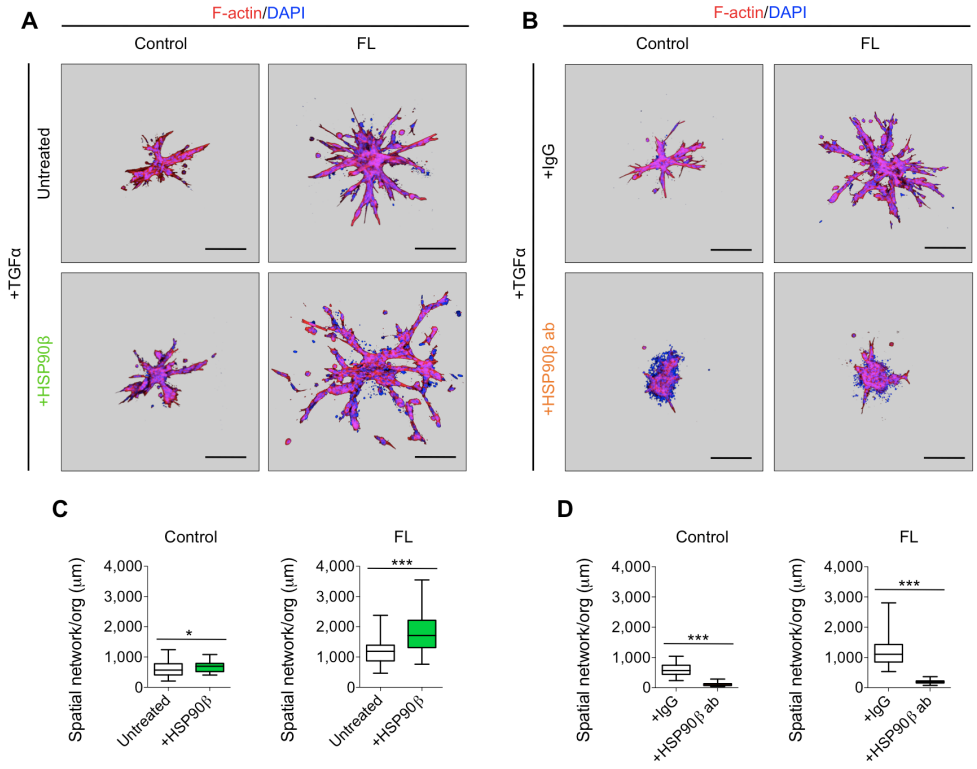


Figure 3.15 Extracellular HSP90β modulates MMP3 function in invasion and branching of mammary epithelial organoids.

(**A**) Recombinant HSP90β added to the medium increases the invasiveness of mammary organoids expressing MMP3. Images of maximum intensity projection from confocal z-stacks of mammary organoids overexpressing FL-MMP3 or control vector embedded in 3 mg/mL Col-1 gels. Organoids were cultured for 3 days in the presence or absence of a recombinant HSP90β. Structures were stained for F-actin (red) and nuclei (DAPI; blue). Image background was pseudo-colored in grey. Scale bars: 100µm. (**B**) Inhibition of extracellular HSP90β abolishes branching ability of mammary organoids. Images of maximum intensity projection from confocal z-stacks of mammary organoids overexpressing FL MMP3 or control vector embedded in 3 mg/mL Col-1 gels. Organoids were cultured for 3 days with a function-

blocking antibody against HSP90β or a control IgG. Structures were stained for F-actin (red) and nuclei (DAPI; blue). Image background was pseudo-colored in grey. Scale bars: 100μm. (**C**,**D**) Quantification of invasion and branching by measuring the 'spatial network' per organoid (50 organoids were counted per culture). *** $P < 0.0001$, * $P < 0.05$ by Student's *t*-test.

The recombinant HSP90β added extracellularly enabled the secreted MMP3 to induce the most exuberant branched structures (Figure 3.15A, bottom right) and the longest spatial network observed so far (Figure 3.15C, right). Importantly, blocking the extracellular HSP90β with inhibitory antibodies added to the medium abolished branching ability in all organoids including controls (Figure 3.15B, bottom; D). Organoids receiving the construct with deleted hemopexin domain were essentially identical to the controls (Figure 3.16A,B). Additionally, there was very little Co-IP of MMP3 with HSP90β in the absence of exogenous HSP90β (Figure 3.16C). These findings identify the crucial role of extracellular HSP90β in mammary epithelial invasion and branching with binding occurring in the presence of the hemopexin domain of MMP3.

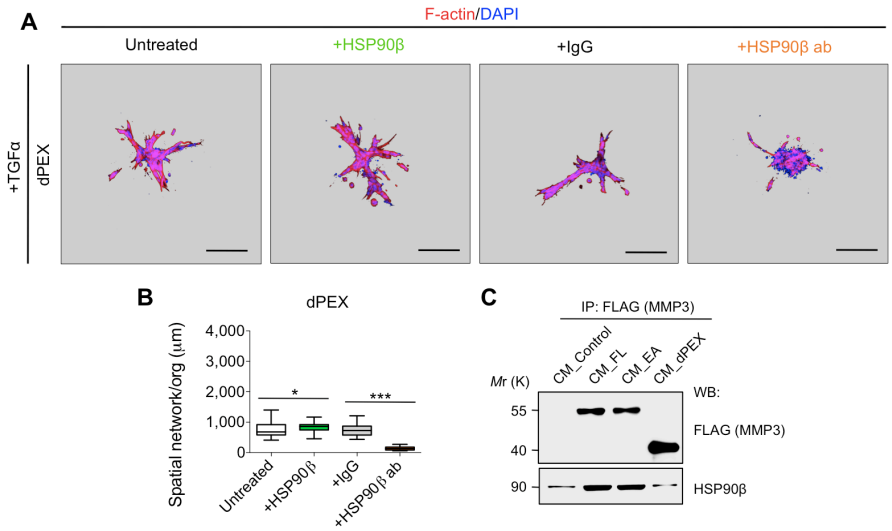


Figure 3.16 Binding of HSP90β to MMP3 occurs in the presence of the hemopexin domain.
(**A**) Addition of recombinant HSP90β to organoid cultures overexpressing MMP3 without the hemopexin domain induces a small but significant increase in invasiveness, whereas blocking of extracellular HSP90β results in complete inhibition of invasion and branching. Images of maximum intensity projection from confocal z-stacks of mammary organoids overexpressing dPEX-MMP3 embedded in 3 mg/mL Col-1 gels. Organoids were cultured for 3 days in the presence of recombinant protein or a function-blocking antibody against HSP90β. Untreated cultures or organoids treated with normal IgG were used as controls. Structures were

stained for F-actin (red) and nuclei (DAPI; blue). Image background was pseudo-colored in grey. Scale bars: 100μm. (**B**) Quantification of invasion and branching by measuring the 'spatial network' per organoid (50 organoids were counted per culture). (**C**) Co-IP of each mutant shows the association between MMP3 and HSP90β via hemopexin domain. FLAG-tagged MMP3 FL, EA and dPEX were immunoprecipitated from CM of organoid cultures with an anti-FLAG antibody, and blotted with an antibody specific for HSP90β. *** $P < 0.0001$, * $P < 0.05$ by Student's *t*-test.

The fact that HSP90α and HSP90β are closely related isoforms of HSP90, showing 86.3% identity in human homologues (Gupta 1995), poses the question of whether HSP90β regulates MMP3 function specifically. Our proteomic screen identified only HSP90β as interacting with MMP3 extracellularly in SCp2 cells (Figure 3.6). Additionally, whereas both isoforms were present intracellularly in organoids from the mammary gland, only HSP90β was found in the extracellular milieu (Figure 3.17). It has been previously shown that MMP2 can interact with HSP90α (Eustace et al. 2004), and this association is increased by the presence of a complex of co-chaperones including HSP70 (Sims et al. 2011). Despite the fact that HSP70 is expressed endogenously in our model, it was not found extracellularly in the medium (Figure 3.17). Since HSP90β and MMP3 interaction occurs extracellularly, we believe that HSP70 is not part of this complex. This is further proof that HSP90β has a specific and crucial extracellular role in the regulation of MMP3 function.

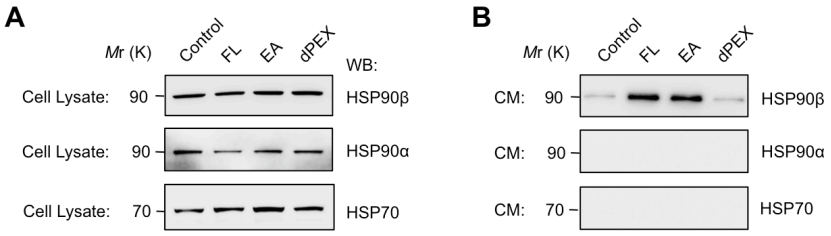


Figure 3.17 HSP90β, but not HSP90α or HSP70, is present extracellularly in mammary organoid cultures.
Blots showing the endogenous expression (**A**) and extracellular presence (**B**) of HSP90β, HSP90α and HSP70 in mammary organoid cultures. Whole cell lysates or conditioned-medium (CM) were isolated from organoids transduced with each of the MMP3 constructs and the control vector. HSP90β, HSP90α and HSP70 were detected with specific antibodies for each protein.

3.5 Coordinated expression of HSP90β and MMP3 regulates epithelial cell function during post-natal mammary gland development

Having shown that HSP90β and MMP3 act together to regulate mammary epithelial invasion and morphogenesis, we asked whether these two proteins are developmentally regulated in the murine mammary gland. We measured the levels of *Hsp90ab1* and *Mmp3* in different stages of mammary gland development (Figure 3.18).

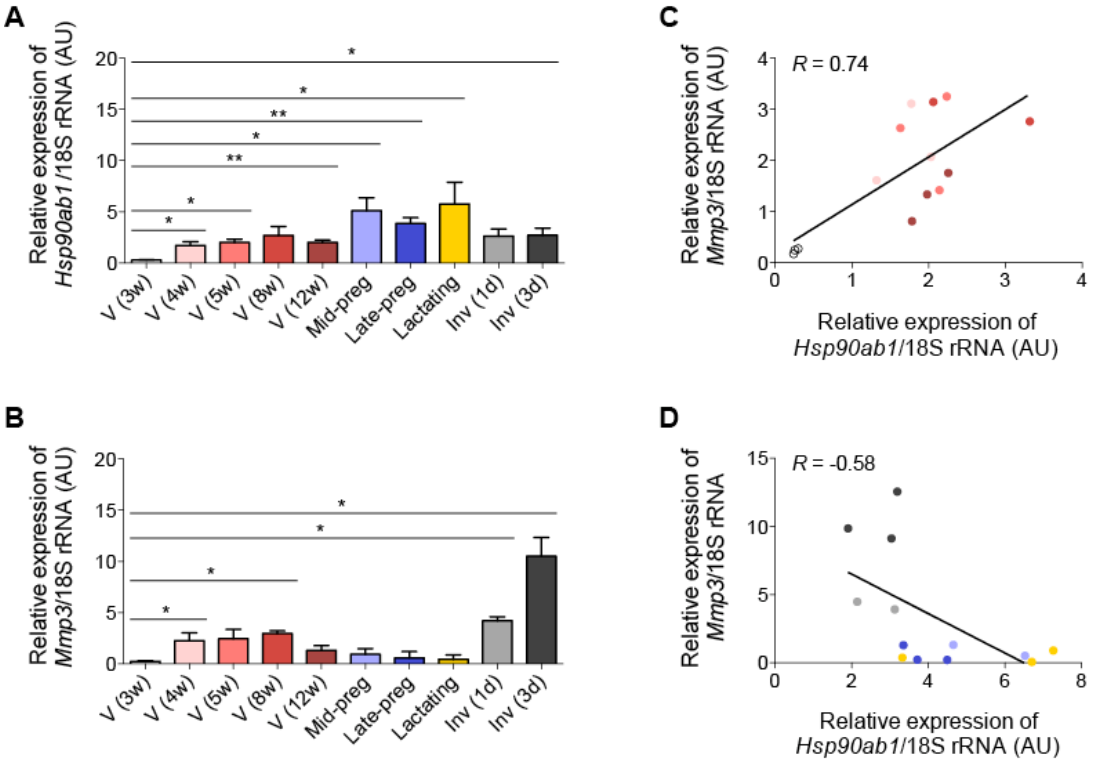


Figure 3.18 The levels of expression of *Hsp90ab1* and *Mmp3* are significantly correlated during mouse post-natal mammary gland development.
(**A**,**B**) qPCR analysis of *Hsp90ab1* and *Mmp3* expression during distinct stages of mouse post-natal mammary gland development: virgin (3-, 4-, 5-, 8- and 12-weeks), mid-pregnant (8-12 days gestation), late-pregnant (16-19 days gestation), lactating, and involuting (1 and 3 days after weaning). Transcript levels of *Hsp90ab1* and *Mmp3* are normalized to 18S rRNA. Results are indicated as mean ± s.d. from three different animals. (**C**) *Hsp90ab1* and *Mmp3* are positively correlated in mammary glands from pubertal females (Spearman correlation $R = 0.74$, $P = 0.0027$). (**D**) *Hsp90ab1* and *Mmp3* are inversely correlated during pregnancy, lactation and involution (Spearman correlation $R = -0.58$, $P = 0.0376$). Dot colors correspond to the different developmental stages plotted in (**A**,**B**). ** $P < 0.001$, * $P < 0.05$ by Student's *t*-test.

We observed that the expression levels of both *Hsp90ab1* and *Mmp3* increased during puberty (Figure 3.18A,B, virgin 3, 4, 5 and 8) and were significantly correlated (Figure 3.18C). However, *Mmp3* levels dropped to nearly zero at the onset of pregnancy, remained minimal until the end of lactation, and peaked during involution (Figure 3.18B, purple, yellow and grey bars, respectively). In sharp contrast, *Hsp90ab1* expression peaked in pregnant and lactating glands, and subsided again at involution (Figure 3.18A, mid- and late-preg, lactating and inv 1 and 3). Nevertheless, the transcript concentration of the two genes was significantly correlated also during this period, but in an inverse manner (Figure 3.18D). Additionally and importantly, we found that *Hsp90ab1* and *Mmp3* exhibited an unusual pattern of expression across different stages of mammary gland development: *Mmp3* expression was only observed in a certain concentration range of *Hsp90ab1* (between 1.2 and 3.2 AU; Figure 3.19). This pattern resembles the performance of an electronic band-pass filter, which passes frequencies within a certain range and attenuates frequencies outside that range. These data suggest that *Hsp90ab1* and *Mmp3* may belong to the same regulatory module, and their temporal expression serves different functions during mammary gland development.

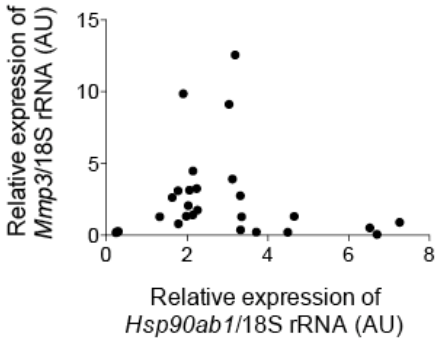


Figure 3.19 The expression pattern of *Hsp90ab1* and *Mmp3* resembles the performance of a band-pass filter.
Dots represent the transcript levels of *Hsp90ab1* and *Mmp3* normalized to 18S rRNA, and correspond to the different developmental stages plotted in Figure 3.18.

We then asked whether the developmental regulation of HSP90β and MMP3 is defined also by differential localization in the mammary gland. Immunostaining on mammary gland sections from different post-natal developmental stages revealed that both proteins were located in the epithelial and

stromal compartments, but HSP90β appeared preferentially in the epithelium (Figure 3.20).

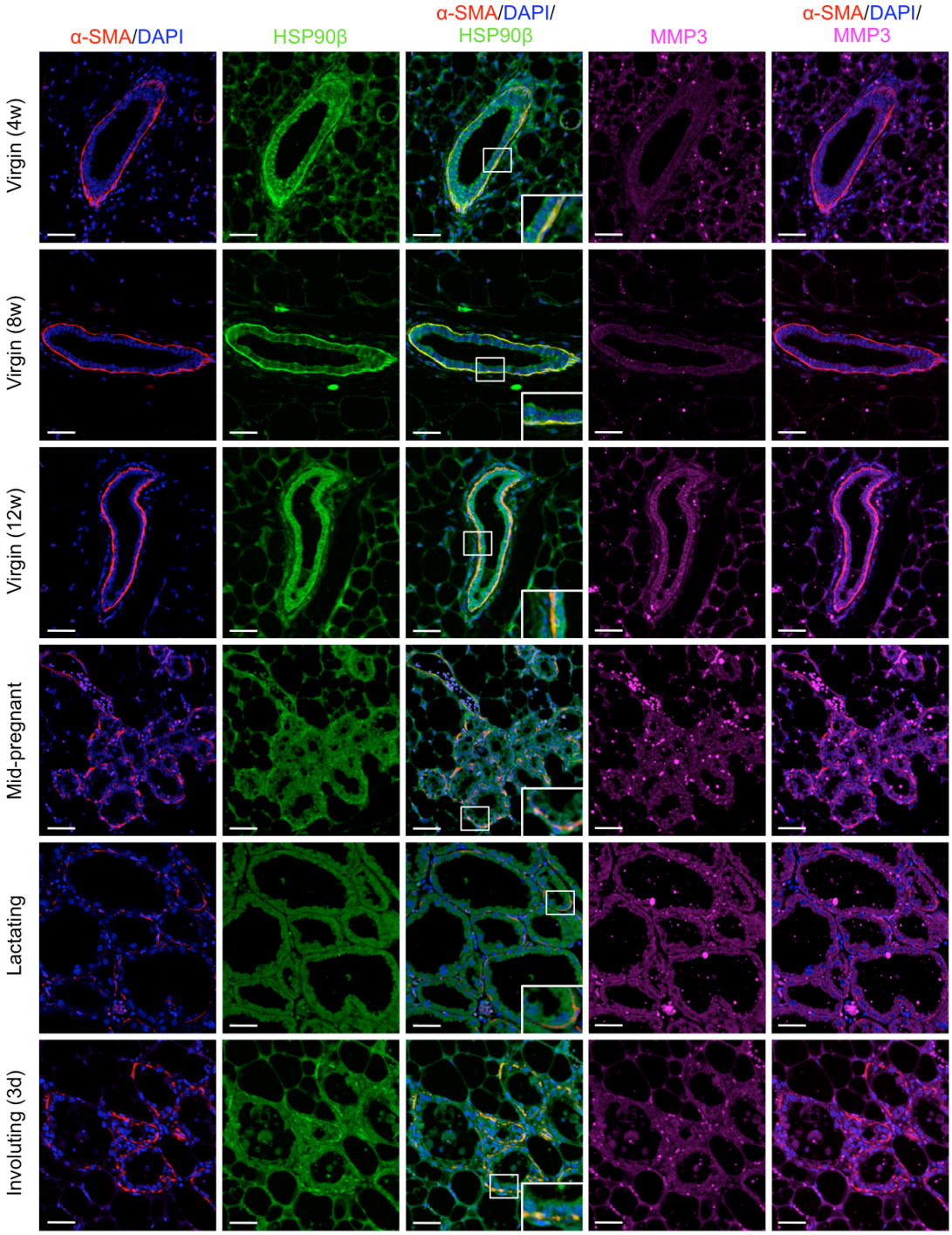


Figure 3.20 HSP90β is predominantly located in mammary epithelium, and shifts localization from the myoepithelial cells (MEPs) during puberty to the luminal epithelial cells (LEPs) in the mature female.

Immunostaining of HSP90β and MMP3 performed on mammary gland sections from different post-natal developmental stages: virgin (4-, 8- and 12-weeks), mid-pregnant (8-12 days ges-

tation), lactating, and involuting (3 days after weaning). Insets show a magnification of the bilayered mammary duct. Images show α-SMA (red) and nuclei (DAPI; blue). α-SMA was used to recognize MEPs, as well as delineate the boundary between the stromal and epithelial compartments. Scale bars: 10μm.

Importantly, the localization of HSP90β shifted from the myoepithelial cells (MEPs) in the pubertal mammary gland to the luminal epithelial cells (LEPs) in the mature female, as illustrated by the loss of co-localization with alpha smooth muscle actin (α-SMA) at age 12-weeks (Figure 3.20). The strong HSP90β immunostaining in LEPs persisted throughout the pregnancy cycle. These observations suggest that a combination of spatial and temporal regulation of HSP90β and MMP3 may determine epithelial function during post-natal mammary gland development.

Chapter 4

Discussion

The importance of MMPs for sculpting the architecture of branched organs is well accepted. This statement is demonstrated in particular in the mammary gland. Others and we showed that overexpression of MMP3 in mammary epithelia enhanced lateral branching and precocious alveolar development in virgin mice (Sympson et al. 1994; Witty et al. 1995). These mice eventually developed tumors that exhibited chromosomal aberrations (Sternlicht et al. 1999) through a mechanism dependent on ROS and RAC1B, a spliced variant of RAC1 (Radisky et al. 2005). Conversely, we showed that MMP3 controls lateral branching *in vivo* (Wiseman et al. 2003) and in Col-1 gels (Simian et al. 2001).

In many of these experiments, others and we had assumed that the catalytic domain of MMP3 was responsible for these functions. More recently, there has been some biochemical evidence that the hemopexin domain of some MMPs has a role in the non-proteolytic function. Mori et al. (2002) and Dufour et al. (2008) examined the role of the hemopexin domain of MMP14 and MMP9 in cancer cells and fibroblasts, respectively, and showed that it is necessary for cell migration (Mori et al. 2002; Dufour et al. 2008). Likewise, the hemopexin domain, but not the catalytic activity, of MMP12 was shown to be required for the antimicrobial function of this enzyme (Houghton et al. 2006). The only clear evidence for the physiological relevance of hemopexin domain in vivo came from a report by Glasheen et al. in *Drosophila* (Glasheen et al. 2009); these investigations showed that

whereas the catalytic domain was still required for all MMP functions, the hemopexin domain was specifically implicated in invasion during metamorphosis.

Neither the requirement for the hemopexin domain of MMP3 nor the surprising interaction with extracellular HSP90β were known or reported previously. Here we show that cells and tissues that overexpress MMP3 but lack catalytic activity can invade and branch easily in 3D Col-1 gels. Additionally and importantly, we show that the functional activity of the hemopexin domain of MMP3 requires extracellular interaction with HSP90β.

The previous literature on functions of HSP90 place its activity essentially within the cell, where it works as a "hub of protein homeostasis" by facilitating the maturation of a wide range of proteins (Taipale et al. 2010). It is only with regard to HSP90α that the extracellular function has been mentioned. A number of investigators has shown that the α isoform of HSP90 is present in CM of either cancer cells or 'wounded cultures' (Eustace et al. 2004; Li et al. 2007; Cheng et al. 2008). Our discovery that the extracellular HSP90β is essential for MMP3-driven invasion and branching adds a new dimension to this chaperone's functions. Despite the fact that HSP90α and HSP70, which was shown previously to increase the association between MMP2 and HSP90α *in vitro* (Sims et al. 2011), are present intracellularly in our model, they are not found in the extracellular milieu. That HSP90β has a crucial extracellular function was shown by addition of specific inhibitory antibodies to the medium, resulting in complete inhibition of branching. These data indicate that the presence of HSP90β in the medium is a selective process and it is not due to cell lysis or apoptosis.

Mice deficient for HSP90β fail to develop a placental labyrinth and die around mid-gestation (Voss et al. 2000). This fact prevented us from characterizing their mammary gland development *in vivo*. Additionally, despite the fact that *Mmp3*-null mice are viable and fertile, they compensate the reduced secondary and tertiary branching phenotype by day 70 (Wiseman et al. 2003). The use of ECM gels, however, has allowed us to elucidate the role of different domains of MMP3, as well as prove that extracellular HSP90β regulates MMP3 function in invasion and branching through interaction with the hemopexin domain. The primary organoids develop into hun-

dreds of mini mammary epithelial trees, thus offering a model of mammary epithelial development in a robust and manipulable format.

