CHAPTER 7

Extracellular Matrix Remodeling in Mammary Gland Branching Morphogenesis and Breast Cancer:

The Double-Edged Sword

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Introduction

That differentiation and malignancy are different faces of the same coin is now almost a cliché.[1-4] Although widely accepted as fact, exactly what are the points of similarity and differences that contribute to normal morphogenesis on the one hand and to neoplastic progression on the other? How can mechanisms that permit, guide and determine differentiation also contribute to malignancy? More specifically, what are the molecules that guide normal morphogenesis yet contribute to neoplastic transformation and progression? These processes probably involve arrays of genetic programs. For the purpose of this review, we will focus on the roles of several genes that appear to fill these contradictory functions.

Breast tissue morphogenesis is unusual in mammals in that it occurs in the adult and is coupled to the periodicity of mammalian reproductive and pregnancy cycles (Fig. 1).[2-8] During the estrous cycle, and to a greater extent during pregnancy and following parturition, breast tissue branching morphogenetic programs dominate over those promoting differentiation or apoptosis and culminate in the expansion of ducts resulting from regulated growth, migration and invasion into the fat pad. Branching morphogenesis is followed in pregnancy by differentiation of ductal cells into lobular alveolar epithelium that produces milk after parturition. This in turn is followed by extensive extracellular matrix remodeling that occurs concomitantly with tissue involution resulting from the regulated apoptosis of lobular alveolar breast epithelium in concert with the proliferation of adipocytes that occurs after weaning. These morphogenetic/differentiation/involution cycles are repeated throughout the reproductive life span of female mammals. The cyclically regenerative capability of normal adult breast tissue is clearly substantial and is currently thought to result from both hierarchies of stem/progenitor cells within the luminal epithelial cell population and myoepithelial cells lining the ducts. These cell types have marked regenerative abilities and can, for instance, give rise to entire mammary trees when they are transplanted into cleared mammary fat pads.[9-12] Progenitor cell types must also survive involution to participate in the future expansion of ducts during subsequent estrous cycles and pregnancies. We will address potential mechanisms that make them resistant to apoptosis-inducing properties associated with a remodeling ("reactive") stroma, and since evidence suggests that breast stem/progenitor cell populations with regenerative capabilities may give rise to highly aggressive tumorigenic cell subsets within breast tumors,[5,9,10,13] how these properties might be utilized by transformed cells to survive and to proliferate.[10,14-19]

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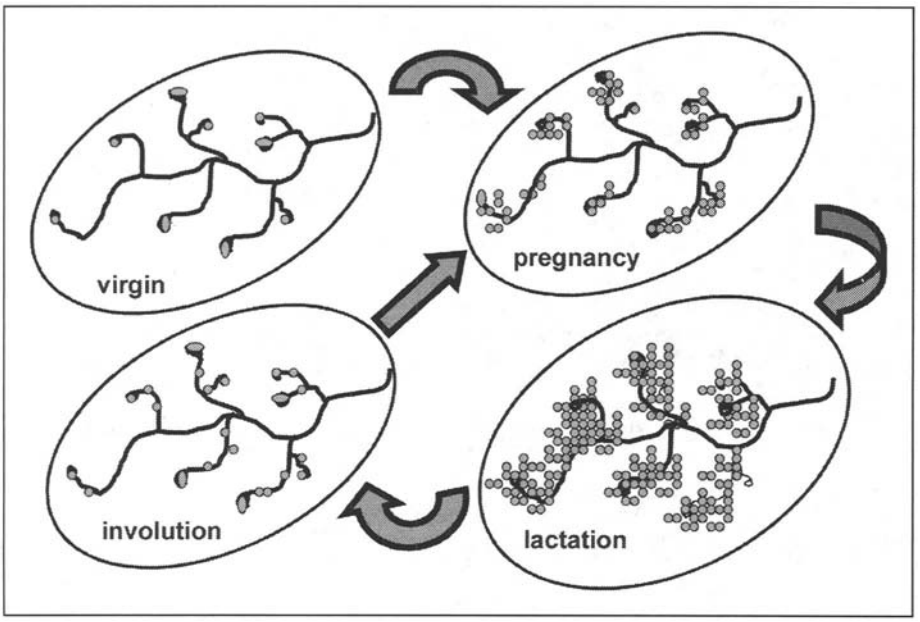

Figure 1. The branching morphogenesis cycles of the mammary gland. Diagrams show the extent of branching that occurs in virgin, pregnant, lactating and involuting mammary glands. In subsequent pregnancies, branching of the mammary glands increases from the involuted state to that typical for pregnancy and lactation, returning to the involuted state again following weaning.

The cyclical morphogenesis of breast tissue results from paracrine morpho-regulatory programs that occur in the context of a clear division of labor amongst the cell types that participate in this program and which include three distinct phenotypes: luminal epithelium, myoepithelium and mesenchyme (Fig. 2). These cell types coordinate branching by two distinct processes: bifurcation of end buds and side branching from the primary ducts.[20,21] Morphogenetic programs are not only spatially regulated but are also temporally confined.[22,23] For example, mesenchymal or stromal cells produce morphogens such as bFGF, HGF, epimorphin, MMPs and proteoglycans that control the expression of gene clusters in luminal epithelial cells involved in coordinating tubule formation and branching.[4,24-27] In addition to their role in cyclical regeneration of the mammary tree, myoepithelial cells provide contractile, inductive and proliferation/tumor suppressive functions in the normal mammary gland.[9-12,28,29] Adipocytes support mammary gland morphogenesis although their inductive and metabolic role(s), while present,[29] have not been as well dissected as those of myoepithelial and stromal fibroblasts.[20,29-31] Disruption of the morpho-regulatory programs that regulate branching results in apoptosis of ductal epithelium that is destined to become lobular alveolar cells.[3,32,33] Selective pressure on such normal cells is therefore towards achieving differentiation. It follows that at least one essential event in neoplastic conversion has to be a resistance to apoptosis, such as must be exhibited by stems cells during involution or must be achieved by transformed epithelial cells.