The signaling pathways and regulatory mechanisms that drive branching in mammalian organs have been described by a number of laboratories including ours, and involve multiple members of the receptor tyrosine kinase (RTK) family (reviewed in (Lu and Werb 2008)). Sustained activation of MAPK ERK$^{-1,2}$, in response to HGF, was shown to be required for kidney epithelial morphogenesis in Col-1 gels (Maroun et al. 2000). We showed that the MAPK ERK$^{-1,2}$ pathway also integrates distinct and antagonistic signals from TGFα and FGF7 to determine the final morphogenetic response of mammary organoids cultured in lrECM; sustained MAPK activation downstream of TGFα and EGFR induces branching whereas its transient activation downstream of FGF7 and FGFR2 stimulates proliferation but not branching (Fata et al. 2007). FGF7 acts in part by suppressing the expression of MMP3, and inhibition of the latter reduces branching significantly both in culture and in vivo (Simian et al. 2001; Wiseman et al. 2003). Our discovery that extracellular HSP90β is critical for MMP3 function in invasion and branching, places HSP90β as an important player in the signaling pathways that determine the final mammary morphogenetic fate.

The presence of HSP90 in murine mammary gland was reported in 1989 (Catelli et al. 1989), therefore it is surprising that its role in functional and morphogenetic aspects of the mammary gland is still poorly understood. The fact that HSP proteins have been postulated as molecular chaperones that mitigate the life-threatening effects of heat and other stresses on the proteome (Taipale et al. 2010), poses the question of whether HSP90β may also play a role in stabilization and maturation of MMP3. We are now beginning to understand that HSP90 functions extend well beyond stress tolerance, and associated changes in its clients can then exert marked effects on the relationship between genotype and phenotype, influencing human health, disease and evolutionary processes (Rutherford and Lindquist 1998; Queitsch et al. 2002; Cowen and Lindquist 2005). The presence of HSP90β in the medium and the functional significance of its interaction with MMP3 is further proof that HSP90-mediated events are above and beyond the heat shock response. Our preliminary data indicate that the

extracellular source of HSP90β for luminal epithelial branching most probably is the myoepithelial cells *in vivo*. These data combined with some evidence that MMP3 is mainly produced by stromal fibroblasts (Witty et al. 1995; Kouros-Mehr et al. 2006) raise the exciting possibility that extracellular interaction of HSP90β with MMP3 may be a way for different cell types to communicate in coordination of the normal processes of invasion and branching.

The fact that the levels of expression of *Hsp90ab1* and *Mmp3* are positively correlated in pubertal mammary glands reaffirms their importance in sculpting the epithelial tree during branching morphogenesis. We show also that the developmental regulation of these two genes extends to the mature female, albeit in an inverse manner: *Hsp90ab1* is positively related with mammary functional differentiation, being remarkably increased in pregnancy and lactation, whereas *Mmp3* peaks at involution, initiating a remodeling program that results in loss of the differentiated lactational phenotype. These observations are consistent with the previously identified role of MMP3 in stage two of mammary involution (Talhouk et al. 1992), and the highest concentration of HSP90 found in lactating mammary glands (Catelli et al. 1989). The regulatory mechanisms underlying the transcription and expression of *Hsp90ab1* and *Mmp3* during mammary gland development remain to be explored. Nevertheless, we found that *Mmp3* is only expressed in a certain concentration range of *Hsp90ab1*, which points to an expression pattern analogous to the performance of an electronic band-pass filter. These data suggest also that *Hsp90ab1* and *Mmp3* may belong to the same regulatory module, and their temporal expression serves different functions during mammary gland development.

In the initial mass spectrometry data we found many additional molecules that appear to be interacting with MMP3. In particular, we show that ANXA2 and MARCKS were co-immunoprecipitated with MMP3, with binding occurring in the presence of the hemopexin domain. Our preliminary data showed also that depletion of each of these proteins reduced invasiveness in SCp2 cells. Unlike the interaction between HSP90β and MMP3 that happened in both directions, the reverse Co-IP of ANXA2 and MARCKS with HSP90β could not be confirmed under these conditions. In addition, our

proteomic screen identified other proteases such as ADAM10, ADAMTS15 and Cathepsins A and L as possible proteins that may interact extracellularly with MMP3. The functional significance of these latter proteins remains to be determined. Our data from the mass spectrometry however, tentatively suggest that a cascade of proteases might function collectively to orchestrate epithelial invasion.

Finally, we have shown most recently that the signaling module for MMP14, a membrane bound MMP, in branching of the end bud of the mammary gland of virgin mice is its transmembrane/cytoplasmic domain in conjunction with integrin-β1 (Mori et al. 2013). Thus the findings presented here along with the above work may provide a compelling explanation for why inhibitors of MMPs failed so dramatically in the clinic (Overall and Kleifeld 2006). Targeting non-catalytic sites of MMPs as well as the interacting partners, with agents such as small inhibitors or antibodies for the binding sites of integrin-β1 and HSP90β, may yield more effective and tissue-specific inhibitors.

Chapter 5

Conclusion and Future Perspectives

The main conclusions of the present study are the following:

- Overexpression of MMP3 constructs without catalytic activity is sufficient to direct mammary epithelial invasion in Col-1 gels.
- The signaling module for MMP3 in invasion and branching is its hemopexin domain.
- The functional activity of MMP3 hemopexin domain requires the surprising interaction with HSP90β in the extracellular milieu.
- The interaction of MMP3 with HSP90β is necessary for invasion and branching, not only in cultured cells, but also in primary organoids where the mammary architecture remains intact.
- Blocking HSP90β with inhibitory antibodies added to the medium abolished invasion and branching.
- The levels of expression of *Hsp90ab1* and *Mmp3* are positively correlated in pubertal mammary glands, reaffirming their importance in sculpting the epithelial tree during branching morphogenesis. However, *Hsp90ab1* and *Mmp3* are inversely correlated during pregnancy, lactation and involution, indicating that their temporal ex-

pression serves different functions during mammary gland development.

- The localization of HSP90β shifts from the myoepithelial cells in the pubertal mammary gland to the luminal epithelial cells in the mature female, suggesting that spatial regulation of HSP90β and MMP3 may determine epithelial cell function during post-natal mammary gland development.

These findings introduce an alternative to the classic paradigm of MMP3 activity and point to an HSP90β-mediated regulation of MMP3 function essential for epithelial invasion and mammary morphogenesis (Figure 5.1).

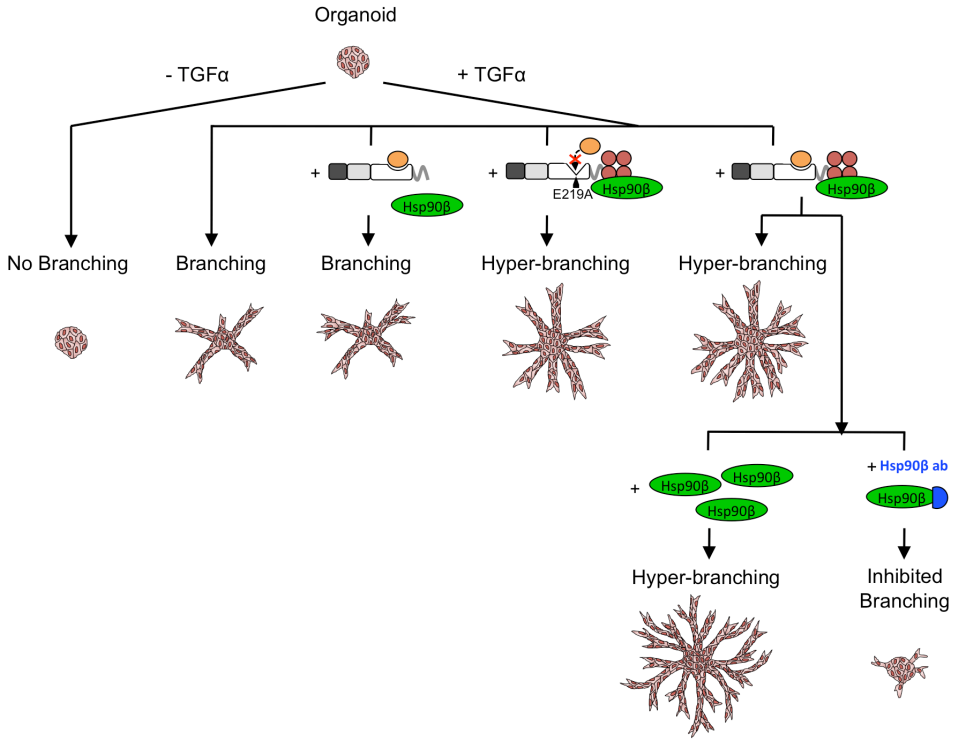


Figure 5.1 Scheme of the essential role of extracellular HSP90β in the modulation of MMP3-driven invasion and branching in mammary organoids.
When organoids from the mammary gland are embedded within 3D gels of Col-1, they undergo invasion and branching morphogenesis upon addition of growth factors. The small endogenous MMP3 activity present in the organoids provides them a baseline of branching to which we could compare the exogenous constructs. The insertion of exogenous MMP3 induces a hyper-branched phenotype only when the hemopexin domain is present. This region mediates the extracellular interaction with HSP90β and is critical for the invasive function of MMP3. Recombinant HSP90β added extracellularly enables the secreted MMP3 to induce the most exuberant branched structures. Conversely, blocking of extracellular HSP90β with inhibitory antibodies added to the medium abolishes branching ability.

This work raises additional questions that should be addressed in future studies:

- The interaction between HSP90β and MMP3 will be fascinating to explore in detail. Solving the structure of this regulatory complex and mapping the binding site of HSP90β to MMP3 would be extremely valuable for designing specific inhibitors of that interaction.

- Given that HSP90α and HSP90β are closely related isoforms of HSP90, showing 86.3% identity in human homologues, it is quite intriguing that only HSP90β appears in the medium of mammary epithelial cells. Why HSP90α is not found extracellularly in this model could be a very interesting subject for a future publication.

- The fact that HSP90β does not have a signal peptide poses the question of how is it secreted outside the cell. We have some evidence suggesting that HSP90β may be exported by a non-classical secretory pathway in exosomes (data not shown), and we intend to explore this more in detail in the future.

- Since MMP3 upregulation seems to be associated with several pathological situations, it would be interesting to study the role of HSP90β-mediated signaling in the processes of MMP3-dependent cancer cell invasion and arthritis.

- The regulatory mechanisms underlying the transcription and expression of *Hsp90ab1* and *Mmp3* during mammary gland development remain also to be explored. Based on our preliminary data suggesting that these two genes belong to the same regulatory module, it would be important to elucidate whether they are regulated by a common upstream gene, or *Hsp90ab1* regulates *Mmp3* directly.

Chapter 6

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Appendices I-III

Appendix I

"The hemopexin domain of MMP3 is responsible for mammary epithelial invasion and morphogenesis through extracellular interaction with HSP90β"

Correia, A.L., Mori, H., Chen, E.I., Schmitt, F.C., and Bissell, M.J.

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The hemopexin domain of MMP3 is responsible for mammary epithelial invasion and morphogenesis through extracellular interaction with HSP90β

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Matrix metalloproteinases (MMPs) are crucial mediators in sculpting tissue architecture, and are required for many physiological and pathological processes. MMP3 has been shown to regulate branching morphogenesis in mammary gland. Ectopic expression of proteolytically active MMP3 in mouse mammary epithelia triggers supernumerary lateral branching and eventually tumors. Using a 3D collagen-1 (Col-1) gel assay that simulates epithelial invasion and branching, we show that it is the hemopexin domain that directs these processes. Using three different engineered constructs containing a variation on MMP3 structural domains, we confirm the importance of the hemopexin domain also in primary organoids of the mammary gland. A proteomic screen of MMP3 binding partners surprisingly revealed that the intracellular chaperone, HSP90β, is present extracellularly, and its interaction with the hemopexin domain of MMP3 is critical for invasion. Blocking of HSP90β with inhibitory antibodies added to the medium abolished invasion and branching. These findings shift the focus from the proteolytic activity of MMP3 as the central player to its hemopexin domain, and add a new dimension to HSP90β's functions by revealing a hitherto undescribed mechanism of MMP3 regulation. Our data also may shed light on the failure of strategies to use MMP inhibitors in cancer treatment and other related disorders.

Keywords: Mammary morphogenesis, epithelial invasion and branching, MMP3, hemopexin domain, HSP90β

Prior to the defining functions of the mammary gland, i.e. pregnancy and lactation, the female mammal develops an epithelial tree through branching morphogenesis. During this process, epithelial cells have to mobilize the necessary machinery for *invasion* of the growing ducts into the fat pad and formation of secondary and tertiary branches to complete the eventual adult mammary architecture. It has been shown that the success of this process relies on activities of a number of MMPs (Fata et al. 2004; Khokha and Werb 2011). Paradoxically, the loss of mammary structure also is dependent on MMPs. Indeed, we showed two decades ago that during the process of involution, up regulation of MMP3 is responsible for the collapse and remodeling of the alveoli of lactating mice, indicating the intimate connection between functional differentiation and tissue structure (Talhouk et al. 1991; Talhouk et al. 1992). Conditional activation of MMP3 in functionally normal mouse mammary epithelial cells led to cleavage of E-cadherin and epithelial-to-mesenchymal transitions (EMT) (Lochter et al. 1997a). We showed also that ectopic expression of constitutively active MMP3 in mammary epithelia enhanced lateral branching and induced precocious alveolar development in virgin mice (Sympson et al. 1994). As these animals aged, the stroma was profoundly altered in both structure and function (Thomasset et al. 1998) and mice eventually developed mammary tumors that exhibited chromosomal aberrations (Sternlicht et al. 1999). The mechanism involved a change in the cytoskeleton and cell shape through induction of RAC1B, a spliced isoform of RAC1 found in human breast tumors (Schnelzer et al. 2000). Addition of MMP3 or the expression of RAC1B also led to formation of reactive oxygen species (ROS) and genomic instability (Radisky et al. 2005).

Because the proteolytic activity of MMPs resides within the catalytic domain, it has been generally assumed that this domain is responsible for all the functions of MMPs. More recently some biochemical literature has indicated that the non-catalytic domains of certain MMPs, such as MMP-9, -12 and -14, may also have activities in mammalian cell lines (Mori et al. 2002; Wang et al. 2004; Dufour et al. 2008; Sakamoto and Seiki 2009). The failure of clinical trials based on inhibitors of MMP catalytic domains (Overall and Kleifeld 2006) suggested to us that the other domains of MMP3 may have functions in invasion and possibly cancer.

Here we show that overexpression of MMP3 constructs without catalytic activity is sufficient to direct mammary epithelial invasion in Col-1 gels. Additionally, the functional activity requires the surprising interaction of HSP90β with MMP3 in the extracellular milieu. This interaction is necessary for invasion and branching, not only in cultured cells, but also in primary organoids where the mammary architecture remains intact. We believe these findings introduce an alternative to the classic paradigm of MMP3 activity and point to an HSP90β-mediated regulation of MMP3 function essential for epithelial invasion and mammary morphogenesis.

Results

The hemopexin domain of MMP3 is required for a change in cell shape in 2D substrata and invasion in Boyden chambers

To investigate the function of different domains of MMP3, we engineered three FLAG-tagged constructs containing different domains of the MMP3 molecule: a wild type (FL) MMP3, a mutant lacking the hemopexin-like domain (dPEX) and a construct containing a point mutation E219A (EA) at the catalytic core (Figure 1A). We overexpressed the distinct MMP3 constructs in SCp2 (Figure 1B), a mammary cell line shown to undergo EMT upon expression of MMP3 (Lochter et al. 1997a; Radisky et al. 2005). SCp2 cells have a low level of endogenous MMP3 activity that resembles that found *in vivo* in mammary epithelia; we chose to maintain this activity advisedly to have a positive control for the overexpression of the human homologues in murine cells. This was additionally useful because we observed that the concurrent knockdown of endogenous MMP3 and the introduction of the exogenous levels of the human constructs would lead to aberrant cell behavior. To compare

the cultures transduced with different constructs to each other and to the control, we ensured that the endogenous as well as the exogenous levels of MMP3 were comparable in all engineered cell lines (Figure S1). Overexpression of the exogenous constructs in SCp2 showed that the proteolytic activity (measured by casein-quenched degradation) in dPEX was similar to FL and they both were higher than EA-SCp2 or control cells (Figure 1C).

Cell scattering is a functional consequence of EMT (Vincent-Salomon and Thiery 2003); overexpression of FL-MMP3 induced scattering in 2D cultures (Figure 1D, first and second rows). The EA mutant also stimulated a spindle-shaped morphology and scattered phenotype, albeit to a lower extent (Figure 1D, third row). In contrast, dPEX-SCp2 did not scatter and resembled the control cultures (Figure 1D, fourth row). Others and we have shown that E-cadherin is a substrate for MMP3 and its loss is associated with scattering (Lochter et al. 1997a; Noe et al. 2001). Consistent with these observations, we found that FL and dPEX-MMP3 both reduced the expression of E-cadherin (Figure 1E, second and fourth rows) by shedding its extracellular domain (Figure S2A). Surprisingly, however, EA-SCp2 cells (which lack the proteolytic activity) still exhibited a stretched phenotype even in the presence of E-cadherin levels similar to control cultures (Figure 1E, third row), suggesting that the ability of MMP3 to disrupt epithelial morphology was due to activities residing in its other domains.

Using changes in cell morphology and reorganization of filamentous actin (F-actin) as additional endpoints, we observed that in dPEX-SCp2 and control cultures, F-actin was predominantly organized in cortical bundles and cells had a classical epithelial morphology in 2D (Figure 1F, first and last rows). In sharp contrast, actin filaments were extended in FL and EA-SCp2 cultures and cells were elongated (Figure 1F, second and third rows). We quantified these morphological changes by calculating the ratio of the longest (length) to the shortest (width) axis of the cell that we refer to as cellular elliptical factor (Figure 1G). Whereas FL and EA-SCp2 displayed elliptical factor >2, cells expressing control vector or dPEX had elliptical factors close to 1. These observations show a critical role for MMP3 hemopexin domain in altering epithelial cell shape.

Despite the small amount of proteolytic activity of SCp2 cells, these exhibit little invasive behavior (Lochter et al. 1997b); the same is true in SCp2 cells transduced with control vector (Figure 1H, control). SCp2 transduced with FL-MMP3 had the highest invasive rate, followed by EA and dPEX-SCp2, respectively (Figure 1H). These data indicate that despite the background proteolytic activity, MMP3 requires the hemopexin domain to induce invasion in SCp2 cells. A similar trend was obtained with EpH4, another mouse mammary epithelial cell line (Figure S2B-E).

Proteomic screen identifies HSP90β as interacting with the hemopexin domain of MMP3

Because MMP3 is a secreted protein, we asked whether the secreted form of this enzyme and its mutants were required to induce the morphological and functional changes observed (Figure S3). Conditioned-medium (CM) from FL-SCp2 was sufficient to induce scattering, elongated shape and a substantial increase in invasion in

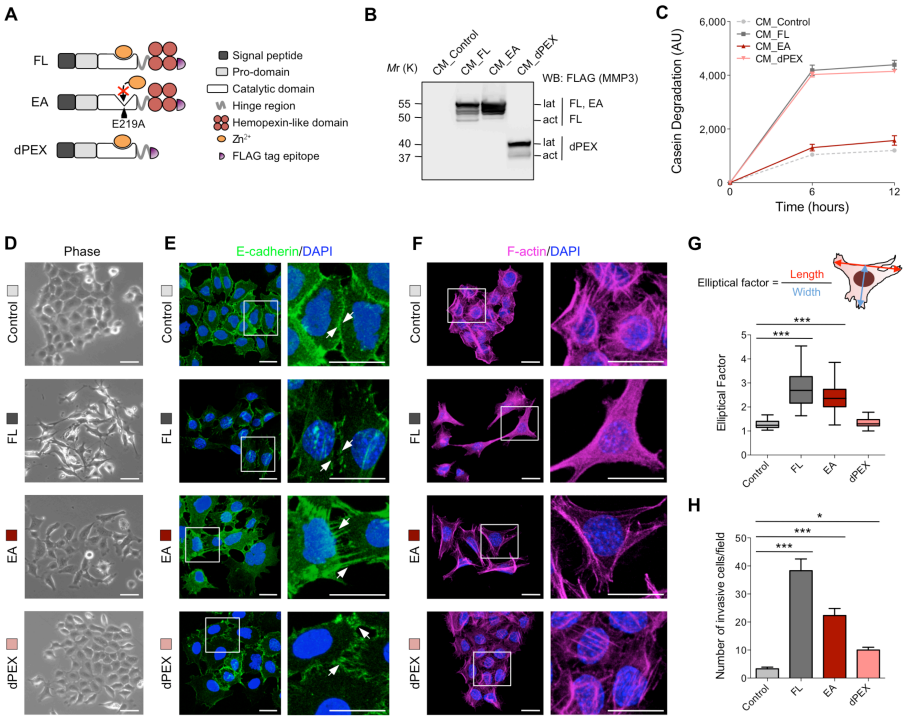

Figure 1. MMP3 hemopexin domain induces altered morphology and invasion in mammary epithelial cells. (**A**) Schematic representation of engineered constructs: the full length MMP3 (FL) and two mutants (EA and dPEX). (**B**) Overexpression of MMP3 and its mutants in SCp2 cells assessed by western blotting (WB). Conditioned-medium (CM) was isolated from cells transduced with each of the MMP3 constructs and the control vector. FLAG epitope tag was detected with anti-FLAG antibody. Both latent (lat) and activated (act) forms of MMP3 were recognized. (**C**) MMP3 proteolytic activity of SCp2 cells overexpressing each construct assayed by casein degradation. CM was incubated with a dye-quenching casein substrate (BODIPY TR-X casein). MMP3-mediated degradation of casein generated fluorescent dye-labeled peptides that were monitored over time. Fluorescence intensity is indicated as arbitrary units (AU). (**D**) Overexpression of MMP3 containing the hemopexin domain induces scattering in SCp2 cells. Scattering ability was evaluated in cells transduced with each construct upon stimulation with epidermal growth factor (EGF). Scale bars: 20μm. (**E**) The presence of the hemopexin domain of MMP3 is required to disrupt adherens-junctions. Immunofluorescence images show E-cadherin distribution (green) in cells expressing each construct. Arrows depict areas of cell-cell contact. Nuclei stained with DAPI (blue). Scale bars: 10μm. (**F**) MMP3 hemopexin domain induces reorganization of filamentous actin (F-actin). Images show F-actin (magenta) and nuclei (DAPI; blue) in each culture. Scale bars: 10μm. (**G**) Quantification of morphological changes in each culture by calculation of the cellular elliptical factor. This is defined as the ratio of the longest (length) to the shortest (width) axis of the cell. The box plot shows the median and the interquartile range, and the whiskers show the extreme values (n = 100 cells for each stable cell line. *** $P < 0.0001$ by Student's t-test). (**H**) MMP3 hemopexin domain is required for invasion. Invasiveness in each condition was assayed in Boyden chambers. Results are indicated as mean ± s.d. from three independent experiments (10 bright field images in x20 magnification were counted. *** $P < 0.0001$, * $P < 0.05$ by Student's t-test).

parental SCp2 cells. Whereas dPEX-SCp2 CM did not trigger scattering or enhance the elliptical factor, there was a small but significant increase in invasion. However when the proteolytic activity of the MMP3 construct was ablated (CM from EA-SCp2) there was still a considerable increase in invasion and cells were elongated. This finding additionally supports the fact that the hemopexin domain is required for invasion in SCp2 cells, and raises the question of whether MMP3 functions alone or depends on other factors being present in CM. The hemopexin domain of MMPs is known to interact with other proteins. MMP14 hemopexin domain was reported to be required for invasion through Col-1 (Tam et al. 2002; Wang et al. 2004) and for binding to the adhesion receptor CD44 and integrin-β1 (Mori et al. 2002; Mori et al. 2013).