The Stroma, Branching Morphogenesis, and Breast Cancer

A considerable literature indicates that stromal extracellular matrix components are major players in determining resistance to apoptosis, and regulation of gene sets that control cell proliferation, migration, and invasion during both branching morphogenesis and tumorigenesis. We define this conundrum of a double-edged sword as follows: Those ECM components

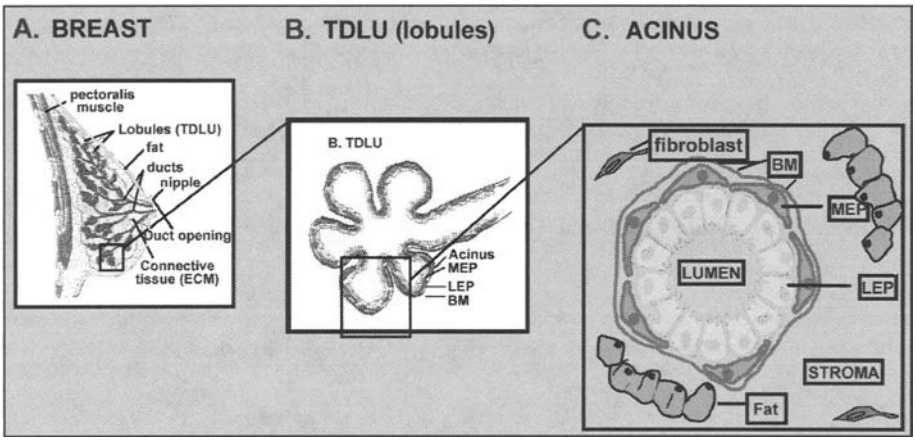

Figure 2. Structure of the human breast. The diagram shows a cross-section of a typical mammary end bud of a mature virgin gland that contains luminal breast epithelial cells, and myo-epithelial cells that are surrounded by a basal lamina. The end bud is incased by stromal fibroblasts and by fat cells. through a mammary duct that includes the different cell lineages that make up the differentiated mammary gland.

that permit cyclical breast morphogenesis and ultimately enable our survival as a species may also predispose us to the risk of breast cancer. The identification of key genes involved in, or associated with ECM remodeling, that are commonly expressed during normal morphogenesis and during neoplastic conversion, can reasonably be expected to yield important markers and possible therapeutic targets for detecting and suppressing breast cancer progression. A number of factors have been identified that commonly regulate breast branching morphogenesis and cancer initiation/progression. Many of these have been well reviewed, and some very recently.[2,3,20,34-36] This review therefore summarizes our current knowledge of how two distinct groups of stromal factors, proteoglycans/glycosaminoglycans (GAG) and metalloproteinases (MMPs), contribute to mammary branching morphogenesis on the one hand and neoplastic conversion/progression on the other hand. These particular stromal factors are functionally and physically interconnected, and they provide an excellent example of how morpho-regulatory stromal factors can interact to coordinate collectively signaling pathways in epithelium necessary for branching morphogenesis, and how changes in the regulation of these associations sustain neoplastic properties of breast epithelium.

Proteoglycans/GAGs As Regulators of Branching Morphogenesis

Hyaluronan

Here, we will review in detail the roles of hyaluronan (HA), a stromal glycosaminoglycan (GAG), and CD44, an HA receptor that is also expressed as a proteoglycan in branching morphogenesis. CD44 also performs docking functions for growth factors such as erbB4 and MMPs such as MMP-9 (gelatinase B), MMP-7 (matrilysin), and MMP-14 (MT1-MMP).[37-40] CD44 thus links signaling pathways regulated by HA/proteoglycans to those regulated by MMPs and growth factors. This integration is essential for efficient branching morphogenesis and appears to be involved also in neoplastic transformation/progression.[37,39,41] Both HA[42-44] and MMPs, such as MMP-3 (stromelysin-1) and MMP–7,[20,45-49] exert effects on mammary stromal tissue that may promote a reliance of breast cells on CD44-mediated signaling for resistance to apoptosis,[50] and that may also predispose to transformation or offer a growth advantage once cells are transformed.[15] Therefore, this group of molecules provides an excellent paradigm for examining the assumptions underlying our concept of a double-edged sword.

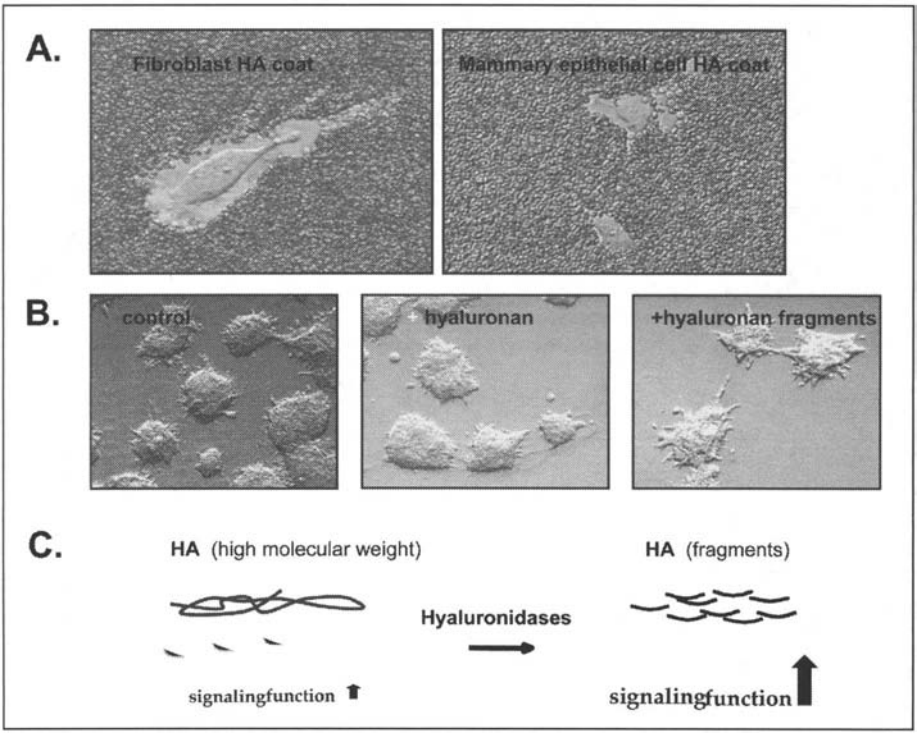

Figure 3. Hyaluronan and breast epithelial cells (EPH4) in vitro. A) Coats of hyaluronan (HA) surround fibroblasts and mammary epithlelial cells (EPH4) and are visualized with particle exclusion. HA is normally produced primarily by the stroma in quiescent mammary glands[68] and normal fibroblasts produce more HA than normal mammary epithelial cells. Magnification X200. Mammary epithelial cell aggregates (EPH4) produce branching structures in response to EGF alone (control) but branching is enhanced when HA fragments (+hyaluronan fragments) are combined with EGF. High molecular weight HA (+ hyaluronan) does not affect EGF-mediated branching. Magnification X120 HA is "activated" to a signaling mode when fragmented. The studies shown in (B) and other reports (see text) suggest that fragmented HA is more active than high molecular weight HA in promoting cell signaling that results, for example, in branching in vitro (B)

GAGs are a class of polysaccharides that typically comprise unbranched chains of repeating units of glucuronic acid and amino sugars. GAG chains rarely exist as free polysaccharides but are covalently attached to proteins forming proteoglycans such as syndecan, perlecan and versican.[51-53] HA, which is composed of disaccharide units of B-glucuronic acid and N-acetyl-glucosamine, is unique in this family of polysaccharides because it lacks sulfated residues, it is rarely covalently attached to proteins (therefore rarely exists as a proteoglycan), and can exceed 10^6 Daltons in mass.[43] Additionally, HA is uniquely synthesized at the plasma membrane by one of three synthase isoforms (HAS 1, 2, 3)[54-58] in contrast to other GAGs which are synthesized in the golgi apparatus.[59,60] A growing nascent HA chain is extruded through the plasma membrane by as yet unknown mechanisms but possibly one that involves oligomerization of the synthase itself.[55,57] HA is retained in the extracellular matrix (ECM) by binding to proteins such as versican and aggrecan, and HA associates with and coats both stromal and epithelial cells by binding to specific cell surface receptors such as CD44, RHAMM, LYVE-1/CSRSBP-1 and layillin;[37,43] this can be visualized in vitro using particle exclusion assays (Fig. 3A), Several recent reviews summarize the mechanisms by which HA binds to

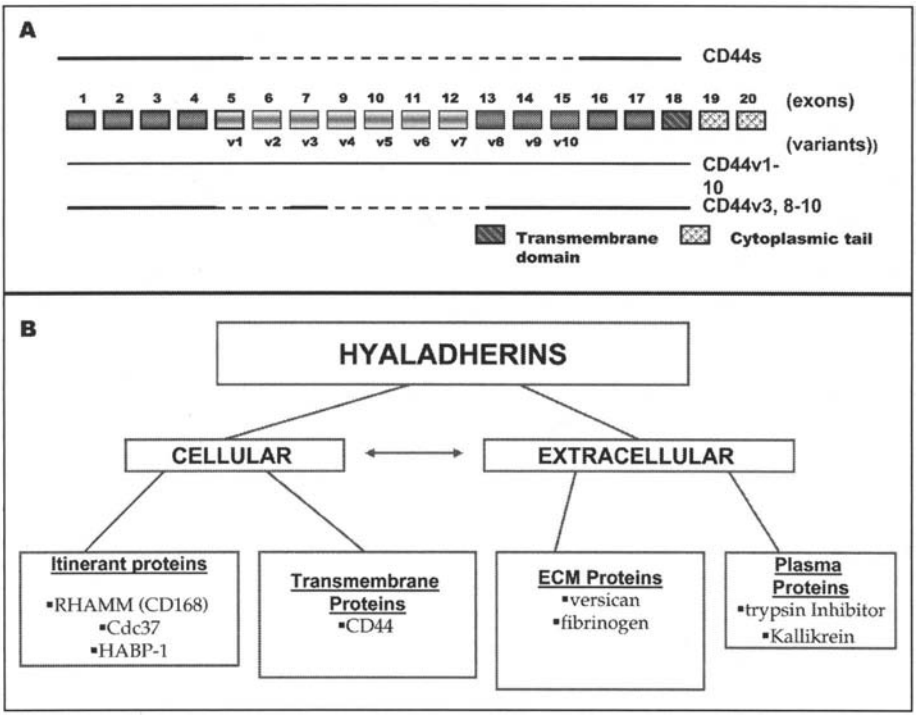

Figure 4. CD44 variants and other hyaladherins expressed in mammary glands. A) Diagram of the splice variants of CD44 that have been documented to be expressed in the mammary glands (see text for references). B) Diagram of hyaluronan receptors/binding proteins (hyaladherins) that have been reported to be expressed in mammary glands.

specific cell receptors such as CD44 and RHAMM, and document details of the signaling cascades regulated by these interactions.[40,46,47] Briefly, the association of HA with cell receptors such as CD44 and RHAMM activates signaling cascades through Src, PI3 kinase, Ras and Erk[36,61-65] pathways that are known to regulate epithelial to mesenchymal transition (EMT), migration/invasion and resistance to anchorage- dependent apoptosis.[66,67] A current summary of HA receptors and of extracellular binding proteins that have been linked to either breast branching morphogenesis or to breast cancer initiation/progression is shown in Figure 4.