To explore what other factors may be required for the functional activities of MMP3, we isolated FLAG-tagged-FL or -dPEX protein complexes from CM and performed a proteomic analysis to identify proteins that interact with MMP3 hemopexin domain (Figure 2A). Based on spectra counts, we selected proteins with abundances greater than 1.5-fold change in FL compared to dPEX (Figure 2B, left; Figure S4). Amongst the 75 proteins that passed the selection criteria, we selected myristoylated alanine-rich C-kinase substrate (MARCKS) and annexin A2 (ANXA2), which were previously implicated in regulation of cell shape, motility and invasion in *Xenopus* embryos and canine kidney cells (Iioka et al. 2004; de Graauw et al. 2008). Additionally we selected heat shock protein 90 beta (HSP90β) detected in both FL and dPEX but much higher in FL (Figure 2B, right). We validated the interaction of the hemopexin domain of MMP3 with these three proteins by co-immunoprecipitation (Co-IP) (Figure 2C).

We then asked whether this interaction is functionally significant and required for MMP3-induced invasion. We generated SCp2 cell lines co-expressing each of the MMP3 constructs and either non-targeting short hairpin RNA (shRNA; negative control) or shRNA selectively targeting each of the three proteins (Figure 2D-F). We treated parental SCp2 with CM from each engineered cell line and screened for invasion using Boyden chambers (Figure 2G-I). Whereas the knockdown did not affect invasion of cells treated with CM from control or dPEX-SCp2, it significantly reduced invasiveness of cells treated with FL or EA-SCp2 CM. These results indicate that binding of each one of these three proteins to the hemopexin domain of MMP3 has functional significance, but the inhibition was much more dramatic when HSP90β was inhibited (Figure 2G). The nature of the complexes containing MMP3 and HSP90β was clarified further by reverse Co-IP of HSP90β protein complexes from CM of control SCp2 cells (Figure S6). Whereas the association of MMP3 and HSP90β was confirmed in reverse, ANXA2 and MARCKS could not be recovered in the immunoprecipitated fraction under these conditions. This suggests that either these proteins do not exist in a single complex at a given time, but may instead represent a network of proteins interacting with one another at dif-

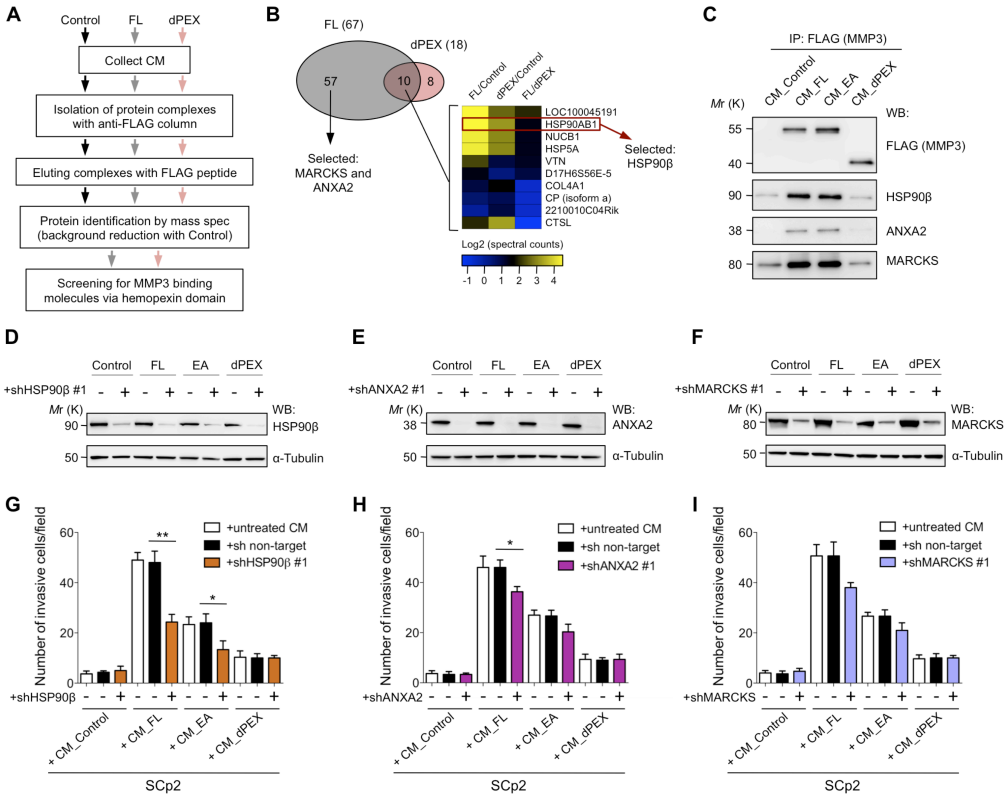

Figure 2. Proteomic screen of MMP3 binding partners reveals an extracellular role for HSP90β, ANXA2 and MARCKS in MMP3-driven invasion via hemopexin domain. (**A**) Strategy for screening MMP3 binding partners through the hemopexin domain. (**B**) Selection of MARCKS, ANXA2 and HSP90β from proteomic analysis. Left: Venn diagram showing the spectrum of proteins detected in FL and/or dPEX FLAG-immunoprecipitated samples. Right: Heat map illustrating the relative difference in abundance of proteins detected both in FL and dPEX but much higher in FL. Proteins were sorted by the highest ratio between FL and dPEX. (**C**) Co-IP of each mutant shows the association between MMP3 and the selected targets via hemopexin domain. FLAG-tagged MMP3 FL, EA and dPEX were immunoprecipitated from CM with an anti-FLAG antibody, and blotted with antibodies for its binding partners. (**D-F**) Blots showing shRNA-mediated silencing of HSP90β (**D**), ANXA2 (**E**) and MARCKS (**F**) in SCp2 cells overexpressing each of the MMP3 constructs and the control vector. Knockdowns were reproduced using two other shRNAs for each one of the targets (Figure S5A-C, G-I). Non-targeting shRNA was used as negative control. Knockdowns were verified by western blotting of whole cell lysates with antibodies specific for each target protein. α-Tubulin was used as loading control. (**G-I**) Silencing of HSP90β, ANXA2 and MARCKS reduces MMP3-driven invasion in SCp2 cells when the hemopexin domain of MMP3 is present. SCp2 cells were co-transduced with each of the MMP3 constructs and either non-targeting shRNA or shRNAs selectively targeting HSP90β (**G**), ANXA2 (**H**) or MARCKS (**I**). SCp2 parental cells were treated with CM from each engineered cell line and assayed for invasiveness in Boyden chambers. Parental cells treated with CM from SCp2 cells expressing each of the MMP3 constructs and the control vector (untreated CM) were used as control. Results are expressed as mean ± s.d. from three independent experiments (10 bright field images in x20 magnification were counted in each experiment. ** $P < 0.001$, * $P < 0.05$ by Student's *t*-test). The biological effects of shRNA-mediated knockdowns were reproduced with two other shRNAs for each of the three interacting proteins (Figure S5D-F, J-L).

ferent times for different purposes, or the interaction of the other two proteins is weak and thus cannot be detected easily by the reverse Co-IP. These data also justify the importance of HSP90β as the major player in regulation of MMP3 function.

The levels of extracellular HSP90β determine MMP3-induced invasion

Given the significance of HSP90β in cellular and tissue function, we concentrated on understanding the role of this molecule in regulating MMP3. The levels of HSP90β in each engineered cell line were tuned by adding either a recombinant protein or a specific inhibitor (CCT018159; (Sharp et al. 2007)). Increasing HSP90β levels enhanced invasion significantly in FL and EA-SCp2 (Figure S7A, second and third panels), but did not raise the invasive potential of dPEX-SCp2 or control cells significantly (Figure S7A, first and last panels). Conversely, inhibition of HSP90β reduced invasion in FL and EA-SCp2 (Figure S7B, second and third panels) and had no significant effect on dPEX-SCp2 or control cells (Figure S7B, first and last panels). The above pattern was reproduced when we used a function-blocking antibody against HSP90β and demonstrated that inhibition of extracellular HSP90β was sufficient to reduce invasiveness of FL and EA-SCp2 (Figure S7C). These data show that MMP3 is unable to perform much of its invasive functions without interacting with HSP90β in the extracellular milieu.

The hemopexin domain is required for the invasive function of MMP3 during branching morphogenesis

The finding of the critical role of the hemopexin domain in the invasion function of MMP3 in cell lines needed to be confirmed in a more physiological context. We used two culture models that simulate the normal processes of mammary invasion and branching: primary mammary organoids (Figure 3A; (Simian et al. 2001)) and cell clusters of a functionally normal mouse mammary epithelial cell line (EpH4) (Figure S8A;(Hirai et al. 1998; Mori et al. 2013)), embedded in Col-1 gels. The physiological relevance of this model is illustrated by the presence of copious amounts of Col-1 in the stroma surrounding epithelial ducts in the murine mammary gland (Williams and Daniel 1983).

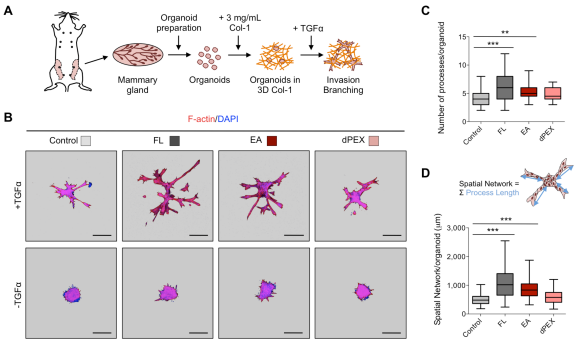

Figure 3. The hemopexin domain of MMP3 is necessary to direct epithelial invasion of mammary organoids in 3D Col-1 gels even without the proteolytic activity. (**A**) Schematic representation of primary mammary organoid preparation and culture in 3D Col-1 gels. (**B**) Overexpression of MMP3 containing the hemopexin domain enhances invasion of mammary organoids in Col-1. Images of maximum-intensity projection of mammary organoids transduced with each of the MMP3 constructs as well as the control vector, and cultured in 3 mg/mL Col-1 gels for 3 days. Organoids invaded and branched only in the presence of the growth factor (TGFα). Structures were stained for F-actin (red) and nuclei (DAPI; blue). Image background was pseudo-colored in grey. Scale bars: 100μm. (**C**) The presence of the hemopexin domain of MMP3 increases the number of extending processes developed from each organoid invading through Col-1 (150 organoids were counted per culture. *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$ by Student's *t*-test). (**D**) The size of the 'spatial network' per organoid is increased by overexpression of MMP3 containing the hemopexin domain. The 'spatial network' per organoid is defined as the sum of the length of all the extending processes of an organoid (50 organoids were counted per culture. *** $P < 0.0001$, * $P < 0.05$ by Student's *t*-test).

There are a number of advantages of using the versatile assay employing organoids from the mammary gland. Cell-cell and cell-matrix interactions remain intact, and the architecture of the tissue is not disrupted. Additionally, we can prepare enough mammary organoids from a single mouse (≈1200) and infect with the four distinct constructs. Even inbred mice are known to change biochemical and morphological characteristics at different stages of estrogen cycle as well as in response to handling and context. In this way we could control for all variations and avoid the excessive use of animals, but also achieve statistical significance. Lastly we could mark them: the presence of the GFP in the constructs indicated that more than 80% of the cells were infected. These cultures allow us not only to create a physiological condition where the organoids regenerate an epithelial tree-like structure, but also to observe and control extracellular events much more robustly.

The functional significance of the hemopexin domain was reproduced in our 3D assays with clustered EpH4 cells (Figure S8B) and most importantly with primary organoids (Figure 3B). We used two different criteria to quantify invasion and branching of organoids: the number of extended sprouts and processes developed from each structure (Figure 3C), and the 'spatial network' per organoid (Figure 3D). As expected, organoids overexpressing FL-MMP3 had the highest number of extending processes and longest spatial network, indicating that the proteolytic activity would be still necessary if the path is obstructed.

For the purpose of the current experiments, we did not distinguish between branches that were more than one cell layer thick and demonstrated basal and apical polarity, and strands that grew as a single file. However, there were very few of the latter in dPEX-overexpressing and control cultures. As mentioned above we advisedly decided against inhibiting the endogenous MMP3 activity using multiple genetic manipulations because both cells and organoids were sensitive to more than one set of viral infections. We therefore used a peptide that has been shown to inhibit MMP3 proteolytic activity effectively and specifically (Fotouhi et al. 1994; Farina et al. 2002). Inhibition of both endogenous as well as exogenous MMP3 proteolytic activity decreased branches in a dose-dependent manner in all organoids (Figure S9). Nevertheless, there still was invasion of cells individually or in a single file, with less branching than untreated cultures (Figure S9A). These data indicate that the hemopexin domain of MMP3 allows epithelial invasion, but that in the presence of proteolytic activity there are more multilayered branches. Additionally, when we knocked down MMP3 in control organoids there was a significant decrease in invasion and branching (Figure S10). This reaffirms the requirement for MMP3 for mammary branching morphogenesis, and provides additional reason for our choice of preserving the endogenous MMP3 intact.

The interaction of HSP90β with MMP3 is required for invasion

Having shown the relevance of the distinct domains for invasion and branching also in organoids, we examined the requirement of HSP90β in organoids transduced with different constructs receiving either recombinant protein (Figure 4A) or a function-blocking antibody against HSP90β (Figure 4B). The recombinant HSP90β added extracellularly enabled the secreted MMP3 to induce the most exuberant branched structures (Figure 4A, bottom right) and the longest spatial network observed so far (Figure 4C, right). Importantly, blocking the extracellular HSP90β with inhibitory antibodies added to the medium abolished branching ability in all organoids including controls (Figure 4B, bottom; 4D). Organoids receiving the construct with deleted hemopexin domain were essentially identical to the controls. Additionally, there was very little Co-IP of MMP3 with HSP90β in the absence of exogenous HSP90β (data not shown). These findings identify the crucial role of extracellular HSP90β in

mammary epithelial invasion and branching with binding occurring in the presence of the hemopexin domain of MMP3.

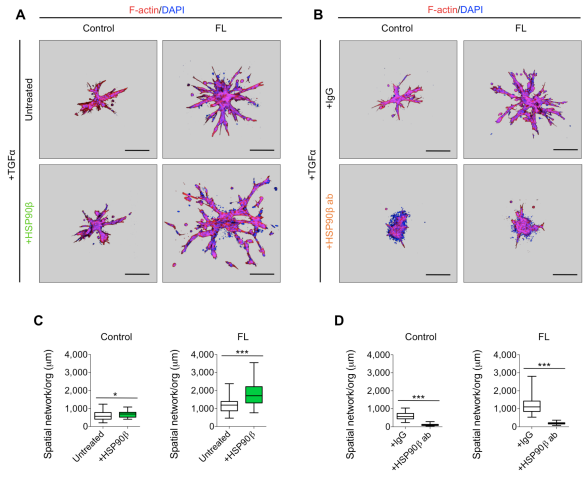

Figure 4. HSP90β enables MMP3 to modulate epithelial invasion and branching in mammary organoids. (**A**) Extracellular HSP90β is required for the invasiveness of mammary organoids expressing MMP3. Images of maximum intensity projection from confocal z-stacks of mammary organoids overexpressing FL-MMP3 or control vector embedded in 3 mg/mL Col-1 gels. Organoids were cultured for 3 days in the presence or absence of a recombinant HSP90β. Structures were stained for F-actin (red) and nuclei (DAPI; blue). Image background was pseudo-colored in grey. Scale bars: 100μm. (**B**) Inhibition of extracellular HSP90β abolishes branching ability of mammary organoids. Images of maximum intensity projection from confocal z-stacks of mammary organoids overexpressing FL MMP3 or control vector embedded in 3 mg/mL Col-1 gels. Organoids were cultured for 3 days with a function-blocking antibody against HSP90β or a control IgG. Structures were stained for F-actin (red) and nuclei (DAPI; blue). Image background was pseudo-colored in grey. Scale bars: 100μm. (**C**,**D**) Quantification of invasion and branching by measuring the 'spatial network' per organoid (50 organoids were counted per culture. *** $P < 0.0001$, * $P < 0.05$ by Student's *t*-test).

Discussion

The importance of MMPs for sculpting the architecture of branched organs is well accepted. This statement is demonstrated in particular in the mammary gland. Others and we showed that overexpression of MMP3 in mammary epithelia enhanced lateral branching and precocious alveolar development in virgin mice (Sympson et al. 1994; Witty et al. 1995). These mice eventually developed tumors that exhibited chromosomal aberrations (Sternlicht et al. 1999) through a mechanism dependent on ROS and RAC1B, a spliced variant of RAC1 (Radisky et al. 2005). Conversely, we showed that MMP3 controls lateral branching *in vivo* (Wiseman et al. 2003) and in Col-1 gels (Simian et al. 2001).

In many of these experiments, others and we had assumed that the catalytic domain of MMP3 was responsible for these functions. More recently, there has been some biochemical evidence that the hemopexin domain of some MMPs has a role in the non-proteolytic function. Mori et al. (2002) and Dufour et al. (2008) examined the role of the hemopexin domain of MMP14 and MMP9 in cancer cells and fibroblasts, respectively, and showed that it is necessary for cell migration (Mori et al. 2002; Dufour et al. 2008). Likewise, the hemopexin domain, but not the catalytic activity, of MMP12 was shown to be required for the antimicrobial function of this enzyme (Houghton et al. 2009). The only clear evidence for the physiological relevance of hemopexin domain *in vivo* came from a report by Glasheen et al. in *Drosophila* (Glasheen et al. 2009); these investigations showed that whereas the catalytic domain was still required for all MMP functions, the hemopexin domain was specifically implicated in invasion during metamorphosis.

Neither the requirement for the hemopexin domain of MMP3 nor the surprising interaction with extracellular HSP90β were known or reported previously. Here we show that cells and tissues that overexpress MMP3 but lack catalytic activity can invade and branch easily in 3D Col-1 gels. Additionally and importantly, we show that the functional activity of the hemopexin domain of MMP3 requires extracellular interaction with HSP90β (Figure 5).

The previous literature on functions of HSP90 place its activity essentially within the cell, where it works as a "hub of protein homeostasis" by facilitating the maturation of a wide range of proteins (Taipale et al. 2010). It is only with regard to HSP90α that the extracellular function has been mentioned. A number of investigators has shown that the α isoform of HSP90 is present in CM of either cancer cells or 'wounded cultures' (Eustace et al. 2004; Li et al. 2007; Cheng et al. 2008). Our discovery that the extracellular HSP90β is essential for MMP3-driven invasion and branching adds a new dimension to this chaperone's functions. Despite the fact that HSP90α and HSP70, which was shown previously to increase the association between MMP2 and HSP90α *in vitro* (Sims et al. 2011), are present intracellularly in our model, they are not found in the extracellular milieu (data not shown). That HSP90β has a crucial extracellular function was shown by addition of specific inhibitory antibodies to the medium, resulting in complete inhibition of branching (Figure 4B,D). These data indicate that the presence of HSP90β in the medium is a selective process and it is not due to cell lysis or apoptosis.

Mice deficient for HSP90β fail to develop a placental labyrinth and die around mid-gestation (Voss et al. 2000). This fact prevented us from characterizing their mammary gland development *in vivo*. Additionally, despite the fact that *Mmp3*-null mice are viable and fertile, they compensate the reduced secondary and tertiary branching phenotype by day 70 (Wiseman et al. 2003). The use of ECM gels, however, has allowed us to elucidate the role of different domains of MMP3, as well as prove that extracellular HSP90β regulates MMP3 function in invasion and branching through interaction with the hemopexin domain. The primary organoids develop into hundreds of mini mammary epithelial trees, thus offering a model of mammary epithelial development in a robust and manipulable format.

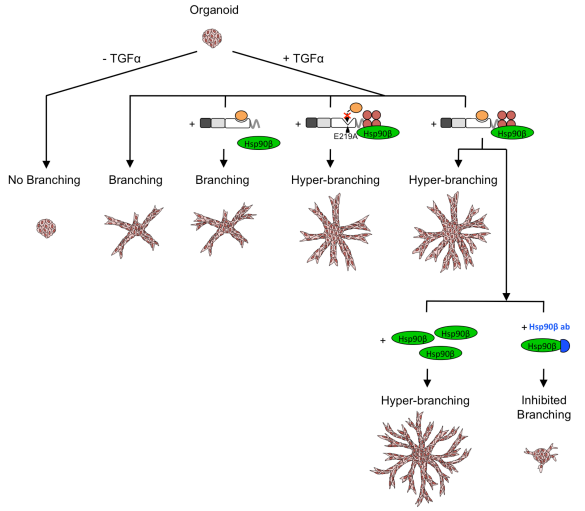

Figure 5. Scheme of the essential role of extracellular HSP90β in the modulation of MMP3-driven invasion and branching in mammary organoids. When organoids from the mammary gland are embedded within 3D gels of Col-1, they undergo invasion and branching morphogenesis upon addition of growth factors. The small endogenous MMP3 activity present in the organoids provides them a baseline of branching to which we could compare the exogenous constructs. The insertion of exogenous MMP3 induces a hyper-branched phenotype only when the hemopexin domain is present. This region mediates the extracellular interaction with HSP90β and is critical for the invasive function of MMP3. Recombinant HSP90β added extracellularly enables the secreted MMP3 to induce the most exuberant branched structures. Conversely, blocking of extracellular HSP90β with inhibitory antibodies added to the medium abolishes branching ability.

The signaling pathways and regulatory mechanisms that drive branching in mammalian organs have been described by a number of laboratories including ours, and involve multiple members of the receptor tyrosine kinase (RTK) family (reviewed in (Lu and Werb 2008)). Sustained activation of MAPK $^{ERK-1,2}$, in response to hepatocyte growth factor, was shown to be required for kidney epithelial morphogenesis in Col-1 gels (Maroun et al. 2000). We showed that the MAPK $^{ERK-1,2}$ pathway also integrates distinct and antagonistic signals from TGFα and FGF7 to determine the final morphogenetic response of mammary organoids cultured in lrECM; sustained MAPK activation downstream of TGFα and EGFR induces branching whereas its transient activation downstream of FGF7 and FGFR2 stimulates proliferation but not branching (Fata et al. 2007). FGF7 acts in part by suppressing the expression of MMP3, and inhibition of the latter reduces branching significantly both in culture and *in vivo* (Simian et al. 2001; Wiseman et al. 2003). Our discovery that extracellular HSP90β is critical for MMP3 function in invasion and branching, places HSP90β as an important player in the signaling pathways that determine the final mammary morphogenetic fate.

The presence of HSP90 in murine mammary gland was reported in 1989 (Catelli et al. 1989), therefore it is surprising that its role in functional and morphogenetic aspects of the mammary gland is still poorly understood. The fact that HSP proteins have been postulated as molecular chaperones that mitigate the life-threatening effects of heat and other stresses on the proteome (Taipale et al. 2010), poses the question of whether HSP90β may also play a role in stabilization and maturation of MMP3. We are now beginning to understand that HSP90 functions extend well beyond stress tolerance, and associated changes in its clients can then exert marked effects on the relationship between genotype and phenotype, influencing human health, disease and evolutionary processes (Rutherford and Lindquist 1998; Queitsch et al. 2002; Cowen and Lindquist 2005). The presence of HSP90β in the medium and the functional significance of its interaction with MMP3 is further proof that HSP90-mediated events are above and beyond the heat shock response. Our preliminary data indicate that the extracellular source of HSP90β for luminal epithelial branching most probably is the myoepithelial cells *in vivo* (data not shown). These data combined with some evidence that MMP3 is mainly produced by stromal fibroblasts (Witty et al. 1995; Kouros-Mehr and Werb 2006) raise the exciting possibility that extracellular interaction of HSP90β with MMP3 may be a way for different cell types to communicate in coordination of the normal processes of invasion and branching.