HA is produced in and primarily associates with the stroma of normal breast tissue[68] and stromal fibroblasts produce more HA, which can be visualized as cell coats in a particle exclusion assay, than do epithelial cells even in vitro (Fig. 3A). In normal breast tissue, stromal production of HA is regulated by stromal branching morphogens including TGF beta, EGF and bFGF,[69] and estradiol.[70] Several in vitro studies have shown that HA can regulate the size and number of tubular outgrowths from ureteric bud and prostate epithelium in collagen gels[71,72] and is required for branching/invasion of a murine mammary carcinoma cell line, TA3.[73] We have shown that HA promotes branching of murine EPH4 breast epithelial cell line, and this effect is dependent upon the size of the HA polymer (Fig. 3B). HA fragments (average MW = 10^4 Daltons) enhance the rate tubular outgrowths from EPH4 aggregates while higher molecular HA (average MW = 10^6 Daltons) has either no effect or has an inhibitory effect (Fig. 3B). These results are consistent with an emerging paradigm for HA-mediated signaling whereby fragmentation of HA enhances its ability to activate signaling cascades (Fig. 3C).[43,74] HA has been shown to promote invasion, enhance motility,[43] promote an

epithelial-to-mesenchymal transition or EMT and is required for HGF- and beta-catenin-mediated EMT.[67,75] In addition to these effects, HA/CD44 interactions mediate the anchorage independent resistance of tumor cells to apoptosis, as demonstrated by the ability of exogenous HA fragments, which compete with endogenous HA for CD44, to reduce tumor cell survival.[63] As with branching of EPH4 cells, apoptosis and EMT of breast epithelial cell lines is regulated by HA fragments.[63,67] Although the molecular basis for the activity of HA fragments vs. higher molecular HA forms has not been established definitively, a number of studies suggest that CD44 plays a role as a signal transducer[37] involved in the activation of transcriptional programs favoring survival/proliferation and migration/invasion. For example, genes that are strongly up-regulated in the ureteric bud epithelium model of branching morphogenesis (see Chapter 8) include the transcription factor C/EBP, PCNA, and Myc-related transcription factor, anti-apoptotic factors such as BAG-1, BID, GAV-1 and HSP84, and invasion related genes such as CD44 itself and MMPs, in particular the stromelysins.[71] In other studies, HA has been reported to regulate expression of MMP2 and MMP9 through binding to CD44[76-78] which directly associates with MMP-14.[79] Complexes of MMP-14/CD44 and MMP-9/CD44 regulate migration and invasion of breast cells in vitro[80,81] and can result in the activation of MMP-2,[82] which also contributes to breast cancer cell invasion.[83] How HA might contribute to the CD44/MMP-mediated invasion has not yet been clarified. The ability of HA to protect growth factors relevant to branching morphogenesis from proteolysis and to present them optimally to their receptors,[84] in a manner that is similar to heparin sulfate[85] probably contributes to the effect of HA on branching morphogenesis. In addition, and of particular relevance to the focus of this review, HA participates in the development of a reactive or fibrotic stroma that is characteristic of involuting mammary gland tissue and of stroma surrounding breast tumors,[68,86,87] and which plays a role in both regulating mammary gland involution following weaning and in promoting breast tumor progression.[49,88] The functions of HA in this remodeling tissue include regulation of collagen fibril formation and neo-angiogenesis,[66,89-91] and regulation of MMP expression including MMP-2, MMP-3 and MMP-9.[92-94]

CD44

CD44 is an HA receptor that also binds to MMP-14,[79] MMP-7[50] and MMP-9.[77] It is expressed as multiple isoforms through alternative splicing of mRNA populations.[37,39,41] The generation of splicing patterns relevant to breast branching morphogenesis (CD44s, CD44v1-10, CD44v3,8-10) is shown in Figure 4A.[95] CD44 is encoded in 20 exons; the first 4 exons and exons 16-18 are constant while exons 5-15 (also called v1-v10) and exons 19-20 are expressed variably. Exon 18 encodes the membrane spanning sequence so that variant exons 19-20 appear to regulate the extent to which the cytoplasmic tail of CD44 can interact with intracellular proteins, for example cytoskeletal proteins such as the cortical actin binding proteins annexin V, cortactin and ERM proteins.[37,95] The standard form of CD44 (CD44s) is most common and is constitutively expressed. This form includes a link-type module that is responsible for binding to HA, a capability that requires activation by post-translational modification.[41,44] CD44s also binds directly to MMP-14 via sequence within the hemopexin domain of this MMP[79] and associates with MMP-7 and MMP-9.[50,77] An important function of a CD44s/HA interactions is to link HA-mediated activation of signaling pathways to the cortical actin cytoskeleton events required for cell motility and invasion.[37,41,95] CD44/MMP-14 and MMP-9 interactions have been linked to promotion of breast tumor cell invasion while CD44/MMP-7 interactions have been linked to breast epithelial cell survival during lactation.

Several CD44 variants are expressed as either heparin sulfate (HS) or chondroitin sulfate (CS) proteoglycans and these are generated by at least three separate mechanisms. CD44 variant forms expressing exon3 (e.g., CD44v3, 8-10) are modified by HS chains in the variable exon 3 and these bind to HB-EGF, HGF, bFGF, MMP-7 and MMP-9. A CS GAG chain can be covalently linked to the variable exon 5 but in addition, CD44 can bind to both HS and CS GAG chains in a noncovalent association via a basic motif encoded in the variable exon 10.[96,97]

The functions of these noncovalent associations with GAG chains have not yet been dissected. The binding of CD44 proteoglycan variant forms to growth factors and MMPs is required for the resistance of breast ductal epithelial cells to apoptosis[50] and for the localization of MMPs to polarized cell lamellae.[79,81]

Like the cell type-specific compartmentalization of growth factors and their receptors in mammary tissue, CD44s and variant forms are only expressed on ductal epithelium and myoepithelial cells.[98] Conversely, stromal cells produce HA, which is the major ligand for CD44.[68] Uniquely, myoepithelial cells can shed CD44, which has been shown to inhibit breast ductal epithelial cell proliferation in vitro, and may act as a tumor suppressor.[99,100] The CD44v6 epitope, which has been linked to breast cancer,[41] is exposed in both ductal epithelium and myoepithelial cells but is increasingly restricted during pregnancy to the myoepithelial cells and reappears in ductal epithelium during involution.[50,98] The v3 epitope is exposed on the luminal surface of lobulo-alveolar epithelium in the lactating mammary gland, and is also expressed by myoepithelial cells.[50,98]

Several in vitro studies have shown that anti-CD44 blocking antibodies inhibit the effects of HA on ureteric and prostate branching morphogenesis[71,72] (see Chapter 10) and that CD44:HA interactions are required for resistance of breast epithelial cell lines to anchorage-dependent apoptosis.[50,71] CD44 also mediates the EMT of breast epithelial cells promoted by HAS-2 overexpression, mutant active beta-catenin expression or exposure to HGF.[67] Genetic deletion of CD44 due to homologous recombination in DBA/1 mice promotes premature involution of the lactating mammary gland immediately following parturition.[50] In this study, the loss of CD44v3,8-10 resulted in premature apoptosis of the differentiated lobulo-aveolar epithelium, and this phenotype was related to reduced maturation of HB-EGF from its inactive pro-form and as a consequence, a reduced activation of the tyrosine receptor kinase ErbB4. CD44v3, 8-10 was shown to bind directly to both MMP-7 and pro-HB-EGF through its HS chain, which functions as a docking site that promotes the close association of pro-HB-EGF with its maturation factor, MMP-7, and also with its cognate receptor, erbB4. CD44/HB-EGF/MMP-7 complexes were concentrated at the apical surface of lobular alveolar cells. In the absence of CD44, MMP-7 underwent a basal redistribution as a result of an atypical association with perlecan, an HS proteoglycan restricted to the basement membrane.[50] Very little mature HB-EGF was detected in CD44-/- mammary glands whereas pro-HB-EGF was abundant throughout the breast epithelium. A similar phenotype was also observed in the uterus of CD44-/- mice.[50] These results do not exclude the possibility that other ligands for CD44 (e.g., HA and MMP-9) play a role in this mammary gland phenotype. For example, transgenic mice expressing a dominant negative erbB4 do not display the same degree of premature mammary tissue involution[101] as observed in these CD44-/- mice, and, further, genetic deletion of MMP-7 does not result in an involution phenotype of the mammary gland[102] although the lack of this MMP is probably compensated for by MMP-3 which has been reported to be up-regulated in MMP-7 -/- mice.[102] Other factors, in addition to the potential role for additional CD44 ligands such as HA, must also affect the mammary gland phenotype observed in this study since premature involution has not been observed in other strains of CD44-/- mice (e.g., BL6).[103]

Matrix Metalloproteinases

MMPs have been extensively reviewed[2,104-107] and only those that associate with, or are regulated by hyaluronan/CD44 interactions, and are involved in mammary gland morphogenesis/breast cancer will be considered in this review. These include the stromal MMPs, MMP-7, MMP-3, MMP-2 (gelatinase A), MMP-9 and a transmembrane MMP, MMP-14 (Fig. 5). All of these MMPs exhibit broad substrate specificity but this is particularly true for MMP-7 and MMP-3, which exhibit overlapping substrate specificities. For example, target proteins for both of these MMPs include ECM proteins such as collagens III, IV, V, IX, X and XI, fibronectin, laminins, tenascin and proteoglycans such as CD44; cytokines and growth factors such as