In the initial mass spectrometry data we found many additional molecules that appear to be interacting with MMP3. In particular, we show that ANXA2 and MARCKS were co-immunoprecipitated with MMP3, with binding occurring in the presence of the hemopexin domain. Our preliminary data showed also that depletion of each of these proteins reduced invasiveness in SCp2 cells. Unlike the interaction between HSP90β and MMP3 that happened in both directions, the reverse Co-IP of ANXA2 and MARCKS with HSP90β could not be confirmed under these conditions. In addition, our proteomic screen identified other proteases such as ADAM10, ADAMTS15 and Cathepsins A and L as possible proteins that may interact extracellularly with MMP3 (Figure S4). The functional significance of these latter proteins remains to be determined. Our data from the mass spectrometry however, tentatively suggest that a cascade of proteases might function collectively to orchestrate epithelial invasion.

Finally, we have shown most recently that the signaling module for MMP14, a membrane bound MMP, in branching of the end bud of the mammary gland of virgin mice is its transmembrane/cytoplasmic domain in conjunction with integrin-β1 (Mori et al. 2013). Thus the findings presented here along with the above work may provide a compelling explanation for why inhibitors of MMPs failed so dramatically in the clinic (Overall and Kleifeld 2006). Targeting non-catalytic sites of MMPs as well as the interacting partners, with agents such as small inhibitors or antibodies for the binding sites of integrin-β1 and HSP90β, may yield more effective and tissue-specific inhibitors.

Materials and Methods

Restriction enzymes, antibodies, proteins and chemical reagents

All restriction enzymes were acquired from New England BioLabs. Bovine dermis acid-solubilized Col-1 solution (IAC-50) was purchased from Koken. Antibodies against the following proteins were obtained as indicated: FLAG (F1804, M2, Sigma, 1:500 for WB), E-cadherin (13-1900, clone ECCD-2, Invitrogen, 1:1,000 for WB, 1:200 for IF), HSP90β (5087, Cell Signaling, 1:1,000 for WB), HSP90β (NBP1-61773, Novus Biologicals, 40 μg/mL for function-blocking experiments, 10 μg for Co-IP experiments), MARCKS (P0370, Sigma, 1:1,000 for WB), ANXA2 (AF3928, R&D Systems, 1:1,000 for WB), α-tubulin (T6074, clone B-5-1-2, Sigma, 1:5,000 for WB) and rabbit IgG (2729, Cell Signaling, 40 μg/mL for function-blocking experiments). Alexa Fluor 594 Phalloidin (A12381, Molecular Probes, 1:400) was used to stain F-actin. DAPI (Sigma) was used to stain nuclei. HSP90β inhibitor CCT018159 (385920), MMP3-specific peptide-based inhibitor (444218) and recombinant HSP90β (385903) were purchased from Calbiochem/EMD Millipore.

Construction of expression plasmids

All MMP3 mutants were constructed using a polymerase chain reaction (PCR)-based method (details in Supplemental Experimental Procedures). The cDNA sequence used as template was cloned from a human breast cell line and sequence confirmed by comparison with gene accession number NM_002422.3. FL contains the full-length MMP3 cDNA. EA is a catalytically inactive mutant, holding a point mutation E219A at the catalytic core. dPEX is a hemopexin-like domain–deleted mutant (ΔN289-C477). A mammalian expression vector, pCDH-EF1-MCS-T2A-copGFP (System Biosciences), was used to express the gene products. To detect MMP3 protein, the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was inserted at the C-terminus of every construct generated. All cDNA constructs were confirmed by DNA sequencing.

shRNA-mediated knockdowns

shRNA constructs selectively targeting HSP90β, ANXA2, MARCKS or MMP3 were purchased from MISSION shRNA library (Sigma) (sequences detailed in Table S1). Control cells were infected with non-targeting shRNA (SHC002, Sigma). Knockdown efficiency was verified by western blotting with appropriate antibodies.

Cell culture and transduction

SCp2 cells were cultured in Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient Mixture (DMEM/F-12) supplemented with 5% fetal bovine serum, 5 μg/mL insulin and 50 μg/mL gentamicin, and maintained as previously described (Desprez et al. 1993). EpH4 cells were cultured in DMEM/F-12 medium supplemented with 2% fetal bovine serum, 5 μg/mL insulin and 50 μg/mL gentamicin, and maintained as previously described (Reichmann et al. 1989). For transduction, cells were seeded in 24-well plates ($1x10^5$ cells/well) and infected with lentiviral particles carrying different expression plasmids using MISSION ExpressMag Beads (Sigma), according to the manufacturer's instructions. Cells transduced with lentivirus carrying shRNA constructs were additionally selected with 2 μg/mL puromycin.

Preparation of primary mammary organoids and transduction

Primary epithelial organoids were isolated from 8-week-old, virgin FVB mice as previously described (Fata et al. 2007). Briefly, inguinal glands were removed, minced with two parallel razor blades and gently shaken for 30 min at 37 °C in a 50 mL collagenase/trypsin mixture (0.2% trypsin, 0.2% type-IV collagenase, 5% fetal bovine serum and 5 μg/mL Insulin in DMEM/F-12). After centrifugation at 80 g for 10 min, supernant was discarded and cell pellet was re-suspended in DMEM/F-12. The suspension was pelleted again, re-suspended in 4 mL DMEM/F-12 containing 80 U of DNase I (Sigma) and incubated for 5 min at room temperature with occasional shaking. After the suspension was spun at 80 g for 10 min, a series of differential centrifugations in DMEM/F-12 was implemented to separate the epithelial organoids from single cells, fibroblasts and fibrillar extracellular matrices. The final pellet was re-suspended in the desired amount of medium. For transduction, organoids were seeded in 24-well polyhema-coated plates (1,000 organoids/well) and infected with lentivirus in the presence of 8 μg/mL polybrene for 24 h.

Preparation of cell clusters and transduction

EpH4 cells suspended in growth medium were plated in 6-well polyhema-coated plates ($1x10^5$ cells/well) and incubated at 37 °C overnight, yielding rounded clusters. Single cells were removed by differential centrifugation, and the final pellet was re-suspended in the desired amount of medium.

Branching morphogenesis assay

Primary organoids or clustered EpH4 cells were embedded in 3D Col-1 gels as previously published (Simian et al. 2001; Mori et al. 2013). In brief, acid-solubilized Col-1 solution was mixed gently on ice with 1 volume of 10x DMEM/F-12, pH adjusted to 7.4 with 0.1M NaOH, and concentration adjusted to 3 mg/mL with DMEM/F-12. A basal layer of 80 μL Col-1 was poured into each well of an 8-well chambered coverglass (155409, Thermo Scientific) and allowed to gel for 5 min at 37 °C. A second layer of 200 μL Col-1 containing 150 organoids or EpH4 clusters was added to each well and placed immediately at 37 °C. After gelation, 400μL of chemically defined medium (DMEM/F-12 containing 1% insulin/transferrin/selenium and 1% penicilin/streptomycin) with 9 nM TGFα (Sigma) or 9 nM bFGF (Sigma) was added to each well (unless stated otherwise) and replaced every other day.

After 3 days of culture, gels were fixed with 4% formalin for 30 min, and stained with phalloidin and DAPI for 1h. Structures were imaged with an upright Zeiss LSM710, using a 0.8 NA 20× air objective. An organoid or cell cluster was defined as invading and branching when it had at least three independent extending processes that were at least half the diameter of the center of the organoid or cell cluster. The number of extending processes and their average length were determined using the Imaris software (Bitplane). We defined a new metric of invasion and branching, which we refer to as the 'spatial network' per organoid. This is defined as the sum of the length of all the extending processes developed from each organoid. 50 structures were counted per condition and the experiments were executed at least 3 times.

Caseinase activity assay

CM was incubated with a casein derivative quenching red-fluorescent dye (BODIPY TR-X Casein, E6639, Invitrogen). Protease-catalyzed hydrolysis released highly fluorescent BODIPY TR-X dye-labeled peptides. The accompanying increase in fluorescence is proportional to MMP3 proteolytic activity and was monitored with a microplate reader. A control without BODIPY casein was used to subtract residual fluorescence background.

Cell scatter assay

SCp2 cells were seeded in 6-well plates at low density ($1x10^5$ cells/well), allowed to form colonies (≈ 48 h) and serum-starved for 24 h. Epithelial cell islets were then stimulated with 9 nM epidermal growth factor (EGF) (Sigma) and imaged at 48 h with a Zeiss Imager Z1 microscope, using a 10x objective.

Immunofluorescence

SCp2 cells were cultured for 72 h on glass coverslips, fixed with 4% paraformaldehyde/PBS for 10 min, washed with PBS and permeabilized in 0.25% Triton X-100/PBS for 10 min. Samples were blocked with 1% BSA and 5% goat serum/PBS for 1 h, followed by incubation with the primary antibody in blocking buffer overnight at 4 °C and the secondary antibody for 1 h at room temperature. Images were acquired with an upright Zeiss LSM710, using a 1.4 NA 63× oil-immersion.

Morphometry analysis

Cell edges were outlined in F-actin stained cells using an "Object Identification Module" from CellProfiler software (Carpenter et al. 2006). Cellular elliptical factors, defined as the ratio of the longest (length) to the shortest (width) axis of the cell, were calculated for 100 random cells per culture.

Invasion assay

Cell culture inserts (8 μm, 24-well format, BD Biosciences) were evenly coated with 20 μL diluted (1:5 in DMEM/F-12 medium) Matrigel (BD Biosciences). $1x10^5$ cells in 200 μL of DMEM/F-12 medium or different CM (as indicated in each experiment) were added to the upper compartment of the chamber. The lower compartment of the chamber was filled with 300 μL medium containing 10% FBS as a chemoattractant. After 48 h of incubation at 37 °C, the topside of the insert was cleared from non-invasive cells with a cotton swab and washed with serum-free DMEM/F-12. The remaining (invasive) cells at the lower surface of the filter were fixed and stained with a solution of Coomassie Blue 0.125% in methanol:acetic acid:H_2O (45:10:45, v/v/v) for 15 min. Invasive cells were scored by counting 10 x20 magnification fields per filter with a Zeiss Imager Z1 microscope, using a 20x objective. Mouse embryo fibroblast NIH/3T3 cells were routinely included as a positive control. Results are expressed as mean ± s.d. from three independent experiments.

Western blotting

Cells were lysed with a buffer containing 1% Triton X-100, 1% NP-40 and protease and phosphatase inhibitor cocktails (Calbiochem/EMD Millipore) in PBS, and the lysates were clarified by centrifugation at 16,000 g for 15 min. Protein concentration was determined using the BCA Protein Assay kit (Thermo Scientific) according to the manufacturer's instructions. Protein samples were mixed with electrophoresis sample buffer containing 5% (v/v) 2-β-mercaptoethanol and 5% (v/v) bromophenol blue and boiled for 5 min at 95 °C. Samples were loaded in equal amounts into pre-cast 4-20% gradient polyacrylamide gels (Invitrogen) and separated by SDS–PAGE. Resolved proteins were transferred to a nitrocellulose membrane (Whatman) at 130 V for 90 min, followed by blocking of non-specific binding with 5% BSA in 0.05% Tween-20/PBS for 1 h at room temperature. The membranes were probed with primary antibodies specific to each protein overnight at 4 °C, and then with HRP-conjugated secondary antibodies (Thermo Scientific and Santa Cruz Biotechnology). Blots were visualized with an ECL detection system (Thermo Scientific) according to the manufacturer's instructions, and chemiluminescent signal was captured with a FluorChem IS-8900 (Alpha Innotech). Each western blot was done at least three times, and here we show representative experiments.

Co-Immunoprecipitation (Co-IP).

For Co-IP of FLAG-tagged MMP3 protein complexes, CM was incubated with anti-FLAG M2 antibody-conjugated agarose beads (F2426, Sigma) for 16 h at 4 °C. The beads were then washed three times with 0.05% Tween/PBS and the immune complexes were directly eluted with electrophoresis sample buffer and analyzed by western blotting. For LC-MS/MS analysis, beads were washed with 0.05% Tween/PBS and protein complexes were eluted with a FLAG peptide (F3290, Sigma) in 0.05% Tween/PBS. Samples were then precipitated with trichloroacetic acid and reconstituted with a buffer (Invitrosol, MS10007, Invitrogen) suitable for mass spectrometry analysis.

For Co-IP of HSP90β protein complexes, CM was incubated with 10 μg of control rabbit-IgG or anti-HSP90β antibody for 16 h at 4 °C. Precipitation was performed with protein G sepharose beads (17-0618-01, GE Healthcare) for 4 h at 4 °C. The beads were then washed three times with 0.05% Tween/PBS and the immune complexes were directly eluted with electrophoresis sample buffer and analyzed by western blotting.

Mass spectrometry analysis

Described in Supplementary Methods. Scaled signal intensities were log2-transformed and analyzed by R software.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0 software. Student's *t*-test (unpaired with Welch's correction, two-tailed, 95% confidence interval) was used to determine statistical significance. Statistical analyses were always performed in relation to vector control cells (unless stated otherwise).

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Appendix II

"The role of the microenvironment in tumor initiation, progression and metastasis"

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Molecular Basis of Cancer, 4th Ed. chapter on "The Tumor Microenvironment", 2013
(in press)

Chapter 16

The Role of the Microenvironment in Tumor Initiation, Progression and Metastasis

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"In the days of seemingly unlimited funding and personnel, the shotgun approach to biology yielded substantial knowledge. Today more taste must be displayed. Because a question can be asked at the molecular level does not mean it is worth asking, any more than just another histological or electron microscopic study of developing tissue or cell is justifiable…Our knowledge of tissue interactions [e.g., cell-extracellular matrix interactions] in embryos [and in adults] is still so primitive that investigations at all levels are necessary if we are to fully explain these processes and their consequences in mechanistic terms." – Norman K. Wessels, 1977[1]

The discovery in the 1970s of proto-oncogenes, genes that become **oncogenic** ("cancer causing") either through genetic modifications or increased expression, and tumor suppressor genes, those that if expressed at the right levels, would suppress progression to malignancy, spurred a revolution. Given the excitement and the implication of these discoveries, it may not be surprising that most cancer researchers have not looked back. As such, much of the work of the early cancer research pioneers such as Paget, Rous, Warburg and Berenblum (see below), which drew attention to other aspects of cancer, became unpopular and considered beside the point.

The subsequent decade brought technologies that enabled automated sequencing of DNA, which eventually made the dream of sequencing whole organismal and tumor genomes a reality. The hope was that pinpointing aberrations in genetic sequence would allow one to understand the origins of cancer (1). Dealing with the mutations by fixing the genes through gene therapy or neutralizing the gene products would thus provide a viable cure. The picture that emerges forty years later is much more complex (2). For breast cancer, we know now that

[1] Bracketed statements added by Bissell, M. J. and Hall, H. G. (1987). Form and function in the mammary gland: the role of extracellular matrix. In The Mammary Gland (eds M. C. Neville and C. W. Daniel), pp. 97-146. New York: Plenum Press.

the frequency of somatic mutations exceeds one per one million DNA base pairs (3). We also know that a single tumor may have hundreds of mutations (3), that some mutations (*TP53*, *PIK3CA*, *GATA3*) are more prevalent than others (4), but that even these are not present in the majority of patients (4). Perhaps most significantly, breast cancer patients have been classified based on their tumor gene expression profiles, which can predict their survival (5) and response to specific treatments (6).

But this approach has also taught us a lot about what the genome may not be able to tell us. For instance, autopsy studies have revealed that the fraction of individuals harboring neoplastic lesions within their breast or prostate is 27- to 142-fold higher than the actual incidence rates of breast and prostate cancer (7, 8). If the initial mutation would be sufficient to cause cancer, why do such a small fraction of these neoplasms progress to frank carcinomas in the general population? Another aspect of tumor progression that cannot be explained by genomic aberrations is why tumors metastasize when they do. The prevailing hypothesis had been that metastases reflect the pinnacle of tumor evolution: tumor cells would have to acquire a set of mutations in order to disseminate from the primary tumor to another tissue (9). Now, it is clear that tumor cells disseminate very early during tumor progression despite few genetic abnormalities (10-12), and that these tumors may emerge even faster than the primary tumor itself (this is referred to as cancer of unknown primary). So, if metastatic outgrowth does not require additional mutations from the primary tumor, what drives the metastatic program? And there are other questions outlined elsewhere (13).

The need to answer such questions has spawned a newfound appreciation that the complexity that governs tumor phenotype cannot be explained only at the genetic level. As a result, and also because a handful of investigators began to probe the role of the **microenvironment** more deeply at the mechanistic level, the focus has slowly begun to shift to the study of the tumor's microenvironment. Whereas this appreciation may be newfound, the concept of the microenvironment's importance is not (see Figure 16-1 for a timeline).

Half of the secret of the cell is outside the cell: a historical perspective on the role of the microenvironment

In 1889, Stephen Paget published results of an autopsy study he conducted on 735 breast cancer patients. His study revealed that these patients tended to have metastases within 4 tissues: lung, liver, uterus and bone. Empowered by these observations, as well as those of peers like Langenbeck and Fuchs, Paget formulated his now-famous **seed and soil hypothesis**: "… every single cancer cell must be regarded as an organism, alive and capable of development. When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil (14)." Perhaps this is where an earnest appreciation for the microenvironment began. Remarkably, the enduring interpretation of Paget's hypothesis is that certain soils are favorable for tumor growth. This has indeed

dominated the landscape of metastasis research, and is the subject of the following subsection ("Promoting Microenvironments"). But inherent in his message was that certain soils are inhospitable to a tumor's seed. In the same article, Paget remarks on a colleague's interpretation that instead of a predisposition to receive a seed, certain organs exhibit "diminished resistance (14)." Thus, the second sub-section ("Suppressive Microenvironments") will deal with this idea.

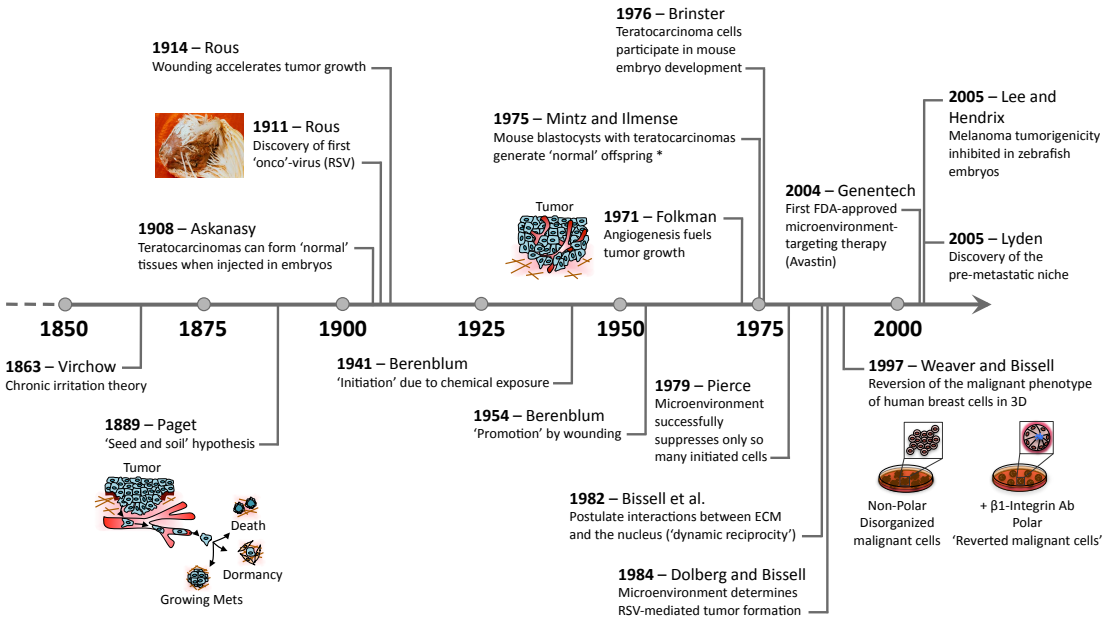

Figure 16-1. A historical perspective on the role of the microenvironment in cancer. A timeline detailing landmark discoveries that showed the dominance of microenvironment over genotype. Space-permitting, a number of other important experiments could have been included, such as Emerman and Pitelka's demonstration that normal mammary epithelial cells on floating collagen gels recapitulate their *in vivo* phenotype (102), Folkman's demonstration that cell shape regulates DNA synthesis (197), and experiments demonstrating that reconstituted 3-D BM gels act as a "blotter" to distinguish the normal from malignant phenotype (38, 198).

Promoting Microenvironments

What makes a given microenvironment favorable for the growth of a tumor cell is a topic that is germane to all tumors, disseminated or not. For instance, the probability of harboring an occult (i.e., hidden) neoplasm increases with age to nearly 100% in an organ like the thyroid gland, yet only 0.1% of individuals are ever diagnosed with thyroid cancer (15). What allows the emergence of these tumors in some, but not others, is a question that has been pursued since the turn of the 20th century. Peyton Rous and others concluded that a transplanted tumor would not take unless there was a **stromal reaction** and immediate vascularization of the implant (16). These properties are common to both **inflammation** and **wounding**, and each has long been suspected of creating a tumor promoting microenvironment. As early as 1863, Virchow noted that chronic irritation and prior injury could precondition tissue for tumor formation (17). Rous was amongst the first to show this conclusively by demonstrating that wounding the peritoneal cavity of mice inoculated with tumor cells accelerated the growth of

tumors within their visceral organs (18). Further evidence for the tumor promoting power of the wounding microenvironment comes from the vast literature on chemical carcinogens. An extensive body of work established that chemicals within coal tar such as benzo(a)pyrene derivatives, despite being known mutagens, were not sufficient to cause skin cancer on their own. Despite '**initiation'** due to chemical exposure taking place, normal skin guards against progression unless the carcinogen dose is so excessive that it damages the tissue in addition to causing mutagenesis (19, 20). This second step, called '**promotion**', is required and is generally caused by wounding or by other toxic agents, many of which are associated with aberrant tissue repair and **fibrosis** (21-23). The discovery of the first 'onco'-virus, referred to as Rous Sarcoma Virus (RSV) (24), would later provide some of the most conclusive evidence that wounding promotes tumor formation. In discovering RSV, Rous took the filtrate of a chicken tumor and noted that this cell-free filtrate would induce sarcomas in recipient chickens (24)[2], thus proving Koch's postulate[3]. Decades later, when experimenting with RSV, Dolberg et al. noted that the injected virus circulated throughout the bird, but tumors tended to arise only at the injection site (25). Was the wound created by the injection needle the key factor? Nicking the contralateral wing of infected chickens caused tumors to arise also at the sites of abrasions (25). This phenomenon was mediated by transforming growth factor **(TGF)-β1**, which was expressed in tissue shortly after wounding and shown to induce tumors on its own even in the absence of wounding (26). Hence, the tumor promoting power of the wounding microenvironment (reviewed more extensively in (27)) was proven.

Of course, processes like wounding and fibrosis are inextricably linked with the formation of new vasculature (e.g., through **angiogenesis**), but it was not until Judah Folkman's work in the early 1970s that a causal relationship between tumor growth and angiogenesis was established. Tumor fragments or tumor cells grafted onto the rabbit cornea were observed to induce sprouting from existing vasculature as they grew (28). Physically preventing microvasculature from reaching the implant resulted in a latent mass where tumor cell proliferation was countered by apoptosis. Folkman's work specified experimentally, for the first time, a non-tumor cell— the endothelial cell— that was critical to the growth of a tumor. His work also started a new field focused on "anti-angiogenesis" based on Folkman's hypothesis that, "Solid tumors can grow to visibility only if they can vascularize themselves. Therefore, the mechanism by which tumor implants stimulate neovascularization must be well understood before therapy based upon interference with angiogenesis can be devised (29)." The angiogenesis inhibitor bevacizumab (Avastin) would become the first therapy explicitly targeting the microenvironment approved by the United States FDA (2004)[4].

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[2] Ironically, despite all of Rous' seminal work establishing the importance of the microenvironment, this discovery perhaps did more to spur the genetic revolution than anything else, since the first oncogene (Src) was cloned from RSV and was shown to cause tumors in chicks.

[3] German physician Heinrich Hermann Robert Koch formulated 4 postulates necessary to prove a causal relationship between a microbe and a disease, one of which was that the isolated agent should cause disease when introduced into a healthy organism.

[4] For further detail, please see Chapter 17 on Angiogenesis

Suppressive Microenvironments

The studies described above established key roles for the microenvironment in promoting tumor growth, which will be the primary focus of this chapter. But it is worth mentioning that much of the milestone research in demonstrating the importance of the microenvironment did so by showing that context could override tumorigenicity; that is, tumor cells could be tricked into thinking they are normal if provided the right cues. The observation that the embryo comprises such a suppressive microenvironment is one that was first made over 100 years ago, when Askanazy showed that ovarian teratomas could form 'normal' tissues composed of the correct embryonic germinal layers when injected into embryos (30). Decades later, a series of studies from different laboratories provided further evidence that the embryonic microenvironment could induce tumors to function normally in development. Mintz and Illmensee injected embryonic teratoma core cells from mice with a steel coat genotype into blastocysts from C57-b/b mice (which have black coats). The blastocysts gave rise to functionally 'normal' offspring (31). The next paper by Illmensee and Mintz (32) reported that the mice born from the initial experiments produced an offspring that was completely normal and had a mosaic (i.e., striped) coat, implying that the teratocarcinoma could pass through the germ line. While it is true that this work has not been confirmed in other laboratories, there are some dramatic variations on the theme. For instance, Brinster injected 2-4 teratocarcinoma cells from agouti brown mice into 4 day old blastocysts of Swiss albino mice. One out of the 60 injected mice retained the teratocarcinoma cells (based on the presence of brown hair patches on the white mouse), and proceeded to develop normally as well (33). Pierce later essentially quantified the balance of power between the embryonic microenvironment and the malignant cell by demonstrating that the embryonic microenvironment could suppress the malignant phenotype of one to a few implanted tumor cells, but that this ability diminished as the number of injected tumor cells increased (34). Perhaps this offers a hint that our bodies are only able to successfully suppress only so many initiated cells, and that this power diminishes with age.

The suppressive effect of the embryonic microenvironment has been demonstrated in species other than mice, as well. Using RSV, Bissell and Dolberg showed that cells within injected chick embryos expressed the virus, but that early embryos failed to form tumors (35). Maintaining embryonic architecture was key, however, as dissociating the embryos and placing the $PP60^{src}$-marked cells (using *LacZ*) in culture resulted in rapid transformation of the blue-labeled cells (36). Hendrix and colleagues more recently reported similar findings for aggressive melanoma cells injected into zebrafish embryos (37). The lasting impact of these studies is that tissue architecture is dominant to the powerful oncogene in embryos, thus overriding tumorigenicity of malignant cells. These studies offered also the clue that the malignant genotype could be suppressed if the interactions between a tumor and its microenvironment could somehow be normalized.

Taking advantage of this insight required an assay that would allow normal and malignant cells to recapitulate their *in vivo* phenotypes in culture. This was achieved by culturing cells in a three-dimensional (3-D) reconstituted **basement membrane** (BM) gel. In 3-D, but not 2-D conditions, non-malignant mammary epithelial cells formed growth-arrested, polarized acini resembling terminal ductal lobular units of the breast, whereas malignant cells formed disorganized masses that continued to grow (38). By examining the expression profiles of **integrins**, heterodimeric receptors on the cellular surface that transduce signals from the **extracellular matrix** (ECM) through traditional and non-traditional pathways to alter gene expression, Weaver, Bissell and colleagues noted aberrant overexpression of integrins and a number of other receptors such as EGFR on malignant cells (39). Suspecting that these receptors were key nodes that integrated signals from the microenvironment to direct cell behavior, the authors began to restore levels of aberrant receptors to normal levels, starting by applying inhibitory antibodies targeting integrin β1 in malignant cells cultured in 3-D gels. The result was a dramatic 'phenotypic reversion' of malignant breast epithelial cells to structures that looked and behaved like their normal counterparts (39). To show that this treatment was not somehow selecting for a non-malignant subpopulation of cells, Weaver et al. dissociated tumor cell clusters from 3-D gels, replated them onto plastic, and then passaged them back into 3-D gels in the absence of blocking antibody. Tumor cells once again formed malignant clusters (Figure 16-2) (39). This strategy led to the discovery of a host of signaling molecules that act in concert to regulate/integrate epithelial phenotype. Many of these molecules effect interactions between a cell and its microenvironment, and include ECM molecules (40), growth/ECM receptors (41, 42), or **matrix metalloproteinases** (MMPs) that digest ECM components (43). Remarkably, targeting only one of these aberrantly expressed molecules restores the levels of all the others back to normal (again, only in 3-D), demonstrating the potential of normalizing aberrant microenvironmental signaling to redirect entire signaling webs and impair manifestation of the malignant genotype (13, 44). Even metastatic cells can be reverted by using any one of these inhibitors, or more completely by using two (45).

The Tumor Organ

The historical studies detailed above demonstrate that a tumor cell is a product of its aberrant genome interacting with its surrounding microenvironment. This concept is easier to appreciate if one considers the tumor as a dysfunctional organ, as suggested by Bissell and Radisky (46). On a basic level, an organ has the following properties:

1. Organs are multicellular and are composed of multiple tissue layers. Functional tissue layers consist of epithelia, which are tube-like structures that carry fluid, and epithelia are separated from surrounding stroma by a specialized ECM called the basement membrane.

2. Organs are governed by properties that emerge as a result of the interactions between the cells, ECM molecules, and soluble factors composing the organ, and the result of these interactions is greater than any one of the individual parts.
3. Organs have unique signatures of functional differentiation; for instance, the mammary gland produces milk, the pancreas produces insulin, and bone marrow is responsible for maintaining homeostasis of the hematopoietic and lymphatic systems.

While tumors lack proper function, they do have the first two of these traits in common with organs. The focus of this section is on the second of these traits: the properties that emerge as a result of a tumor's interactions with its microenvironment. There are two ways to illustrate this concept. The first would be to consider the parallels between a developing organ and a developing tumor. The scope of this discussion would extend well beyond this chapter, however, since just as organs develop differently, tumors of these organs develop distinctly as well. Instead, a more general way to illustrate the concept of the tumor organ is to consider what happens when an organ is injured— it attempts to heal. The wounding microenvironment shares a great deal in common with the tumor microenvironment (illustrated in Figure 16-3), the difference being that wounds eventually stop healing, whereas a tumor's microenvironment persists. This is why tumors have been called, "wounds that do not heal (47)."

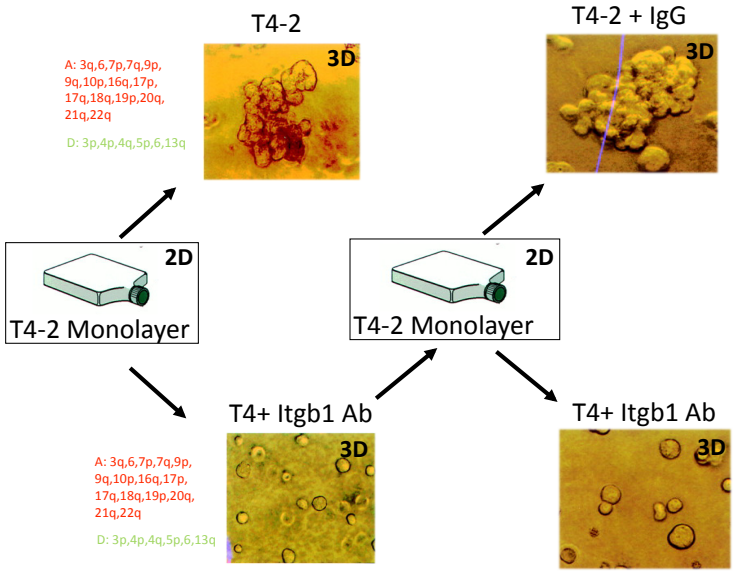

Figure 16-2. Serial passaging from 3D to 2D cultures (and back) demonstrates phenotypic reversion as opposed to selection. Phase contrast micrographs of T4-2 cells grown in the presence of mock antibody (IgG) or anti–β1-integrin function blocking antibody (Itgb1 Ab) within 3-D reconstituted BM gels. Despite two rounds of treatment, reverted cells were able to resume their original tumorigenic phenotypes after being passaged and re-cultured in the absence of antibody. Note that despite displaying a non-malignant phenotype, reverted tumor cells retain an aberrant genome (genomic amplifications (A) shown in red, deletions (D) shown in green). Figure adapted from *ref.* (39).

The wounding microenvironment

Upon wounding, the goal is to restore function of injured tissue, which means that damaged epithelia must be resealed. Damaged blood vessels leak plasma and platelets into the wound site (or simply hemorrhage blood due to more serious injury) (46, 48). Tissue procoagulants such as tissue factor initiate a cascade that results in a clot rich in ECM molecules fibrin and **fibronectin** that entraps platelets and blood cells. Platelets are a rich initial source of clotting factors, mitogens and chemoattracting cues that lure cells into the wound site. Inflammatory cells are amongst these cells, releasing extracellular proteinases such as MMPs and cysteine cathepsins that cleave the provisional ECM to facilitate migration. Fibroblasts follow, and become activated by TGF-β and fibronectin splice variants within the clot to become **myofibroblasts**—muscle-like cells with an enhanced ability to exert contractile force (49, 50). These cells are charged with cinching the wound, and in doing so deposit copious amounts of ECM, consisting primarily of **type I collagen** (Col-1), to provide scaffold to the tissue in its contracted state (49). Endothelial cells are stimulated to invade from nearby microvasculature by angiogenic factors (e.g., VEGF, FGF-2) secreted by platelets, immune cells and fibroblasts, and also released from the provisional ECM by MMPs (e.g., MMP-9), to rapidly vascularize and feed the new tissue (51). Meanwhile, this complex cascade of events reduces adhesiveness of adjacent epithelial cells, which undergo an **epithelial-mesenchymal transition** (EMT) in order to migrate to reseal the epithelium, and later revert back to their normal state by re-depositing BM, reengaging each other through cell-cell junctions, growth-arresting, and functionally differentiating (46).

Thus, much as the tumor microenvironment (see below), the wounding microenvironment is populated by circulating cell types such as immune cells and platelets, and resident tissue cells like fibroblasts and endothelial cells. These cellular constituents engage in a dynamic and reciprocal chorus via secreted factors and ECM molecules to activate nearby epithelial cells to close the wound via proliferation and migration. The biggest difference between a tumor and the healing wound is what happens next. In the proper wounding context, not only does the epithelium differentiate, but activated cell types do not persist, the provisional ECM is remodeled, and secretion of stimulatory growth factors and cytokines is dampened. There is still much to be learned about what comprises the 'homeostatic switch,' that is, the cues that tell a tissue to stop remodeling presumably because it is now fully functional. For instance, what exactly happens to all of the activated myofibroblasts is not known. They may deactivate, transdifferentiate or undergo apoptosis (this fails to occur in individuals with chronic wounding disorders, and it should not surprise the reader that these individuals are also more susceptible to cancer(27)). The stimuli that cause myofibroblasts to undergo any of these programs are also unknown. Perhaps uncovering these cues will offer insight as to how the tumor microenvironment itself can be phenotypically reverted so that it does not persist.

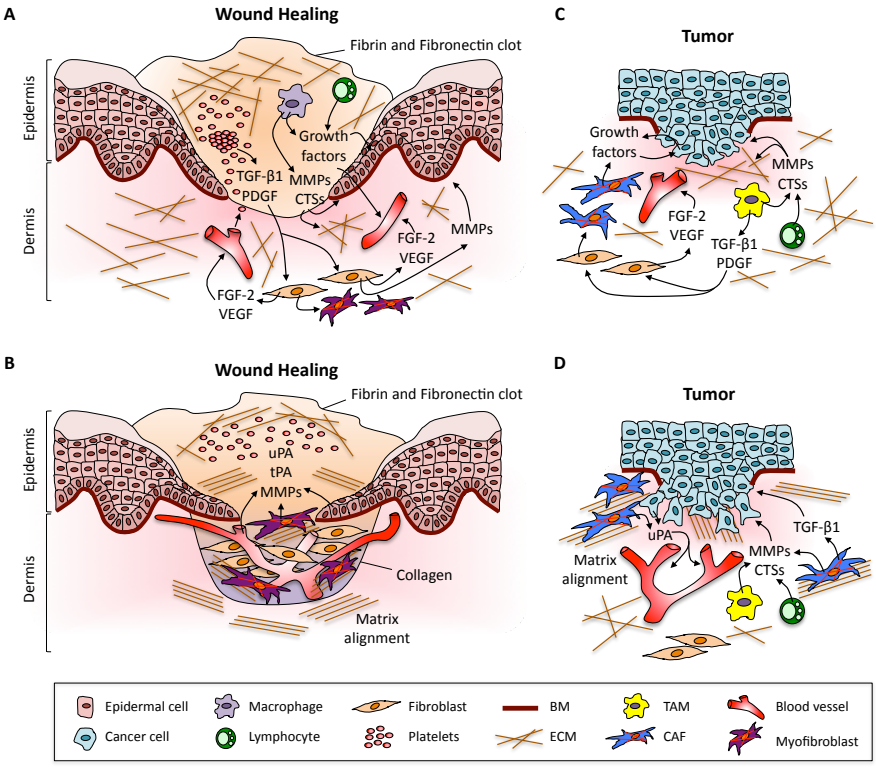

Figure 16-3. Tumors: "Wounds that do not Heal." (**A**) Immediately after injury, damaged blood vessels leak plasma and platelets, which form a hemostatic plug and release vasoactive mediators to increase vascular permeability and enable the influx of serum fibrinogen to generate a fibrin and fibronectin-rich clot. Platelets produce chemotactic factors such as TGF-β and PDGF, which lure inflammatory cells and fibroblasts into the wound site. These cells produce extracellular proteases, including MMPs and cathepsins, which remodel ECM to facilitate cell migration. Recruited cells also secrete a number of growth factors, such as FGF-2, which promote the development of new blood vessels. Many of the fibroblasts take on a myofibroblast phenotype to facilitate wound contraction. **(B)** In order for the wound to close, myofibroblasts deposit and align abundant amounts of ECM, mainly Col-1. The intricate reaction to wounding reduces epithelial adhesiveness and increases epithelial-cell mobility to re-form an intact sheet of tissue over the wound. Production of MMPs, uroplasminogen activator (uPA) and tissue plasminogen activator (tPA) facilitates the re-epithelialization. Blood vessels can then enter the fibrin and fibronectin-rich clot to rapidly vascularize and feed the new tissue. The lateral migration of the epidermal cells is followed by a reversion to their normal state by re-depositing basement membrane (BM), reengaging intercellular adhesions, growth-arresting and functionally differentiating. **(C)** Similarly, the tumor microenvironment is populated by immune cells, fibroblasts and endothelial cells, and tumor cells produce many of the same growth factors that activate the adjacent stromal tissues in wounding in order to create a reactive stroma. **(D)** Tumor cells, cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs) increase production of MMPs and uPA at the invasive tumor front to stimulate angiogenesis and proliferation. CAFs exert also contractile forces to generate tracks within the ECM that epithelial cells subsequently invade through in a collective fashion.

The tumor microenvironment

Whether a tumor creates its own microenvironment or the aberrant microenvironment causes a tumor is a conundrum of tumor biology. There is, in fact, evidence for both. Regardless, what manifests is a dynamic interplay between a tumor and its surroundings that ultimately results in loss of organ structure and architecture. Indeed, this is how oncologists and pathologists diagnose tumors and determine their stage or grade (52-54). Loss of architecture

is a hallmark of cancer that reflects behaviors such as deregulated growth, enhanced survival, new blood vessel formation, stromal activation, and inappropriate migratory and invasive behavior of cells (55). The microenvironment becomes a runaway train of sorts, and activation is heightened as entropy (loss of cell and tissue architecture) increases. As the reader goes through the remainder of this chapter, keep the following questions in mind: at what stage of progression do tumor cells or cells in the vicinity of tumors begin to secrete and deposit factors that cause aberrant growth and invasion? At what stage of progression do epithelial cells (transformed or not) respond? Once a tumor disseminates, what type of microenvironment is necessary for it to grow? And finally, once a reactive stroma has formed, can it be reversed?

Initiation

Perhaps the most convincing demonstration of the microenvironment's influence is that its disruption causes not just aberrant growth, but *de novo* genetic lesions and full blown malignancy (Figure 16-4A). This has been shown in mouse models in which ECM remodeling enzymes MMP-3 and MMP-14 are overexpressed in the murine mammary gland (56, 57). Both of these proteases are expressed normally during mammary epithelial morphogenesis, and function in part to execute the branching program (58, 59). However, sustained ectopic expression of MMP-3 in luminal epithelial cells (via a MMP-3 transgene driven by a milk protein promoter (WAP), which is primarily active in the murine mammary gland (59)), led to a dramatic upregulation in murine MMP-3 expression in the mammary stroma. This was sufficient to cause formation of a **reactive stroma** characterized by increased blood vessel density, accumulation of collagen, and expression of ECM molecules typically observed only during development or wounding (e.g., Tenascin-C) (60). By six months, these mice exhibited substantially more epithelial hyperplasia than their wild-type counterparts, and a small percentage of these mice eventually formed full-blown carcinomas (56) (Figure 16-5A). Amazingly, MMP-3 does not act only on the stroma; epithelial cells are affected directly as well. Exogenous MMP-3 causes oxidative DNA damage through production of reactive oxygen species (ROS), resulting in oxidative damage which causes genetic instability and aneuploidy in epithelial cells (61) (Figure 16-5B). Additionally, ROS stimulate expression of Snail, which negatively regulates E-Cadherin, causing loss of cell-cell adhesion and EMT (61). In sum, forcing the overexpression of a single ECM remodeling enzyme— without addition of an oncogene or knockdown of a tumor suppressor gene in the mammary gland—results in manifestation of all of the putative hallmarks of cancer (55).

More sophisticated transgenic models allow tissue-specific recombination events to facilitate genetic deletion in specific tissue compartments (62). This strategy was utilized to demonstrate that introducing a genetic aberration to fibroblasts is sufficient to cause cancer in adjacent epithelium. For instance, driving TGF-β receptor type II (TGF-βRII) deletion by the fibroblast-specific protein 1 (FSP1) promoter (which is expressed ubiquitously by fibroblasts)

renders fibroblasts unresponsive to TGF-β signaling, and results in a three- to four-fold increase in hepatocyte growth factor (HGF) secretion in the prostate and forestomach of these mice (63). The ultimate consequence is the induction of proliferative, intraepithelial neoplasms within the prostates of young mice, and of invasive squamous cell carcinomas in the forestomachs of these mice. Similarly, engineering human fibroblasts to overexpress either HGF or TGF-β is sufficient to induce tumorigenic growths from ostensibly normal co-implanted human epithelial tissue (64).

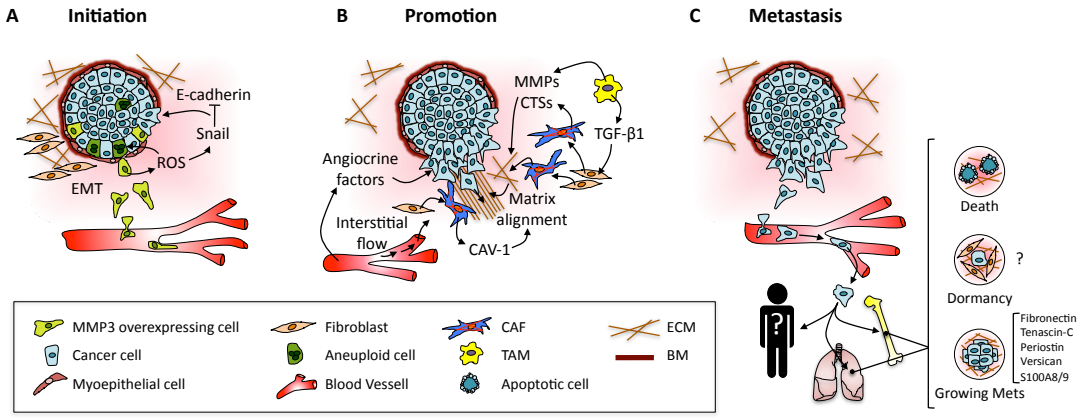

Figure 16-4. The microenvironment is a dominant force in tumor initiation, promotion and metastasis. (A) Forcing the expression of a single ECM remodeling enzyme, MMP3, in mammary epithelial cells *in the absence of any additional mutations* initiates genetic lesions and full-blown malignancy. Activation of MMP3 leads to epithelial-to-mesenchymal transitions (EMT), as well as genomic instability through formation of reactive oxygen species (ROS). In addition, ROS stimulate the expression of Snail, which negatively regulates E-cadherin, loosening cell-cell adhesion and facilitating invasion. **(B)** The microenvironment unleashes initiated epithelium. An increase in the interstitial flow combined with TGF-β1 released by the tumor and by TAMs induces dermal fibroblasts to differentiate into CAFs. These cells secrete several growth factors and cytokines that enhance proliferation of nearby tumor cells. In addition, CAFs produce proteases to remodel and align the ECM, thus creating tracks that epithelial cells subsequently invade through in a collective fashion. **(C)** The microenvironment is also a potent enhancer of distant metastatic spread. Invasive cancer cells enter local microvasculature and travel through hematogenous and/or lymphatic routes to distant organ sites. Formation of a metastatic niche, characterized by elevated expression of ECM molecules such as fibronectin, tenascin-C or periostin may be required in order for disseminated tumor cells to colonize distant tissue.

Of course, fibroblasts also play key roles during tissue development by producing ECM and other molecules that induce growth, branching and tissue-specific gene expression of resident epithelia. Thus it stands to reason that transforming fibroblasts to re-express a developmental marker in the mature gland could severely impact epithelial homeostasis. This was tested by co-implanting urogenital sinus mesenchymal cells overexpressing the global epigenetic regulator Hmga2, which is expressed primarily during embryonic development, in a sustained fashion. When implanted with adult prostate cells, Hmga2-overexpressing mesenchyme fostered formation of frank carcinomas primarily by acting on the basal stem cell population of prostate epithelia (65).

These studies demonstrate that inducing aberrations within the stromal compartment is sufficient— in and of itself— to induce genomic instability, sustained growth, and the transition to an invasive phenotype by uninitiated and untransformed (i.e., normal) epithelia.

However, even when the epithelia has been initiated (e.g., mutation or radiation), the activated microenvironment is sufficient (and perhaps necessary) to accelerate progression of transformed epithelia into frank carcinomas (66, 67).

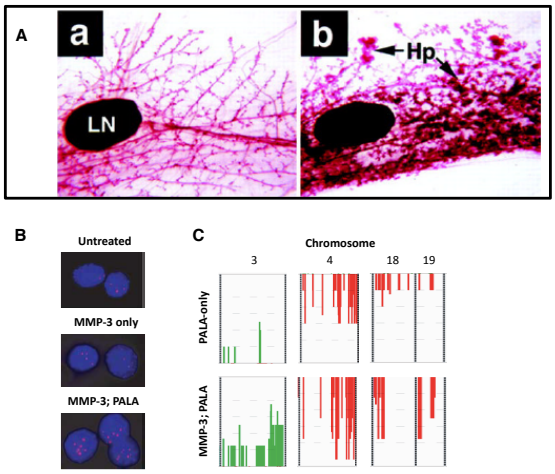

Figure 5. Forced overexpression of MMP-3 in the murine mammary gland results in tumor formation in part by causing genomic instability in mammary epithelial cells. (A) As opposed to non-transgenic control mice (a; *left*), WAP-*MMP3* mice (b; *right*) exhibit multifocal hyperplasia (Hp) by 16 months of age. MMP-3 functions to induce tumorigenesis via reciprocal overexpression of MMP-3 in the mammary stroma to induce a reactive stroma (*not shown*), and by acting directly on mammary epithelial cells to induce reactive oxygen species, which cause genomic instability. This is shown by **(B)** amplification of the *CAD* locus (red), which confers resistance to *N*-(phosphonacetyl)-L-aspartate (PALA) treatment, in MMP-3 treated mammary epithelial cells and by **(C)** comparative genomic hybridization analysis (green, amplifications; red, deletions) of MMP-3 treated cells (vs. control (PALA)-treated cells). Figure adapted from *refs.* (56, 61).