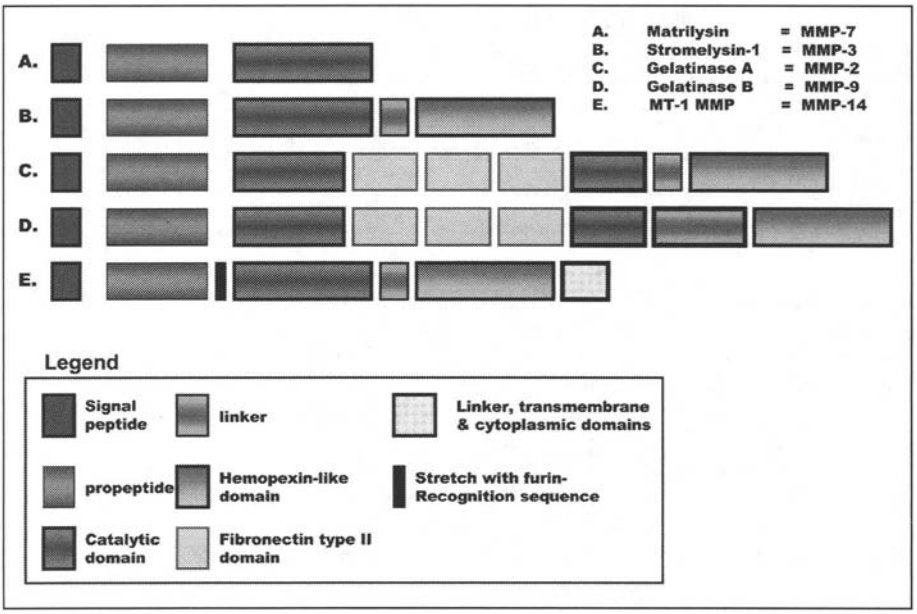

Figure 5. Diagram of domain structures and nomenclature of matrix metalloproteinases (MMPs) associated with hyaluronan and CD44. The MMPs that either associate with or are regulated by CD44 or hyaluronan include MMP-7, MMP-2, MMP-9, MMP-14 and MMP3. The domain structure of these MMPs is shown in this diagram.

TGF-beta and chemokine 4; MMPs such as MMP-2 and 9, and cell adhesion proteins such as E-cadherin. MMP-3, 7 and MMP-14-mediated proteolysis of target proteins often results in their activation. For example, MMP-promoted degradation of ECM proteins such as laminin-5 or growth factors such as TGFβs can expose cryptic, active sites that permit their interaction with specific cell surface receptors.[20,46,108-110] These activated proteins can regulate expression of other MMPs, for example, activated TGFβ promotes expression of MMP-2 or MMP-9 during branching morphogenesis.[111,112] The action of MMPs on their substrates may also release protein fragments that block function. For example MMP-3 released E-cadherin fragments block the cell-cell adhesion functions of intact E-cadherin in mammary epithelium, permitting invasion of these cells into collagen type I gels.[113] These promiscuous effects of MMPs predict that their involvement in branching morphogenesis will be multifactorial. Indeed, the morphogenetic effects of such "downstream" targets of MMP-3 or MMP-7 such as MMP-9,[21] have been related to activation of signaling pathways/gene sets involved in promoting EMT[114] and the invasion/migration of breast epithelial cells.[115-119] The development of 3-dimensional(3D) culture methods for culturing mammary epithelium, which were developed in this laboratory[120-122] and are increasingly used by other laboratories, as well as analysis of animals in which specific MMPs have either been genetically deleted or aberrantly expressed as a transgene,[21,49,88,102,123,124] has greatly aided progress in this area. In particular, specific actions and overlapping functions of MMP-3, MMP-2 and MMP-7 during breast branching morphogenesis and tumor progression have been identified using these experimental approaches.[21,49,88,102] For example, aberrant and constitutive expression of MMP-7,[49] MMP-3[88] and MMP-14[123] in mammary ductal epithelium driven by either the whey acidic protein (WAP) or MMTV promoters[49,88,125] results in tumor formation. MMP-3-mediated neoplastic transformation was noted to be associated with a chronically altered or "reactive" stroma having typical characteristics in common with stroma during involuting mammary glands.[33] MMP-2

and MMP-14, which are localized primarily to the periductal stroma of pubertal branching mammary glands but are reduced near side branches or where buds are initiating, regulate the early events in primary duct invasion of the mammary fat pad following puberty. Thus, virgin MMP-2 -/- mice exhibited an initial transient lag in duct invasion that is compensated for over time. The lag is accompanied by increased apoptosis of ductal epithelium suggesting that MMP-2 affects ductal invasion by regulating survival of end-bud cells. These effects of MMP-2 can be compensated for by other MMPs only during a brief window of morphogenesis. Unexpectedly, MMP-2 appears to repress side branching from primary ducts since a transient enhancement of side branching was observed in pubescent virgin MMP-2-/- mice. In contrast, analysis of MMP-3-/- mice suggest that it is transiently required for secondary duct formation and expansion but not primary duct expansion.[21] Consistent with these observations, an activated MMP-3 transgene expressed in ductal epithelium promotes precocious secondary branching, proliferation and differentiation during puberty but results in premature apoptosis during pregnancy. The premature involution is linked to the appearance of a reactive stroma characterized by elevated expression of tenascin, other MMPs such as MMP-9, as well as enhanced collagen deposition, and increased angiogenesis.[33] These particular studies of MMP-3 function illuminate an important principle that is relevant to our thesis of a double edged sword: the consequence of MMP activity to the ductal epithelial cells is stage- and therefore context- dependent and is associated with measurable changes in the cell's microenvironment. As is the case for MMP-3, an MMP-7 transgene expressed in mammary ductal epithelial cells also affects apoptosis during mammary tissue involution. However, the consequences of transgene expression differ in single vs. multiple pregnancies. Thus, during the first pregnancy, transgenic mice exhibited increased apoptosis of the mammary ductal epithelium during involution, a reduction in apoptosis of ductal epithelial apoptosis was observed following the third pregnancy. A resistance to apoptosis was associated with loss of FasL expression, an apoptosis-promoting gene.[126] Interestingly, CD44v3/MMP7/HB-EGF/erbB4 complexes appear on lobular alveolar epithelium during lactation[50] when expression of MMP-3 and MMP-7 are repressed.[33,102] Whether or not these CD44 complexes, which permit alveolar cell survival, affect the expression of MMPs hasn't been reported but is an intriguing possibility. In any case, an intricately timed regulation of MMP expression in the stroma (and also possibly HA accumulation), together with timed CD44 complex formation in mammary ductal epithelium, is required for the normal expansion and differentiation of the mammary gland to a lactating phenotype, and at least the MMPs are also required for the reversal to a quiescent tissue during involution. Is there any evidence that these specific molecular processes, which are clearly required for branching morphogenesis, are also integral to the malignant transformation of breast cells? If so, how do transformed cells utilize these signals to proliferate and how do they escape apoptosis that is a normal consequence of a remodeling microenvironment controlled by these MMPs?

HA, CD44 and MMP Interactions in Breast Branching Morphogenesis and Cancer

Our understanding of how malignancies originate and progress has recently undergone several important paradigm shifts that must be incorporated into any meaningful molecular analysis of events that control both breast branching morphogenesis and initiation/progression of breast tumors. These are the demonstration that the malignant phenotype is plastic even in the presence of multiple activating mutations in regulatory genes or oncogenes,[34,45] that the microenvironment can be dominant over these mutations[34,45,127] and that most of the tumorigenic capacity of tumors may reside in a minor tumor cell subset, which exhibits stem cell characteristics.[19,128,129] The studies focused upon in this review suggest that, at least in experimental models, specific stromal MMPs, HA and CD44 coordinately regulate branching morphogenesis at a minimum by controlling the growth and survival of ductal epithelium. What is the evidence for a role of these stromal genes in breast cancer, particularly in humans, and specifically in breast stem cells that may give rise to aggressive tumors?

Although a role for stroma in regulating tumor progression is less well appreciated than its role in regulating branching morphogenesis,[2] clinical links between the stroma and tumorigenesis have often been reported.[20,23,34,130-133] For example, heritable gene defects in stroma with a predisposition to cancer have been known for some time (reviewed in ref. 45). Furthermore, an association with changes in stromal characteristics, such as a predisposition to wound-like fibrosis or tissue inflammation, with increased susceptibility to a variety of cancers including breast cancer have often been cited e.g.[134,135] Experimentally, links between modification of stromal characteristics and cancer initiation has been strongly made by studies showing that aberrant expression of MMP-3, MMP-7 or MMP14 in ductal epithelium promote tumorigenesis.[49,88,123,136,137] Hyper-expression or de-regulated expression of these MMPs is also common in human breast tumors.[20,34,138,139] The accumulation of HA, which is produced in breast tumors by both the stroma and tumorigenic epithelial cells, is associated with poor differentiation of tumors, axillary lymph node positivity and short overall survival of breast cancer patients.[68] Aberrant regulation of CD44 in breast cancer biopsy samples has also been linked to patient outcome although a consistent relationship with disease progression has yet to emerge. For example, hyper-expression of CD44 has been linked to both poor and good outcomes and this has been interpreted to suggest that CD44 can act both as a tumor-progressing factor and a tumor-suppressing factor depending on context.[95,140,141] Thus, the consequence of aberrant CD44 expression to malignant progression may depend upon a variety of additional properties of the tumor cells that make assessment of expression per se an inadequate measure to assess for its role in tumorigenesis. For example, interplay amongst combinations of variant forms may determine overall CD44 function,[41,95,141] retention of myofibroblasts in tumors that express and shed a growth suppressing form of CD44 may contribute to a positive outcome,[99,142] CD44 expression in small tumor subsets (e.g., transformed progenitor cells) vs. the entire tumor[15] may be more important to tumor aggressiveness, and expression of other proteins by key malignant cells may affect CD44 function. Examples of the latter include E-cadherin, which suppresses CD44-mediated breast tumor cell invasion,[73] and RHAMM, which can counter anti-invasive properties of CD44.[143]

Evidence is increasing to suggest that progenitor cells known to be present in normal breast tissue[14,19,128,129] may be targets for malignancy in breast cancer, as is now accepted for hematopoeitic malignancies such as AML or CML.[14,144] For example, one recent study has identified a small population (2%) of tumor cells obtained from primary breast cancer biopsies that contained virtually all of the breast cancer initiating activity, as defined by an ability to form tumor cells to give rise to breast tumors when serially transplanted into severely immuno-suppressed SCID/NOD mice.[15] These highly tumorigenic cell subsets gave rise to tumors that were similar in heterogeneity, as defined by surface phenotypes, to the original primary tumor, suggesting that they arose from progenitor cells that retain the ability to differentiate into heterogeneous lineages. These results also provide clinical evidence that confirms the plasticity of the tumorigenic phenotype. Of particular relevance to the focus of this review, a high expression of CD44 was a defining surface phenotype of the tumorigenic stem cell subset surface phenotype.

The above reports are intriguing since progenitor cells from other tissues typically express CD44, utilize CD44 to adhere to the HA that is produced by stromal cells, and require an HA rich environment for their survival. This characteristic is retained with neoplastic transformation.[89,145] These results are also consistent with our thesis and considerable data[23,146] that aberrant expression of ECM receptors, such as CD44, provides both morphogenetically active normal as well as proliferating transformed breast cells with a selective advantage for growth and survival. Although the roles of MMPs during breast branching morphogenesis and breast tumor progression have been more thoroughly characterized than that of HA or CD44, evidence to date supports the existence of a functional relationship between these two classes of molecules in both branching morphogenesis and cancer. We summarize one model for how these functional interactions might contribute to branching morphogenesis and how they also