Progression

The previously described wounding studies performed by Bissell and colleagues with RSV-injected chickens (25, 26) were amongst the first to show conclusively that the wounding microenvironment is sufficient to push transformed cells (in this case, those expressing the Src oncogene) to form full-blown tumors. More recent works have pinpointed precise roles for specific cell types, growth factors, cytokines, ECM molecules, and associated physicochemical properties in creating a biochemical and mechanical signaling milieu that unleashes initiated/transformed epithelium (Figure 16-4B).

Before delving into all of the ways that the microenvironment conspires to promote loss of architecture, growth and invasion, it is important to note that we likely harbor initiated cells throughout our body, which accumulate over the course of our lives due to diet, radiation, etc (13, 15). Yet on a per cell level, progression to malignancy is actually a rare event. The reason is that normal tissue architecture supersedes an aberrant genome, as detailed above. For epithelial tissues, the BM is the principle biochemical and physical scaffold that must be compromised for *in situ* disease to become invasive. This specialized, layered ECM typically consists of at least one member of the laminin family, type IV collagen, nidogens, and heparin sulfate proteoglycans, which signal in a tissue-specific

fashion to confer architecture and function to the epithelium (68, 69). Depriving normal cells of BM can have drastic consequences. Not only does adhesion to BM protect epithelial cells from apoptosis (70), it confers proper tissue polarity to epithelium. For instance, luminal epithelial cells from the mammary gland "reverse polarize" when removed from BM and cultured in Col-1 (which is found mainly in the stroma); that is, they express apical proteins basally, and vice versa (71). Addition of **myoepithelial cells**, which lie basal to the luminal epithelium and secrete laminin-111 *in vivo*, restores proper polarity to luminal epithelial cells (71). Laminin-111 is critical also to tissue-specific gene expression within the mammary gland (72-75), and its organized presence is quite important for preventing carcinoma progression. Co-implantation of myoepithelial cells with a cell line that forms ductal carcinoma in situ-like lesions *in vivo* restrains progression of these cells even in the presence of tumor-associated fibroblasts or fibroblasts taken from a chronically inflamed microenvironment (76). Myoepithelial cells derived from cancer patients, which fail to express laminin-111 (71), cannot confer proper polarity to luminal epithelial cells in culture and fail to prevent fibroblast-mediated invasion of in situ lesions (76). This establishes a vicious cycle, as loss of epithelial polarity results in upregulated expression of MMP-9, which degrades remaining BM and effects of loss of tissue architecture (43). Thus, loss of laminin-111, or inability to produce laminin-111, results in loss of tissue architecture, accelerated degradation of pre-existing laminin-111 and if additional necessary signals are present, progression to malignancy. Taken together, these data demonstrate how disruption of tumor suppressive components within tissues renders cells sensitive to the coercive elements of the tumor microenvironment.

CAF-derived soluble factors promote tumor progression.

Carcinoma-associated fibroblasts (CAFs) are one such element, accounting for up to 80% of the fibroblast population in a tumor (77). They arise from fibroblasts resident to the tissue, as well as vascular smooth muscle cells, pericytes (78), mesenchymal stem cells (79), and even epithelial (80) and endothelial cells (via mesenchymal transitions (81)). CAFs phenotypically resemble myofibroblasts—they express fibroblast activation protein, incorporate alpha smooth muscle actin within their actin stress fibers, and deposit copious amounts of fibronectin, including the extra domain (ED)-A-containing splice variant (49, 82). CAF phenotype is stimulated initially, at least in part, by tumor-derived TGF-β1 (83), and the soluble nature of this stimulation is reflected by the graded pattern of fibroblast activation observed in tumors (84). Fibroblasts closest to the tumor exhibit the highest level of activation, as judged by the expression of myofibroblast markers and the ability to induce increased MMP production and invasion of co-cultured breast tumor cells (78, 80, 84).

The demonstration that fibroblasts associated with tumors actually promote tumor progression first was reported in prostate cancer. Recombinant tissues composed of CAFs isolated from prostate tumors and SV40-transformed prostate epithelial cells displayed loss of

epithelial architecture, and formed masses more than 10-fold larger than those formed by recombinants composed of normal human prostate fibroblasts and initiated epithelial cells (85). CAFs from other tissues effect similar outcomes; there is evidence that CAFs play a role in accelerating tumor progression in breast (86), ovarian (87), pancreatic (88), and liver (89) cancers, as well as others. Sustained secretion of growth factors and cytokines such as TGF-β, HGF, SDF-1, and IL-1β by CAFs enhance proliferation and invasion of nearby tumor cells, promote angiogenesis (including recruitment of circulating endothelial progenitor cells), and stimulate a tumor-promoting inflammatory response (83, 86, 90, 91). However, it should be cautioned that fibroblasts from different organs are not the same, since fibroblasts exhibit substantial heterogeneity between organs (92). Hence, the mechanisms by which CAFs from a given tissue promote tumor progression are likely to differ as well. Case in point, analysis of CAFs from skin, cervical, mammary and pancreatic tumors revealed that each have unique expression signatures of a pro-inflammatory gene set (90).

What is clear from these experiments, which by-in-large require the isolation and expansion of CAFs, is that the CAF phenotype persists in culture despite the absence of a tumor. What is unclear, however, is why. Interestingly, CAFs rarely exhibit somatic genetic alterations (93); however, on a population level, they do have reduced expression of the well-known tumor suppressors p53 and PTEN. Deletion of PTEN in mammary fibroblasts is sufficient, all on its own, to steer these cells towards a desmoplastic, pro-inflammatory, pro-angiogenic phenotype that drastically accelerates tumor growth in mice (94). Therefore, epigenetic changes, perhaps caused by sustained over-stimulation by TGF-β and SDF-1 (91), or even effected by direct transfer of genetic material from the tumor itself (95), may drive altered expression profiles in local fibroblasts that are sufficient to induce and sustain the CAF phenotype. Since CAFs persist in culture without the tumor (although the tissue culture plastic and milieu is analogous to wounding), the likelihood is that they persist also *in vivo* once the tumor has been removed. What role residual CAFs play in tumor recurrence has yet to be defined.

CAF-mediated ECM remodeling promotes loss of tissue architecture and tumor invasion.

In addition to soluble factor-mediated effects, CAFs influence tumor progression by remodeling the ECM. This remodeling can be constructive or destructive, force-mediated or not. Destructive remodeling refers to proteolytic breakdown of ECM. Using organotypic skin reconstructs, Gaggioli et al demonstrated that CAFs utilize a combination of MMP- and force-mediated remodeling of Col-1-rich ECM to promote invasion of squamous cell carcinoma (SCC) cells. The authors reached this conclusion after first observing that SCC cells required a physical association with CAFs in order to invade subjacent ECM. Remarkably, pre-conditioning the underlying ECM with CAFs was still sufficient to induce SCC cell invasion. How? CAFs secrete MMPs and exert contractile forces to generate tracks

within the ECM that epithelial cells subsequently invade through in a collective fashion (96). Once these tracks are generated, it is quite possible that trailing cells no longer require the proteolytic function of MMP molecules to invade the ECM (58, 97, 98). Thus, depending on the amount and type of ECM remodeling that has taken place, inhibiting the proteolytic function of MMPs (which was hailed as a promising strategy for targeting the microenvironment, but has failed to live up to that promise (99)) may prove inadequate to prevent invasion of tumor cells.

Activated fibroblasts are the principle mediator of **desmoplasia** or excessive ECM deposition. Not only does the altered profile of deposited ECM molecules (e.g., Col-1, tenascin-C, fibronectin ED-A) alter cellular behavior, but increasing concentrations of these molecules results in stiffer tissue that drives integrin clustering and enhanced signaling potential. Tumors exhibit up to 10-fold increases in collagen concentration over physiologic conditions (100), corresponding to a 24-fold increase in tissue stiffness in a tissue like the mammary gland (101). Elevating stiffness out of physiological range is sufficient to alter the function of normal mammary epithelial cells by altering cell shape (102), increasing intracellular elasticity, inhibiting tissue-specific gene expression (103) and causing disorganization of non-malignant epithelia (101). Once epithelia are initiated by any means, pathological ECM stiffness drives integrin clustering, focal adhesion formation, ERK activation, and ROCK-mediated contractility, ultimately resulting in disrupted tissue architecture and an invasive phenotype (101, 104, 105). Blocking integrin clustering or Rho signaling in 3-D culture is sufficient to restore proper epithelial architecture, while inhibiting collagen cross-linking in MMTV-Neu mice through lysyl oxidase (LOX) blockade results in enhanced preservation of epithelial architecture and slowed tumor progression (101, 104).

Cell-intrinsic and –extrinsic forces also have a significant effect on ECM alignment, which in turn profoundly influences tumor invasion. "**Tumor-associated collagen signatures**" were first described by Provenzano and Keely (106), and refer to three possible alignments of fibrillar collagen observed at the tumor-stroma interface: random, perpendicular/belt-like, or radially aligned. The latter is observed at invasive tumor fronts, and patients with radially aligned collagen at the tumor-stroma interface have significantly diminished disease-free and overall survival (107-109). One potential mediator of collagen alignment is the tumor itself, particularly in instances where stiffening of the stroma enhances contractility of tumor cells (104, 110). Physical stimulus from the microenvironment also triggers ECM alignment by fibroblasts. Hydrostatic pressure drives water out of capillaries into the interstitial (tissue) space to be collected, in part, by lymphatic vessels present within the tissue. This **interstitial flow** can increase by an order of magnitude in pathological conditions such as cancer (111). When subjected to pathological flow rates, dermal fibroblasts differentiate into myofibroblasts and align themselves and their surrounding ECM perpendicular to the direction of flow (112). Strain from the aligned ECM may potentiate TGF-β1 activation by allowing fibroblasts to physically pull apart the molecule from its ECM-bound latent complex (113), creating a feed-forward loop to sustain the activated

phenotype. The aligned ECM, in conjunction with factors secreted by activated fibroblasts, can then direct tumor invasion.

Recently, a molecular mediator of CAF-mediated ECM alignment was uncovered. This molecule, known as Caveolin-1 (Cav-1), is a scaffolding protein essential to the structure of caveolae, or "little caves" in cellular plasma membranes (113). Cav-1 assists with focal adhesion maturation (114) and promotes force-dependent remodeling of surrounding ECM (115) by embryonic fibroblasts. Whereas *loss* of Cav-1 in patient stroma is associated with increased breast tumor size (116, 117), Cav-1-mediated ECM remodeling by fibroblasts enhances tumor invasion and distant metastatic spread. In co-culture assays consisting of breast tumor cells and embryonic fibroblasts, Cav-1 expression within the fibroblast compartment potentiates directional alignment of Col-1 and fibronectin-rich ECM to facilitate tumor cell invasion (115). Cav-1-null fibroblasts are unable to align ECM in culture, and fail to promote distant metastasis of co-implanted breast cancer cells *in vivo* (115). These findings open the door for therapies that target both CAF-derived soluble factors that promote tumor growth, and molecules such as Cav-1 that promote disruption of tissue architecture to facilitate tumor invasion. However, while CAFs are representative of how non-tumor cells function in the tumor microenvironment, they are only part of the story. It is important to realize that other cell types can be induced to aid tumor cell survival, growth, and invasion as well.

Other cell types contribute to the tumor microenvironment.

Our bodies consist of roughly 300 different cell types, a subset of which constantly engage each other in any given organ to direct development and maintain homeostasis. One would expect that few, if any of the cells in an organ act as silent bystanders during tumor initiation, formation and progression. Thus, while we describe known roles for immune cells and endothelial cells below, the reader should not construe this to mean that those cell types not mentioned are not involved. Instead, the reader should consider these unknown interactions as potential avenues of exploration, and an opportunity to define new connections that shape the ecological landscape that is the tumor microenvironment.

Because the immune system is known to protect the host, it was expected that immune cells would protected against cancer. But it is now clear that a subset of immune cells in fact promote tumor progression. No immune cell type embodies this paradigm shift better than the **macrophage**, which was long pigeon-holed as a phagocytic cell tasked with rejecting a tumor until its trophic functions in development and disease became clear (118, 119). Macrophages have been classified based on their mode of activation: classically activated/M1 macrophages respond to interferon-g by releasing pro-inflammatory cytokines and are involved in T helper 1 cell-mediated resolution of acute infection, whereas alternatively activated/M2 macrophages respond to cytokines from T helper 2 cells, and are involved in wounding and fibrosis (118). To some, this classification is overly restrictive, and ignores the phenotypic

diversity displayed by macrophages as they maintain bone homeostasis (120), promote ductal branching or involution of the mammary gland (121, 122), function in different steps of the angiogenic cascade (123-127) and guide neural networking (128). These diverse functions are executed by a number of discrete macrophage subtypes in a tissue- and context-specific fashion, which aid these developmental processes by remodeling collagen and secreting a host of other factors, including VEGF, TGF-β1, TNF-a, and a number of MMPs (reviewed in (118, 119, 129)).

Tumor-associated macrophages (TAMs) are M2-like in function, and their presence correlates with increased vascular density and worse clinical outcome for a number of human cancers, including breast, lung, and ovarian cancer (118). A principle mediator of macrophage recruitment to the tumor microenvironment is colony stimulating factor (CSF)-1. Once there, CD4-positive ($CD4^+$) and $CD8^+$ T cells steer recruited macrophages towards an M2-like phenotype via IL-4. Diminishing CSF-1 levels (130) or neutralizing IL-4 (131) have similar effects on mammary tumor progression. Whereas neither inhibit tumor growth, tumor progression is slowed and mice have significantly fewer metastases (130, 131). TAMs promote progression and metastasis through ECM remodeling and by secreting many of the same trophic factors released by M2-like macrophages during development, such as extracellular proteases that degrade BM, disrupt epithelial architecture, and enable invasion (130, 132), angiogenic factors that promote new blood vessel formation (133, 134) and epidermal growth factor (EGF) as part of a chemotactic EGF-CSF1 paracrine loop that mediates co-invasion of tumor cells and macrophages (131, 135).

It is important to note also that there is a tissue-specificity to immune cell distributions that extends beyond macrophage subsets to a host of other **leukocytes**. The distribution of B cells, T cells, and other white blood cells changes change from tissue-to-tissue, and shift also in a tissue-specific fashion during tumor progression (136). Thus, it is likely as important to understand how leukocyte populations shift during tumor progression as it is to understand how phenotypic characteristics of different leukocytes change during tumor progression (e.g., the shift from M1- to M2-like macrophages) in order to design therapies that effectively target tumor-associated inflammation.

Blood vessels are generally regarded as conduits for oxygen, nutrients, and hematopoietic cells, and as such regarded as passive participants in the tumor microenvironment (137). However, this is not correct. **Endothelial cells** are active participants in the dynamic interactions that occur between cells in any tissue. This was established first in development; endothelial cells secrete soluble factors that stimulate liver growth and tissue-specific gene expression from the pancreas in developing embryos (138, 139). More recently, endothelial-derived '**angiocrine factors**' have been shown to comprise niches that maintain stem cells in both brain (140, 141) and bone marrow (142-144). Increasing evidence suggests also that NOTCH ligands and specific BM molecules expressed in the brain perivasculature mediates the survival of glioma-initiating cells and disseminated tumor cells in the brain (145-148).

Tumor-associated endothelial cells display a host of phenotypic abnormalities, including aneuploidy (149), BM irregularities (150), and disrupted mechanosensing due to hyperactivation of the Rho/ROCK pathway (150). Elevated MAPK signaling is a symptom of disrupted mechanosensing. Interestingly, the suite of factors secreted by endothelial cells shifts upon activation of MAPK signaling to a set that promotes proliferation (144), so angiocrine factors derived from tumor-associated endothelial cells may also promote proliferation of tumor cells. In addition to these direct influences on tumor growth, endothelial cells may also modulate tumor behavior indirectly by promoting the polarization of macrophages towards a M2-like phenotype (151).

Significant tumor-promoting roles have been defined also for other immune cells (152), as well as endothelial progenitor cells (153), mesenchymal stem cells (79, 154), neurons (155), and adipocytes (156). We direct the reader to the references listed to learn more about the contributions that these cell types make to the tumor microenvironment.

Metastasis

The study of the microenvironment's role in metastasis returns us to the beginning of the chapter, and harkens back to Paget's observation regarding the spread of breast cancer that was the basis of his "seed and soil" hypothesis (14). The mechanistic underpinnings of this hypothesis are perhaps the hottest topic in metastasis research today (157). Whereas it has been postulated that tumor cells actively "home" to a given organ site, there is also evidence that tumor cells spread indiscriminately (158). In this latter case, certain secondary tissue microenvironments, or "soils", must be particularly hospitable for growth of disseminated tumor cells (DTCs). This could happen in three ways: 1) Tumor cells preferentially remodel target organ sites *before* they get there (i.e., they form a pre-metastatic niche); 2) Tumor cells bring their own microenvironment with them; or 3) DTCs are dependent on formation of a niche that favors their survival and growth upon arriving to the secondary site. The dynamics of metastatic relapse displayed by cancer patients suggest that all three of these mechanisms are plausible (159), and experimental data from spontaneous and experimental metastasis assays in mice offer some insight. We discuss these three possibilities in more detail below:

The concept of the **pre-metastatic niche** refers to the priming of distant organs by tumor-derived factors. This was demonstrated first in immune-competent mice inoculated subcutaneously with either B16 melanoma or Lewis lung carcinoma cells. In these mice, $VEGFR1^+$ bone marrow-derived progenitor cells (BMDCs) are recruited to future sites of metastasis before even the first tumor cells arrive (160). Upon arriving to target organs, BMDCs secrete the chemokine SDF-1 to recruit tumor cells (161), upregulate fibronectin expression in these tissues to promote engraftment and growth of the recruited tumor cells, and activate MMP-9 to destroy BM, disorganize epithelial tissues, and liberate VEGF from the ECM (43, 162). In addition to promoting angiogenesis within the tissue, VEGF functions also to enhance permeability of the microvasculature and to recruit $VEGFR2^+$ BMDCs that

contribute to new blood vessel formation. The ultimate result is more rapid activation of the angiogenic switch and hence accelerated metastatic outgrowth (160). Subsequent studies have pinpointed induction of MMP-2, S100A8 and S100A9 at secondary sites as other principle constituents of the pre-metastatic niche (163, 164).

Tumor-derived agents that induce formation of the pre-metastatic niche continue to be uncovered. In the study by Kaplan et al. mentioned above, media conditioned by B16 melanoma cells, which metastasize to the lung, liver, testis, spleen and kidney, primed Lewis lung carcinoma cells, which metastasize only to the lung and liver, to metastasize to a wider array of organs (160). What is it in the tumor conditioned medium causing this affect? Whereas tumor-derived factors such as VEGF-A, VEGF-C and LOX have been implicated in modulating distant microenvironments (163, 165, 166), secretion of soluble factors, which ostensibly circulate systemically and thus have the potential to modulate any organ site, does not account for the organ-specificity of pre-metastatic niche formation.

A breakthrough study revealed that tumors execute tissue-specific remodeling of distant microenvironments through **exosomes**, small (30-100 nm) cell membrane-derived microvesicles packed with a selected number of molecules. Cargo carried by exosomes includes cytokines, growth factors, ECM proteins, mRNA, microRNAs and even phosphorylated signaling proteins (167). The exciting discovery that exosomes can be transferred from cell-to-cell would indicate that tumor-derived exosomes can activate or deactivate a number of signaling pathways in recipient cells to effect tissue remodeling and other processes from near or far (167).

Specific exosome cargos evolve with tumor progression. For instance, a number of proteins are upregulated in exosomes from metastatic melanoma (compared to non-metastatic disease), including the oncoprotein MET (168). Pre-conditioning mice with exosomes from highly metastatic melanoma cells causes subsequently injected poorly metastatic melanoma cells to home to a greater number of organs and rapidly form lethal metastases (168). How? Melanoma exosomes home to target organs (possibly influenced by expression of specific integrins on their surface) and induce vascular permeability to promote entry of circulating tumor cells. Additionally, exosomes are taken up by BMDCs, resulting in a MET-directed education towards a pro-vasculogenic phenotype. Pro-vasculogenic BMDCs promotes metastatic outgrowth of disseminated cells upon arriving to target organs (168). Accordingly, exosomes derived from metastatic tumor cells can prime distant organ sites for tumor cell extravasation, adhesion, and growth. Thus inhibiting exosome production, engraftment or specific exosome contents could prove effective in blocking or disrupting formation of the pre-metastatic niche.

Whereas tumor cells and tumor-derived exosomes have been observed in the blood, only recently has the possibility been raised that stromal cells from the tumor microenvironment may also wind up in the body's circulation. Is it possible that tumor cells could be bringing their own fibroblasts (soil) to distant organs? A small-scale study (11 prostate cancer patients with metastatic disease and 10 with localized disease) revealed that non-epithelial, non-

lymphocytic, fibroblast-like cells were present in blood more than 50% of patients with metastatic disease, but absent in all participants with localized cancer (169). Experiments in mice have shown the potential significance of circulating fibroblasts, as tumor cells in heterotypic tumor-fibroblast fragments exhibit enhanced survival and enhanced efficiency of metastatic outgrowth upon reaching target organs (170, 171).

Aside from pre-conditioning metastatic sites before their arrival or bringing activated stromal cells with them as they metastasize, tumor cells are also able to disrupt homeostasis in target organs and induce stromal cells to deposit factors that create a favorable **metastatic niche** (172) (Figure 16-4C). Metastatic tumor cell lines can be enriched for lung-, bone- and brain-tropism by serial passaging through mice, and these cells reveal distinct gene expression signatures that enable more efficient metastasis to these target organs (173-175). The products of a number of these genes mediate interactions between the tumor and its microenvironment. Follow-up studies have defined a number of ECM molecules and extracellular factors such as tenascin-C (176), periostin (177), versican (178), col-1 (179), interleukin-6 (180), and tissue factor (181) that comprise the metastatic niche[5]. From these studies, two themes emerge: 1) it is not necessary for both the tumor and the stromal cells to express these factors-- either will do, and induction could be reciprocal; and 2) these molecules are also commonly expressed during development or wounding in a temporally regulated fashion. As a result, the metastatic niche comprises a familiar, hospitable milieu that supports DTC survival and growth.

The Frontiers of the Microenvironment: Where to from here?

There are a number of conclusions that can be made from the newly found field of the microenvironment. One is that the microenvironment can have both positive and negative influences on aberrant cells. The second is that the normal microenvironment may indeed protect against tumor progression (for review, see (13)). In addition, two of the most crucial directions in this research are understanding how the microenvironment contributes to chemotherapeutic resistance, and how therapeutic regimens could be devised that simultaneously target tumor cells and their surrounding microenvironment.

The tumor microenvironment promotes drug resistance.

A popular theory about how tumors become chemoresistant is that a given chemotherapeutic kills all of the susceptible cells. The fraction that survives then clonally expands, and the genetic or epigenetic alterations that rendered these cells resistant largely remain, resulting in a tumor that may no longer respond to that given therapy (182). However, it is increasingly appreciated that the microenvironment can also significantly impact treatment response.

[5] With the caveat that these studies were mostly focused on breast cancer metastasis to the lung.

One way is by influencing the delivery and distribution of administered drugs. Blood vessels in tumors are highly heterogeneous in terms of blood flow and permeability (150, 183). Thus, depending on its molecular weight, a drug be able to enter one portion of a tumor, but never reach another (183). Even if it exits the vasculature, drug transport through the bulk of the tumor is greatly influenced by the density of cells and the ECM molecules. Cell density impacts the effective concentration of drug taken up by tumor cells, while ECM molecules restrict the effective diffusion of a drug due to binding interactions and/or by physically restricting mobility through the proteinacious mesh (69, 183, 184). One may thus conclude that attenuating the stromal reaction and associated fibrosis and desmoplasia, is a promising approach to improve the distribution and efficacy of subsequently delivered chemotherapeutics. Indeed, initial attempts at this approach by pre-treating tumors with anti-fibrotic agents prior to administrating chemotherapeutics appear promising (185, 186).