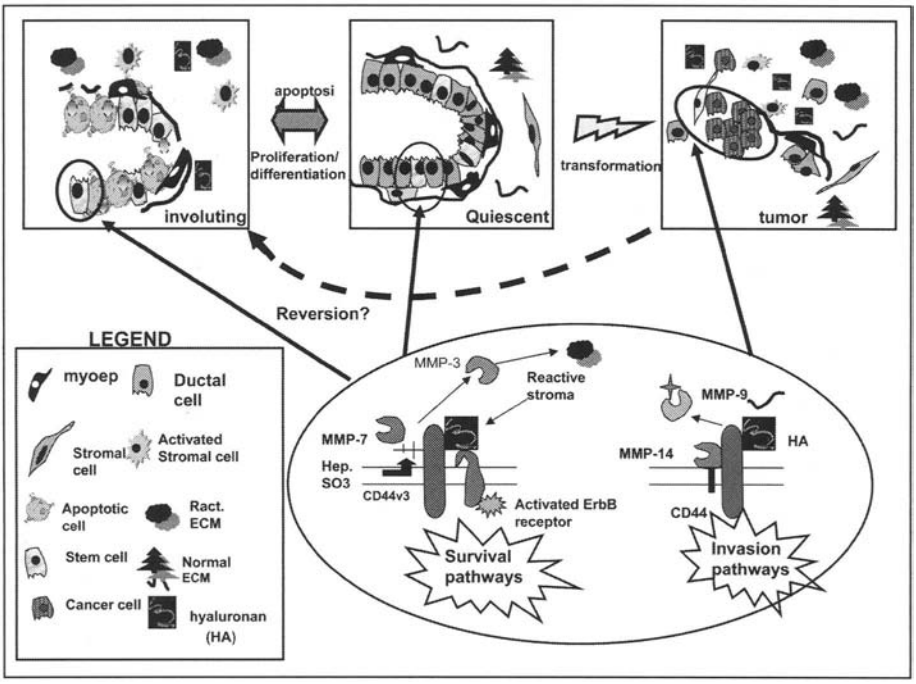

Figure 6. Model of molecular interactions proposed to regulate both morphogenesis and neoplasia. A model of some of the molecular mechanisms regulated by CD44, HA and MMPs that contribute to normal mammary gland morphogenesis yet appear to be utilized by transformed cells to form aggressive tumors. Quiescent or lactating ductal lobular aveolar cells are able to survive due to signaling through CD44v3, which also requires the functioning of MMP-7 and Erb B4. During involution that follows weaning, these normal ductal cells lose the ability to signal through this pathway. We propose that stem cells, which will replenish the ductal epithelium in subsequent pregnancies retain signaling activity through this pathway and therefore survive. As mammals age, somatic mutations accumulate in the cells of mammary tissue, and if or when mutations occur in stem cells, aggressive tumors will arise partly due to the selective advantage provided by the CD44v3/HA/MMP-7 survival pathway and partly to an ability to utilize CD44/HA/MMP-9/MMP-14 for activating invasive signaling pathways.

may promote neoplastic progression of this same tissue (Fig. 6). In this model, expression of CD44 variants that bind to HA, MMP-14 and MMP-9 are predicted to be involved in promoting side and/or primary bud branching that contribute to mammary tree expansion following puberty and during pregnancy. MMP-3 and MMP-2 activity is also transiently required for side and primary duct branching respectively.[21,33,147] The presence of HA and activation of signaling cascades through CD44 are predicted to contribute to the regulation of expression of these MMPs. Expression and release of CD44 variant forms by myofibroblasts provides one mechanism for controlling the extent of ductal epithelial cell proliferation. Expression of CD44v3, 8-10 (Fig. 4) and its association with MMP-7/erbB4/EGF complexes is upregulated as breast tissue differentiates into lobular-alveolar cells during pregnancy and following parturition. Formation of this complex is required to prevent apoptosis and to sustain a differentiation mammary tree.[50] Expression of these complexes decreases as MMP-3 expression (and possibly HA) increases following weaning, contributing to the remodeling of ECM into reactive stroma characteristic of involuting breast tissue. This modified ECM favors the apoptosis of terminally differentiated lobular-alveolar breast epithelial cells while selectively permitting the survival of progenitor cells. We speculate that these progenitor cells continue to

express CD44 variant forms and this allows their survival in the presence of a remodeling or reactive stroma. The surviving progenitor cells subsequently regenerate the mammary tree of future pregnancies. These same genes could contribute to neoplastic transformation and progression of breast tumors as modeled in Figure 6. Aberrant MMP-3 expression can initiate neoplastic transformation of murine breast epithelia via an action on the stroma. The MMP-3-altered stroma has been proposed to both contribute to neoplastic transformation as a tumor promoter and permit the survival and growth of cells that become neoplastic. In a remodeling ECM, growth of even a minor population of aberrant cells could lead to de-regulation of interactions amongst breast tissue cell types, with consequent hyperplastic and dysplastic events that precede neoplastic transformation. Since mutations occur continuously in key oncogenes in most tissues, the step from a dysplastic state to a frankly neoplastic state might not be great, particularly in a chronically remodeling MMP-and HA-rich tissue.[34,45,148-150] Although it is unlikely that transformation of progenitor cells is responsible for all breast cancers, several properties of these cells could contribute to a particularly aggressive and persistent tumors. For example, if progenitor cells express CD44 isoforms that associate with key MMPs that promote a remodeling environment, they will have a selective survival advantage over other cells and also have the machinery for metastasis such as invasion and translatability. These cells would not have to undergo a lengthy evolution to become malignant but would generate highly aggressive tumors. The model shown in Figure 6 is one example of how specific genes, whose products functionally interact to regulate branching morphogenesis and are therefore essential for mammalian survival, can, with a small shift in regulation, act as seeds for individual destruction particularly in the presence of activating mutations in other genes which inevitably occurs with age.

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