Aside from directly impacting delivery of drugs, it is apparent also that privileged niches within the microenvironment protect tumor cells from the damaging effects of therapeutics. One such niche is the BM. It has long been established that β1 integrin-mediated binding of BM suppresses apoptosis of normal mammary epithelial cells (70, 187). Binding of a principle constituent of BM-- laminin-111-- via integrin-β1 has been shown also to mediate resistance of small cell lung carcinoma to cytotoxic agents (188). BM also directs formation of polarized acini that render mammary epithelial cells resistant to a host of cytotoxic drugs and death receptor ligands *independent of growth status*. This laminin-induced resistance to apoptosis is transmitted via integrin $\alpha_6\beta_4$ through formation of mature hemidesmosomes to direct cytoskeletal organization and polarity (189).

Adhesion to other ECM molecules also plays a role in therapeutic resistance. For instance, engagement of the fibronectin ED-A splice variant via integrin $\alpha_5\beta_1$ diminishes radioresponse of breast cancer cells (190), and association with type VI collagen renders ovarian cancer cells resistant to cisplatin (191). These molecules as well as others are likely to act by hyperactivating integrin-mediated survival pathways (e.g., integrin->focal adhesion kinase->Akt) (192), thus providing tumor cells with a survival advantage.

It is also important to consider the effects that chemotherapeutics exert on non-tumor cells, and how their response alters the tumor microenvironment. For instance, normal epithelial cells and stromal fibroblasts respond to the chemotherapeutic mitoxantrone by shifting towards a senescence-associated secretory phenotype. This phenotype is characterized by secretion of a host of molecules associated with angiogenesis, immune cell recruitment, and EMT (193). Hence, a possible byproduct of treatment is that the reactive stroma secretes factors that stimulate nearby tumor cells to grow, invade, and spread. This applies not just to resident tissue cells, but to recruited myeloid cells also (194-196).

From these studies, it is apparent that single therapies are highly unlikely to retain their efficacy long-term, as tumor cells are able to successfully seek refuge in a variety of protective niches. An attractive solution is to devise therapeutic regimens that target the

tumor and its microenvironment simultaneously or in step-wise fashion to deprive the tumor of the interactions that sustain it in the face of chemotherapy.

Targeting the tumor microenvironment. In this chapter, we have provided a number of examples for how reciprocal interactions between cells and their microenvironment function to initiate tumors, force progression, facilitate metastatic outgrowth, and compose a privileged niche that confers therapeutic resistance. A logical extension of these findings is that inhibiting critical interactions between tumor cells and their microenvironmental constituents could slow tumor progression and render these cells more susceptible to treatment. The growing literature on the importance of microenvironment that spans now more than a century offers a blueprint as to how this can be accomplished, including:

1. Targeting reactive elements of the tumor microenvironment. This notion has spurred a number of studies aimed at testing new therapies (and revisiting others) that target angiogenesis, fibrosis, inflammation, desmoplasia, and other aspects of the tumor microenvironment. A summary of these drugs is presented in Table I.
2. Inhibiting signals that impart chemotherapeutic resistance, e.g., β1-integrin mediated signaling, prior to or coincident with administration of chemotherapeutics.
3. Reverting the tumor microenvironment. By coming to a more complete understanding about the signals that cease the development of tissues, or those that deactivate wounding stroma, therapies based on these same cues will perhaps have the same effect on tumor stroma.

In the end, it is important to remember that genes, ECM, forces, or other components of the tumor and the microenvironment do not function in a vacuum. They are constantly engaged in dynamic and reciprocal interactions, and it is the balance of signaling between all of these components that is key. When this balance is disrupted, so is tissue homeostasis, and we have provided examples above for how this can result in tumor initiation and accelerated progression. Remember also that there is likely no one way to restore the balance of signaling in a tumor. Just as tumor types are different, tumor microenvironments differ from tumor-to-tumor and patient-to-patient. Accordingly, one should investigate multiple therapeutic avenues, and remain open to how they can be applied concert, as this is likely to provide the best means to manage tumors and improve patient outcome.

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Appendix III

"The tumor microenvironment is a dominant force in multidrug resistance"

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The tumor microenvironment is a dominant force in multidrug resistance

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ABSTRACT

The emergence of clinical drug resistance is still one of the most challenging factors in cancer treatment effectiveness. Until more recently, the assumption has been that random genetic lesions are sufficient to explain the progression of malignancy and escape from chemotherapy. Here we propose an additional perspective, one in which the tumor cells despite the malignant genome could find a microenvironment either within the tumor or as a dormant cell to remain polar and blend into an organized context. Targeting this dynamic interplay could be considered a new avenue to prevent therapeutic resistance, and may even provide a promising effective cancer treatment.

1. Introduction

Despite the large repertoire of therapies available and the continuing efforts to incorporate new drugs into clinical practice, it is generally realized that we still have a long way to go to control cancer. This is particularly evident in patients with metastatic solid cancers, frequently resistant to first-line chemotherapy, the approach thus merely palliative, most often leading to progression of the disease and ultimate demise. Many factors conspire to limit treatment effectiveness, including restrictions in drug distribution and penetration (Jain, 1989), and a certain degree of selectivity for the very cells drugs are designed to eradicate. One of the most challenging of these limiting factors is multidrug resistance (MDR), reflected in our lack of clear understanding of how cells evolve to ensure their survival and facilitate metastasis when challenged by therapeutic intervention.

The conventional assumption, based on single cell studies of drug-resistant clones selected after prolonged exposure to cytotoxic agents, has been that multiple mutations are sufficient to fuel both tumor growth and clinical MDR (Vogelstein and Kinzler, 2004). Although this may reflect dispersed tumor cell systems such as leukemia, there is substantial data indicating that such unicellular drug resistance mechanisms represent but one cause of the effective clinical resistance expressed by multicellular solid cancers *in vivo*. These tumors are more than just a clonal expansion of mutant cells; they are organ-like structures (Bissell and Radisky, 2001; Radisky et al., 2001) and as such exist in intimate relationship with other cells within the tumor and the surrounding microenvironment. It is thus reasonable to hypothesize that the dynamics of this rich and ever changing ecosystem encloses additional, but crucial information for mutated genes to exert their influence, and can itself determine the overall sensitivity to anti-cancer drugs. Here we briefly describe how the solid tumor microenvironment/architecture may in fact significantly contribute to the emergence of therapeutic resistance, and discuss the possibility of targeting and manipulating this complex symbiotic interplay to overcome MDR.

2. Cells and their microenvironment: the reciprocal communication that defines normal and malignant contexts

Maintaining the *status quo* in adult tissues requires that newly generated cells adopt the appropriate fate and contribute to the structure and function of the organ to which they belong. Two-way communication therefore has emerged as the organizing principle that enables "dynamic and reciprocal" exchanges of information between cells and their surroundings (Bissell et al., 1982, 2002). According to this model, tissues and organs are embedded in extracellular matrix (ECM)/basement membrane (BM) that provide them structural support and contextual information together with soluble factors. The model of Bissell et al. (1982) took the bidirectional cross talk between the ECM and the cell membrane (Bornstein et al., 1982), and extended it to the level of control of gene expression, by connecting ECM–ECM receptor interactions to the cytoskeleton and to the nuclear matrix and chromatin. Indeed Bissell and Hall (1987) argued that in the last analysis the organ (or indeed the organism) is the unit of function in all organisms. Cells respond then to cocktails of soluble and insoluble signaling molecules and, in turn, tune their microenvironment. It is the result

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of this harmonious combination that governs tissue dynamics and function.

The importance of 'tissue interaction' to formation of organs was first hypothesized by Pander (1817). Over a century later, seminal work of early developmental biologists demonstrated that cells of distinct embryonic lineages engage in a highly organized cross talk that ensures proper cell sorting and directs tissue and organ morphogenesis and differentiation (reviewed by Nelson and Bissell, 2006). It is important to mention that phenotypic plasticity is implicit to this normal differentiation (Bissell, 1981), as within an individual, genotype does not specify a strictly defined phenotype, but instead a range of phenotypic manifestations within a norm of reaction. In an example of the dominance of the microenvironment over even a potent oncogene, Dolberg and Bissell injected Rous sarcoma virus (RSV), encoding the oncogene v-*src*, into the wings of chick embryos, and observed the initial normal development of the embryos, despite the presence of the active oncogene (Dolberg and Bissell, 1984; Howlett et al., 1988). However, when these same embryonic wings were removed from the greater context of the embryo, they quickly displayed a transformed phenotype in a tissue culture dish (Stoker et al., 1990), suggesting the embryonic environment or context was dominant over the pp60 Src. This suppression was not absolute, and a profound microenvironmental change, such as the one occurring when the embryos got closer to hatching, favors aberration and disintegration in blood vessels and other tissues as was seen also in experiments of Hochedlinger et al. (2004). Subsequent experiments showed that the wound-healing response is a critical event that creates a permissive environment also for RSV tumorigenesis in chickens (Dolberg et al., 1985). Together, these studies demonstrated that oncogene expression was compatible with an apparently normal tissue morphogenesis in the embryo presumably since the wound healing response is different in the embryo, and that the tumorigenic phenotype could be revealed after microenvironmental perturbations, such as those induced by culturing cells on plastic with serum or wounding in the adult chicken.

Evidence of the coexistence of normal and malignant cell populations within the same tissue, without resulting in a frank malignant tumor, has been reported also in human tissue specimens. Studies of large autopsy series have revealed that the majority of middle-aged and older people who die from causes other than cancer have frequent precancerous lesions throughout their bodies (Rich, 1979; Nielsen et al., 1987; Harach et al., 1985; Folkman and Kalluri, 2004). Analyses of 'normal' epithelial tissue adjacent to tumors have shown that similar patterns of mutations can be found in both, yet tumor growth is restrained by normal contextual cues (Deng et al., 1996; Washington et al., 2000). These and other related findings led Bissell and Hines recently to propose the microenvironment as the attenuator of both tumor onset and malignant progression, providing a rational framework to explain why the majority of people live cancer-free lives for decades, yet harboring a number of harmful mutations they accumulate over time (Bissell and Hines, 2011). Another example is that despite the fact that people with familial BRCA or APC mutations have these in all their cells yet they develop tumors only in a few of cells in specific organs.

If reciprocal communication between a normal context and ECM defines the normal tissue homeostasis, the opposite should also be true: abnormal context should lead to abnormal conversation allowing cells to disregard sorting rules and violate normal tissue boundaries, setting the stage for cancer progression. That this indeed is the case has long been obvious to pathologists, as judged by common reports of fibrotic tissue, ECM deposition, and immune and inflammatory infiltration, collectively called 'reactive' tumor stroma. As early as 1938, Orr observed that morphological changes in the microenvironment of the skin of carcinogen-treated mice appear long before neoplastic alterations in epithelial cells (Orr, 1938). Subsequently, Tarin showed that complex sequential changes occur at the epithelial–mesenchymal boundary during mammary tumor progression (Tarin, 1969), and insights into the nature of these reciprocal tumor–stromal interactions have gradually accumulated. The presence of cancer-associated fibroblasts (CAFs) has been reported in many cancer types, and bidirectional CAF–epithelial interactions were shown to precede invasion and stimulate tumor growth and progression (Picard et al., 1986; Camps et al., 1990; Hayashi et al., 1990; Skobe and Fusenig, 1998; Thomasset et al., 1998; Olumi et al., 1999; Cunha et al., 2003; Bhowmick et al., 2004). Concomitantly, cancer cells overproduce proteolytic enzymes, particularly metalloproteases (MMPs) (Chambers and Matrisian, 1997), which generate fragments with pro-migratory and pro-angiogenic functions (Folkman and Kalluri, 2002) as well as activate cell-surface and ECM-bound growth factors (Egeblad and Werb, 2002), reflecting the extensive crosstalk between the microenvironment and the malignant cells. Other examples include overexpression of an autoactivated form of MMP3 in mouse mammary gland epithelium where the MMP disrupts the integrity of the BM, leading to the development of a reactive stroma and eventually genomically unstable mammary tumors (Sympson et al., 1994; Thomasset et al., 1998; Sternlicht et al., 1999). Recently, adipocytes have been recognized as important mediators of normal context disruption as well, since they produce a host of biologically active molecules that promote the inflammatory process and angiogenesis (Iyengar et al., 2005; Motrescu and Rio, 2008; Cao et al., 2010; Dirat et al., 2011). Preference for metastatic colonization is heavily influenced also by communication between circulating tumor cells and bone marrow-derived cells (BMDCs), which home to the tumor and promote progression, escape from the tumor, survival and ultimately metastatic growth (reviewed in Joyce and Pollard, 2009).

3. Tumor microenvironment facilitates the emergence of MDR

As discussed above, tumors exist in intimate relationship with the surrounding microenvironment, and it is the dynamics of this heterogeneous and ever changing ecosystem that provides additional but crucial information for mutated genes to exert their function. In addition to initiating and supporting the tumorigenic process, a permissive microenvironment can also affect the sensitivity of tumor cells to drug treatment. The composition and organization of the ECM and stromal components contribute to marked gradients in drug concentration, increased interstitial fluid pressure and metabolic changes, all of which can strongly enhance the resistance of tumor cells to drug agents (Dang and Semenza, 1999; Heldin et al., 2004; Di Paolo and Bocci, 2007). That the three-dimensional structure of the tissue itself could also account for tumor resistance to radiation and chemotherapy was first recognized by Sutherland and co-workers in the early 1970s. Using Chinese hamster lung fibroblasts and EMT-6 mammary tumor cells, they showed that multicellular spheroids were markedly more resistant to radiation and distinct doses of adriamycin than the same cells cultured in monolayer (Durand and Sutherland, 1972; Sutherland et al., 1979). This finding led Teicher and colleagues to hypothesize that resistance to anticancer drugs could develop through mechanisms operative only *in vivo*. By deriving a series of alkylating agent-resistant variants of the EMT-6 mouse mammary tumor, they surprisingly found that those cells plated on plastic culture dishes were no more resistant than the parental EMT-6 cell line, but they would reexpress their drug resistance properties upon reinjection in mice or when grown in three-dimensional conditions (Teicher et al., 1990; Kobayashi et al., 1993). Further work demonstrated that this rapid reappearance of resistance represents

a physiological strategy of adaptation implicit to a multicellular tissue, involving cell-cell and cell-ECM interactions, and it may be one reason to explain the seemingly rapid development of drug resistance in some patients who are initially responsive to chemotherapy (Graham et al., 1994; Durand and Olive, 2001; Kerbel et al., 1996).

3.1. Cell adhesion-mediated drug resistance (CAM-DR)

While adhesion is essential for normal cells to grow and survive, anchorage-independence for growth and survival is considered an essential feature of malignant cells (Frisch and Francis, 1994). We demonstrated in 1995, that loss of β1-integrin-mediated adhesion in non-malignant mammary cells leads to apoptosis and that Laminin-111 specifically was needed for survival (Boudreau et al., 1995). Some tumor cells lose β1-integrin altogether (Howlett et al., 1995), others dramatically upregulate the level, but driving the level down using inhibitory antibodies allows these cells to reversibly 'revert' to a 'normal' phenotype, and reduce tumor take and size appreciably despite the malignant genome (Weaver et al., 1997; Wang et al., 1998, 2002; Weaver and Bissell, 1999; Bissell et al., 2005). Adhesion to ECM *via* β1-integrins can also enhance the tumorigenicity and resistance of multiple myeloma and small cell lung cancer (SCLC) cells to chemotherapeutic agents doxorubicin and melphalan (Fridman et al., 1990; Sethi et al., 1999). Conversely, preventing tumor cell adhesion by blocking integrin binding to ECM and stromal cells results in a dramatic reduction in tumor burden and increases considerably the overall survival in a mouse model of multiple myeloma (Mori et al., 2004). The combination of this anti-adhesion approach with conventional cytotoxic melphalan proves even greater efficiency, reducing tumor load substantially more than either treatment alone. Similar observations by Park et al. (2006, 2008) show that inhibition of β1-integrin allows also for a significant reduction in tumor volume and increases sensitivity to ionizing radiation (IR) in human breast cancer xenografts. Recent work has also showed that inhibition of β1-integrin significantly increases the sensitivity of *HER2*-amplified breast cancer cell lines to Trastuzumab and Pertuzumab. This study has also reported dramatic differences in response to therapeutic agents for cells grown in monolayer as opposed to three-dimensional matrices, highlighting again that cellular response to drugs is context dependent (Weigelt et al., 2010).

Simple culture models have been used to delineate specific molecular mechanisms of cell adhesion-mediated drug resistance (CAM-DR) – the term coined to describe a rapid form of drug resistance mediated by adhesion. For example, allowing adhesion of human SCLC to the ECM components fibronectin or laminin confers those cells a survival advantage under acute exposure to cytotoxic drugs, by inhibiting drug-induced apoptosis (Sethi et al., 1999). Not unique to SCLC, resistance-promoting effects by integrin-mediated adhesion to ECM were also observed in cancers of the pancreas (Cordes and Meineke, 2003), ovary (Maubant et al., 2002; Sherman-Baust et al., 2003), prostate (Miyamoto et al., 2004), breast (Aoudjit and Vuori, 2001; Menendez et al., 2005), liver (Zhang et al., 2002), brain (Uhm et al., 1999) and leukemia (Damiano et al., 1999; de la Fuente et al., 2003). Further studies in leukemia cell lines showed that β1-integrin-mediated adhesion could modulate cellular localization and availability of several apoptotic regulators (such as CASP8, c-FLIP$_L$ and BIM), preventing tumor cells from apoptosis and favoring MDR (Shain et al., 2002; Hazlehurst et al., 2007). Interestingly, this mechanism of CAM-DR identified in cell culture models is consistent with patterns of low expression of apoptotic promoters in patients with resistant acute lymphoblastic lymphoma or acute myeloid leukemia (Flotho et al., 2006, 2007; van Stijn et al., 2005).

Integrin binding to ECM and stromal cells can also control cell cycle progression in both hematological and epithelial malignancies. Work by Hazlehurst et al. (2003) reported that G1 arrest of myeloma cells induced by β1-integrin adhesion to fibronectin correlates with upregulated levels of cell cycle regulator p27, and enhanced resistance to etoposide (Hazlehurst et al., 2003). Later studies showed that integrin-mediated adhesion could also interfere with ubiquitin-proteasome proteolytic pathways. For example, preventing p27 proteosomal degradation induces cell cycle arrest in non-Hodgkin B cell lymphoma and hepatocellular carcinoma cell lines, leading to extreme drug resistance (Lwin et al., 2007; Fu et al., 2007). In addition to being significantly less chemosensitive, tumor cells grown on certain ECM components show prolonged radiation-induced cell cycle arrest in contrast to cells growing on non-specific substrates (Cordes and van Beuningen, 2004; Kremer et al., 2006; Dimitrijevic-Bussod et al., 1999). This delay appears to provide more time for DNA damage repair at distinct cell cycle checkpoints after genotoxic injury (Bartek and Lukas, 2001). Experiments in non-tumorigenic lung endothelial and hemopoietic cancer cell lines demonstrated that integrin-ECM interactions can strongly affect the machinery of DNA damage recognition and repair (Hoyt et al., 1997; Hazlehurst et al., 2003; Jones et al., 2001). Activation of these pathways by adhesion of tumor cells to ECM is likely to accelerate and optimize the efficacy of DNA damage repair after irradiation, providing for a more stable genome and thus cell survival.

The absence of unit tissue architecture inherent in two-dimensional (2D) cell culture systems used in the majority of the studies referred to above explains why so many of these cells do not express tissue specific functions (for review see Bissell, 1981; Bissell et al., 2005). When normal mammary cells were cultured in a laminin-rich ECM gel (3D lrECM) (Barcellos-Hoff et al., 1989) the cells reorganized, and both form and function were restored. This concept was used to develop an assay that could distinguish between non-malignant and malignant cells on the basis of their structural integrity. Whereas non-malignant cells formed polarized growth-arrested acini in lrECM, primary breast tumor cells or breast cancer cell lines formed highly disorganized and proliferative colonies (Petersen et al., 1992; Weaver et al., 1995). Under these conditions the balance of signaling pathways are deranged in tumor cells. Antagonizing one or more of the many signaling pathways that are deregulated in tumor cells causes them to functionally revert to a 'normal' phenotype, despite their malignant genome (Howlett et al., 1995; Weaver et al., 1997; Wang et al., 1998, 2002; Kirshner et al., 2003; Weaver and Bissell, 1999; Muschler et al., 2002; Liu et al., 2004; Weir et al., 2006; Itoh et al., 2007; Kenny and Bissell, 2007; Beliveau et al., 2010). Interestingly, there is a reciprocal interaction between any oncogenic pathway and all the rest in 3D and the changes do not occur in 2D (Anders et al., 2003). Together, these data show that tissue architecture can override the proliferative and invasive malignant phenotype of breast tumor cells, but that reversion to a 'normal' phenotype is dependent upon sensing of the appropriate spatial and biochemical cues from the microenvironment.

The same concepts were later used to demonstrate that survival and sensitivity to drugs used in the clinic of human breast cells is dependent on cell and tissue polarity as well as integrin-mediated adhesion to BM and does not correlate with the rate of growth or quiescence (Weaver et al., 2002) (Fig. 1). Briefly, when non-malignant and malignant cells were treated with three immunomodulators (Trail peptide, anti-FAS antibody and tumor necrosis factor TNF-α) and three chemical drugs (the topoisomerase II inhibitor etoposide, the microtubule modulator paclitaxol and actin cytoskeleton disruptor cytochalasin B) on 2D, the rate of apoptosis was equivalent in both cell types with high statistical significance. However, when placed in 3D lrECM, the cells that become polarized (either non-malignant or reverted tumor cells) were resistant to all six agents, whereas disorganized cells

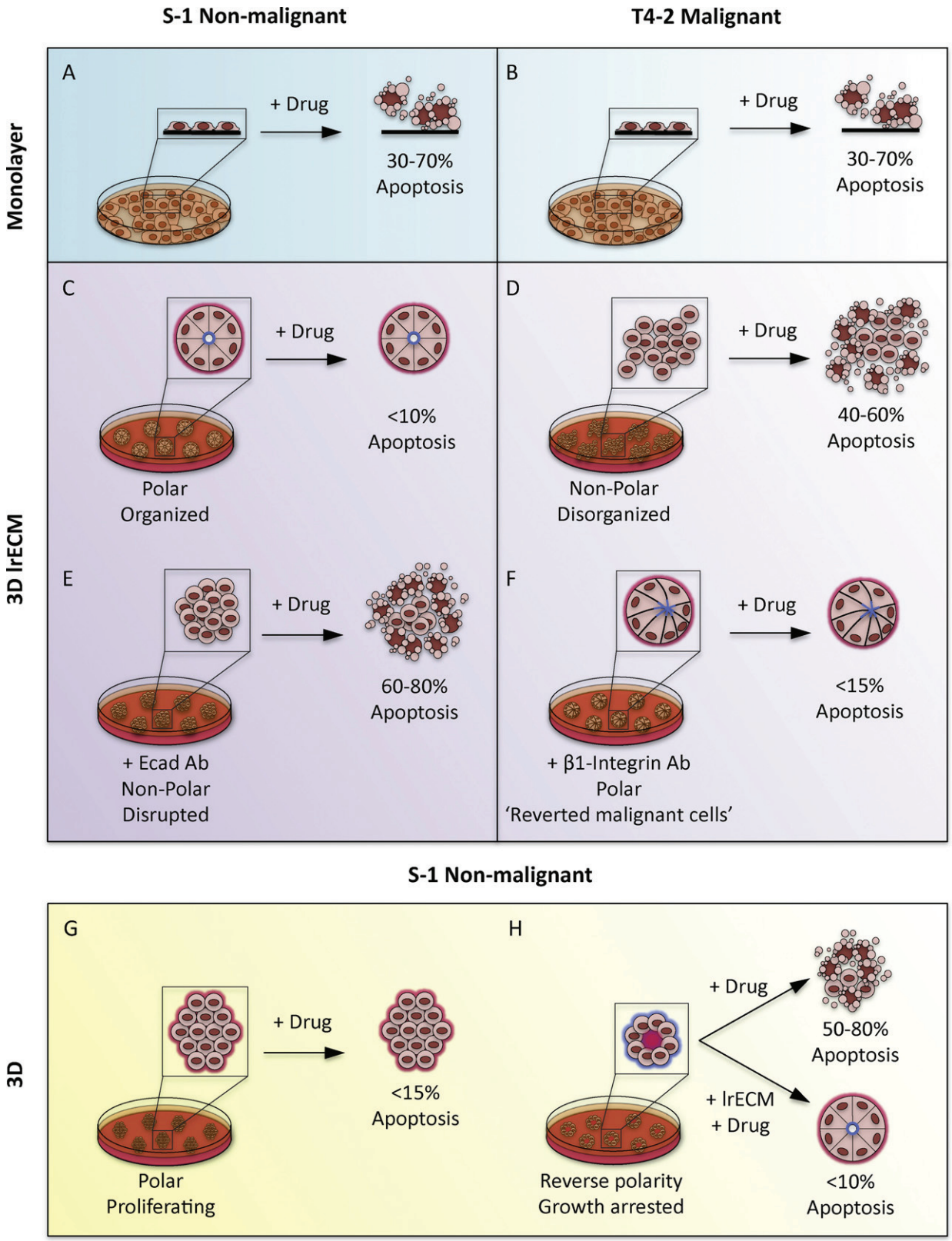

Fig. 1. Polarized mammary structures are resistant to apoptosis induced by chemotherapeutics. When cultured on 2D monolayer, both non-malignant (A) and malignant (B) human breast cells show a similar rate of apoptosis upon treatment with distinct immunomodulators and chemical agents. However, when placed in 3D lrECM, S-1 non-malignant cells form polarized growth-arrested acini resistant to drug cytotoxic effects (C), whereas T4-2 malignant cells appear highly disorganized, proliferative and sensitive to therapeutic drugs (D). Perturbing apical–basal polarity of S-1 acini, by treatment with E-cadherin function-blocking antibody, results in a dramatic increase of sensitivity to drug agents (E). Conversely, restoring cell and tissue polarity in T4-2 structures, by treatment with β1 integrin inhibitory antibody, induces malignant cells to 'revert' and provides them resistance to chemotherapeutic agents (F). Polarized mammary epithelial cells are resistant to apoptosis induced by cytotoxic agents, either growth-arrested (C) or proliferating (G). In contrast, growth-arrested but reversely polarized S-1 cells grown in collagen I ECM undergo apoptosis (H, upper panel); once exposed to lrECM, these S-1 non-polar structures polarize and become resistant to apoptosis (H, lower panel).

both normal and malignant were equally sensitive. It was shown that the resistance to apoptosis depends upon the 3D organization of the acini and is functionally linked to β4-integrin-directed hemidesmosome formation and NFκB activation. Expression of a β4-integrin that lacked the hemidesmosome-targeting domain disrupted tissue polarity and triggered apoptosis by all drugs tested (Weaver et al., 2002).

Aside from determining cell and tissue architecture, the way cell surface adhesion molecules perceive ECM also affects nuclear structure and chromatin organization. Experiments in mammary epithelial cells demonstrate that ECM can modulate the transcription of the β-casein gene by activating an ECM/response element inducing rapid histone modifications (Schmidhauser et al., 1992; Myers et al., 1998). Studies by Maniotis et al. (1997) in fibroblasts and endothelial cells have also confirmed that alterations in surface-adhesion receptors are channeled along cytoskeletal filaments and ultimately concentrate at the nucleus to reorganize chromatin structure and gene expression. The work that followed brought the first demonstration that cells experience a complete and global reorganization of chromatin in response to a certain ECM composition and thickness (Maniotis et al., 2005; Sandal et al., 2007). Laminin specifically, but not fibronectin or Type I collagen, greatly increased the resistance of chromatin digestion by *Alu*I restriction enzyme in breast cancer cells. This suggests chromatin reorganization as another mechanism by which cells develop CAM-DR, particularly to drug agents that bind to or disrupt DNA.

3.2. 'Forcing' malignant progression and therapeutic resistance

Cells and ECM exert positive and negative tension on each other. Cells sense force through mechanoreceptors and respond by generating mechanical tension in their actin cytoskeleton and adhesions to ECM (Ingber, 1991, 1997). This phenomenon of mechanoreciprocity maintains tensional homeostasis in the tissue and is crucial for normal tissue-specific development (Krieg et al., 2008). Each tissue has a particular 'stiffness phenotype' and each cell type is finely tuned to the specific tissue in which it resides. An increase in ECM protein concentration, matrix crosslinking or reorientation of matrix fibrils can stiffen a tissue locally to alter cell growth or direct cell migration (Discher et al., 2005). This has important implications for development and frequently leads to disease progression, including cancer. For example, malignant transformation of the breast has been associated with a dramatic and chronic increase in mammary gland tension and ECM stiffening (Krouskop et al., 1998; Plewes et al., 2000) (Fig. 2). Here we describe the variety of physical stresses experienced by transformed mammary epithelial cells (MECs) within a breast tumor, which can dramatically enhance cell growth, survival, motility, invasion, and ultimately compromise therapeutic response.

At the tissue level, actively proliferating transformed MECs exert gradually increased compression stresses in the ductal tree. Once the tension becomes large enough, the tumor mass compresses intratumoral vessels, and prevents the blood flow, producing regions of tissue hypoxia and compromising the efficacy of tumor therapy (Roose et al., 2003; Shannon et al., 2003). Likewise, compression stress also increases the interstitial pressure, blocking tissue vasculature and lymphatic networks, and impairing drug delivery and immune cell infiltration and clearance (Jain, 2001; Padera et al., 2004). When a tumor forms within a breast, even non-malignant cells within that breast experience fields of increased resistance force (increased stiffness) in their ECM microenvironment that alternate with pockets of high compliancy (decreased stiffness). Such fluctuations in the ECM elastic properties likely arise from the activation of resident stromal fibroblasts and infiltrating immune cells, as well as the increased deposition, processing and cross-linking of ECM proteins (Ebihara et al., 2000; Chiquet et al., 2009). All these changes can strongly influence the behavior of transformed MECs, either by directly activating mechanotransduction pathways or by indirectly stimulating resident mammary gland stromal fibroblasts to release various cytokines, growth factors and ECM degrading enzymes (Decitre et al., 1998; Yeung et al., 2005; Wozniak et al., 2003). For example, increases in matrix stiffness that enhance cell contractility have been found as sufficient to induce transformation of mammary epithelial cells (Paszek et al., 2005; Samuel et al., 2011). Conversely, a decrease in tissue stiffness by inhibition of collagen crosslinking prevents malignant growth and tumor progression in a murine model of breast cancer (Levental et al., 2009). Increased matrix stiffness has been recently implicated also in the modulation of chemotherapeutic resistance in hepatocarcinoma cells (Schrader et al., 2011). The relation of the biomechanical properties of the microenvironment with the emergence of therapeutic resistance is still in its infancy and requires much more attention.

3.3. Plasticity of cell phenotype and the emergence of MDR

The tumor microenvironment is extraordinarily heterogeneous: different numbers and types of infiltrating normal cells, distinct densities of blood and lymphatic vasculature, and singular composition of extracellular matrix. For this reason, cells within a given tumor are expected to experience an array of microenvironmental cues, which will in turn translate into several phenotypic manifestations. In epithelial cancers, these adaptive changes may involve, at least in part, a stepwise cycle of epithelial plasticity, governed by epithelial to mesenchymal transitions (EMT) and the reverse mesenchymal to epithelial transitions (MET). It is now believed that this state is a reversible change of cell phenotype (Petersen et al., 2003), characterized by loose cell–cell adhesion, disruption of apical–basal polarity and cytoskeleton reorganization. Cells become isolated, motile and resistant to apoptosis (Thiery et al., 2009). Although EMT was initially defined to support normal tissue remodeling and diversification during development, an intermediate EMT-like process, meaning transient plasticity, is also evoked during tumor progression, metastasis and recently drug resistance (Lee et al., 2006). For instance, an EMT-like signature was identified as a determinant of insensitivity of non-small cell lung carcinoma (NSCLC) cell lines and xenografts to the small molecule-EGFR-inhibitor Erlotinib (Tarceva) (Yauch et al., 2005; Thomson et al., 2005). These results were also confirmed in other types of tumors, such as head and neck squamous cell carcinoma (HNSCC) and hepatocellular carcinoma, as well as for treatment with other EGFR inhibitors such as Gefitinib (Iressa) (Frederick et al., 2007) and Cetuximab (Erbitux) (Fuchs et al., 2008). The implication of EMT in therapeutic drug resistance has been increasingly reported, for example gemcitabine resistance in pancreatic tumor cell lines (Shah et al., 2007), Oxaliplatin resistance in colorectal cancer cells (Yang et al., 2006), Lapatinib resistance in breast cancer (Konecny et al., 2008) and Paclitaxel resistance in both breast (Cheng et al., 2007) and epithelial ovarian carcinoma (Kajiyama et al., 2007).

3.4. Microenvironment-induced protective quiescence

The selective pressure imposed by conventional chemotherapy regimes eliminates certain cells within the tumor population. This surviving population following chemotherapy is referred to as minimal residual disease; these cells either can stay within the tumor or most likely have already found refuge in protective microenvironments in specific organs, depending on the origin of the primary tumor. These dormant cells remain in a state of quiescence until they sense signals to start a burst of growth. Failure of the initial therapy to eradicate these cells to prevent tumor recurrence is clearly one of the main barriers to effective cancer treatment.

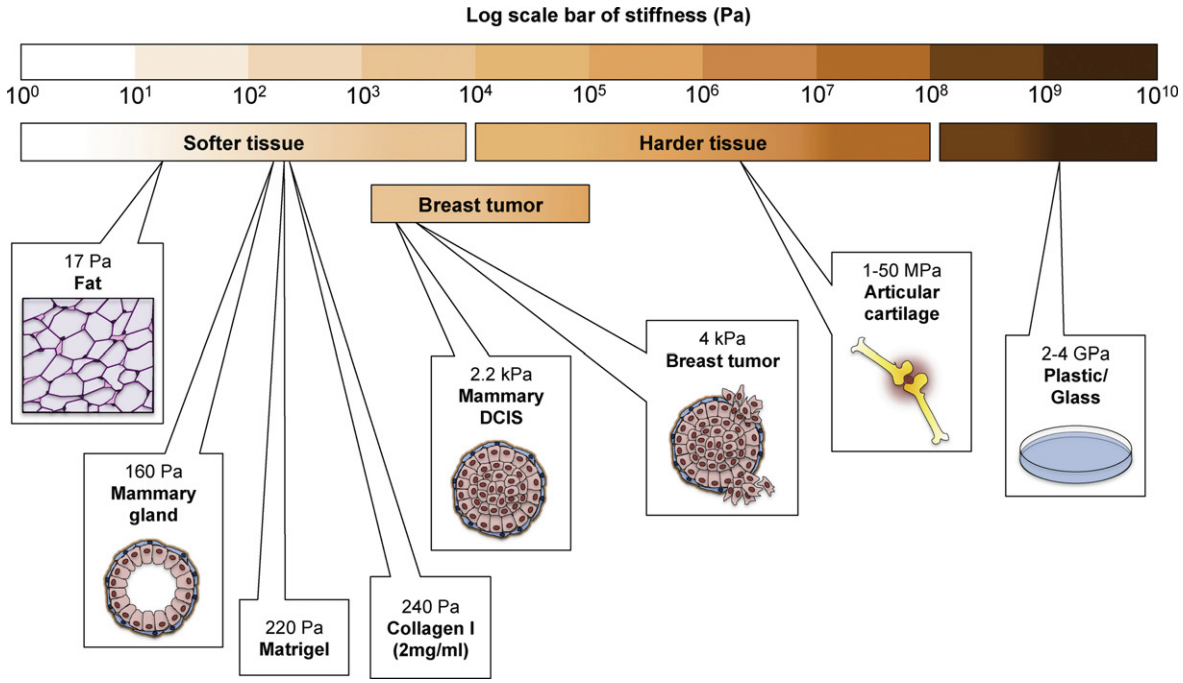

Fig. 2. Mammary gland tissue becomes increasingly stiffer during tumor progression. Each tissue has a particular 'stiffness phenotype' (stiffness measured in Pascals – Pa) and each cell type is finely tuned to the specific tissue in which it resides. For example, fat tissue is much softer than cartilage. Thus, a highly compliant matrix favors adipogenesis, whereas osteoblast differentiation is optimal on stiffer ECM. Similarly, normal mammary gland development is optimally supported by interaction of epithelial cells with a soft matrix. During tumor progression, breast tissue becomes increasingly stiffer and tumor cells become significantly more contractile and hyper-responsive to highly compliance signals. Although breast tumors are much stiffer than the normal mammary gland, the material properties of a breast tumor or any other physiological environment remain significantly softer than those of glass or plastic culture dishes.

For example, the presence of bone marrow micrometastases in about 30% of patients with breast cancer at the time of diagnosis is a strong predictor of disease recurrence (Karrison et al., 1999; Bidard et al., 2008). It is reported that 15–20% of patients still have disseminated tumor cells in the bone marrow regardless of the aggressiveness of the treatment (Wiedswang et al., 2004). But how do some cells manage to become quiescent to survive the selective pressure of therapeutics? We suggest that, despite the malignant genome, some tumor cells can find a microenvironment to allow them to remain polar, blend into an organized context and survive in a quiescent state similar to our studies on reversion described above. Polar cells are quiescent. However, cells with reverse polarity in 3D cultures are also quiescent, and yet they die when treated with chemotherapeutic agents, whereas the polar cells do not (Gudjonsson et al., 2002; Weaver et al., 2002). Studies with metastatic hematopoietic, colon adenocarcinoma and breast cancer cells show that tumor-ECM interactions indeed determine a state of quiescence associated with CAM-DR (Nefedova et al., 2003; Korah et al., 2004; Schmidt et al., 2001). Similar observations in mouse models of breast cancer micrometastasis confirmed that microenvironments that induce dormancy harbor cells that become quiescent and tolerant to doxorubicin (Naumov et al., 2003; Goodison et al., 2003). Further support for the concept of microenvironmental-induced quiescence comes from data from bone marrow specimens from breast, gastric and colorectal cancers, in which micrometastasis with marked signs of quiescence were found in 34% of the patient cohort (Pantel et al., 1993). Whereas some cells interpret the microenvironment as conducive to quiescence, others may remodel and/or reproduce a microenvironment that would be permissive for growth of dormant cells. Experiments in myeloma cells showed that a dynamic interaction with their surrounding stroma allows tumor cells to proliferate in response to IL-6 stimulation while still adhered to a fibronectin matrix (Shain et al., 2009). *In vivo* studies demonstrated that single mammary epithelial tumor cells can remain dormant in metastatic sites for long periods of time, but retain their ability to proliferate when transplanted to their tissue of origin (Naumov et al., 2002). A similar switch between proliferation and growth arrest controlled by the tumor cell–microenvironment crosstalk was observed in head and neck carcinoma (Aguirre Ghiso et al., 1999). In this model, the metastasis-associated urokinase receptor (uPAR) drives tumor growth by interacting and activating $\alpha5\beta1$-integrins, whereas blocking this interaction results in tumor suppression due to induction of dormancy. A more detailed analysis of the mechanisms and markers of dormant cancers will be important for the choice of therapy when patients are known to have minimal residual disease.

4. Treating the tumor microenvironment to overcome MDR

We believe that the same mechanisms that help us not to develop more cancers (Bissell and Hines, 2011) can also help keeping dormant cells dormant (Fig. 3). The initial attempts to reconstruct the correct microenvironment were based on co-culture assays. Analyzing combinatorial products of human prostate epithelial and fibroblast cells, Olumi et al. (1999) showed that normal stromal cells inhibit the progression to epithelial malignancy. Similarly, Javaherian et al. (1998) were able to suppress early stages of neoplastic progression of malignant keratinocytes by introducing an excess of normal keratinocytes. However, it became evident that the 3D architecture and the complex network of interactions that characterize both organs and tumors were traits simply not possible to recapitulate in traditional 2D cultures. In the last two decades, engineered animal models and 3D culture systems have become commonplace, making it possible to start dissecting the plasticity of the tumor ecosystem and mechanisms by which microenvironmental signals could lead to tumor cell reprogramming and 'reversion'. In addition to the work from

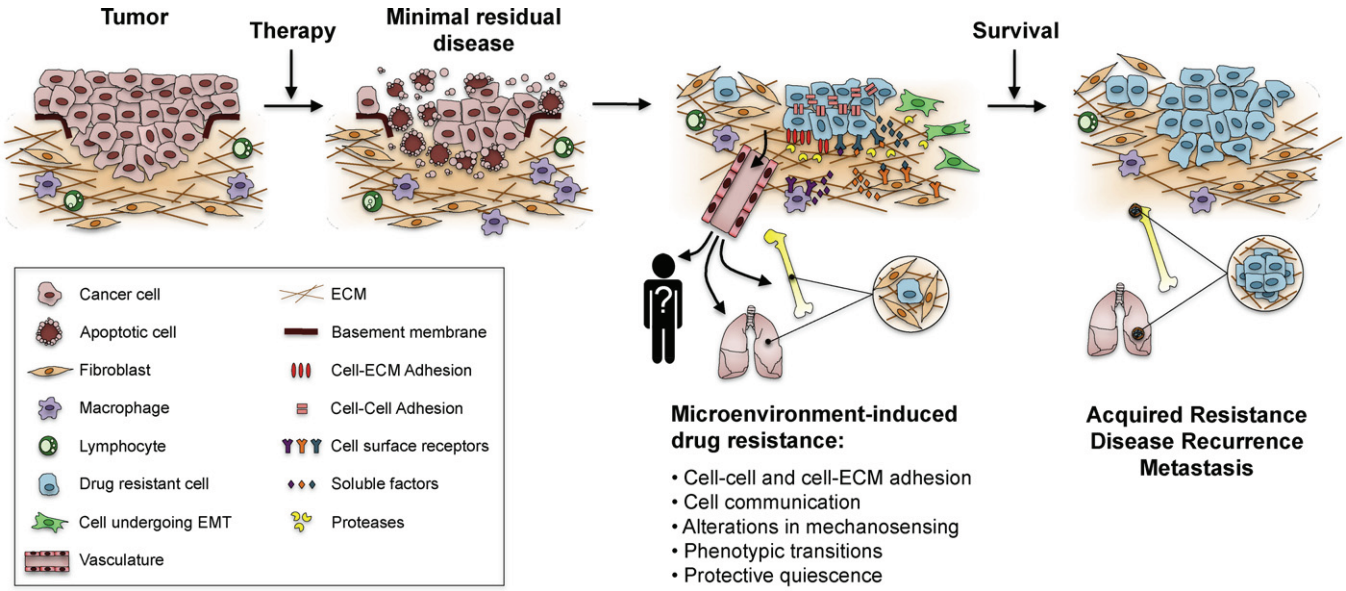

Fig. 3. Postulated steps in drug resistance and dormancy. Cancer cells exist in intimate relationship with other cells within the tumor and the surrounding microenvironment. This dynamic coalition ensures tumor survival and proliferation, but may determine also the overall sensitivity to anti-cancer drugs. The selective pressure imposed by conventional chemotherapy regimes eliminates certain cells within the tumor population. The surviving population following chemotherapy is referred to as minimal residual disease; despite the malignant genome, these cells can find a microenvironment to allow them to remain polar, blend into an organized context and survive therapeutic insults. These protective microenvironments facilitate the development of drug resistance by distinct molecular mechanisms, including: intercellular and cell-ECM adhesion; cell communication by various soluble factors and overproduction of proteolytic enzymes; alterations in mechanosensing that disrupt tensional homeostasis in the tissue; phenotypic transitions for cells to become isolated, motile and resistant to apoptosis; and a state of protective quiescence, either within the tumor or in specific organs depending on the origin of the primary tumor. Over time, drug resistant cells develop even more permanent mechanisms of resistance (acquired resistance), and eventually cause disease recurrence and metastatic growth.

Bissell laboratory described above, Hendrix and colleagues conduced a series of elegant studies elucidating the regulation of tumor cell plasticity by an embryonic milieu of human stem cells (hESCs), zebrafish or chick (Topczewska et al., 2006; Kulesa et al., 2006). Her laboratory has also developed a 3D model to demonstrate that the microenvironment of hESCs can reprogramme aggressive melanoma cells toward a less aggressive melanocytic-like phenotype (Postovit et al., 2006). Work from Gil Smith laboratory has demonstrated that human carcinoma cells could be redirected to produce progeny capable of typical mammary epithelial cell function by interaction with the microenvironment of a mouse mammary gland developing *in vivo* (Bussard et al., 2010).

Collectively, the above observations reaffirm the dominance of tissue microenvironment and architecture over the genotype, and suggest that differentiation therapy, a concept used in treating some forms of leukemia by administration of retinoic acid, vitamin D compounds and PPARγ agonists (reviewed in Nowak et al., 2009), may also be a powerful strategy for therapeutic intervention in solid cancers. This 'microenvironmental therapy' might potentially reverse subtle, but critical, imbalances in tumor–microenvironment interactions, and provide a higher specificity that can help minimizing collateral toxicity to normal adjacent tissues. Additionally, stromal cells are not as genetically unstable as cancer cells, and are therefore less likely to develop drug resistance. There already have been several exciting reports of success in the clinical targeting of tumor stroma. For example, inhibition of inflammatory cells and cytokines by treatment with non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to lower the risk for colon and breast cancer, and might help preventing lung, oesophageal and stomach cancers (Ricchi et al., 2003). Likewise, the angiogenesis inhibitor bevacizumab (Avastin) has proven successful in the treatment of colorectal (Salgaller, 2003) and kidney tumors (Mass et al., 2004). However, there have been also some disappointments, such as the inefficacy and severe intolerable side effects of MMP inhibitors in patients with late-stage cancers (Stetler-Stevenson and Yu, 2001). This may be due to the many contradictory roles that MMPs play in modulating tumor microenvironment, and that were not taken into account when the broad-spectrum MMP inhibitors were designed (Coussens et al., 2002; Morrison et al., 2009). In addition, selecting patients in advanced stage of the disease is not likely to be successful.

In summary, normalizing tumor microenvironment represents an important new direction for cancer therapy. Despite the fact that the microenvironment comprises many different components, it is still possible to reduce the severity of malignant cells using a single effective agent. Of course it is best always to combine drugs that target distinct aspects of the reactive stroma with the conventional cytotoxic drugs designed the kill tumor cells, thereby treating the tumor as the organ we now recognize it to be.

5. Concluding remarks

We now appreciate tumors as true ecosystems, harboring a plethora of cells and stromal components that coexist and engage in dynamic and reciprocal interactions. It is the product of these interactions from a very early stage of the disease that clearly determines the fate of the tumor as well as the patient. The data we have summarized here suggests that tumor microenvironment also is a prominent shelter for the population of surviving tumor cells following initial chemotherapy. As such, the microenvironment can facilitate the development of therapeutic resistance. Given the increased knowledge of the signaling cues and components that comprise the tumor and its microenvironment, it would be important to incorporate this knowledge into organ-specific and physiological culture models of human cells together with appropriate animal models for drug testing. These systems represent the toolkit to more successfully translate fundamental research findings into therapies in the clinic, and may have great potential in providing answers before proceeding into costly clinical trials. So

far, most 3D models available allow co-culture of epithelial cells only with one other cell type. Thus heterotypic culturing systems that more closely mimic the heterogeneity of the tumor microenvironment still need to be developed. Tailoring drugs to target the tumor microenvironment represents a new direction for anticancer drug development, and may hold significant therapeutic promise.

Conflict of interest

The authors declare no competing financial interests.

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