# Extracellular Matrix Composition Reveals Complex and Dynamic Stromal-Epithelial Interactions in the Mammary Gland

**Ori Maller · Holly Martinson · Pepper Schedin**

**Abstract** The mammary gland is an excellent model system to study the interplay between stroma and epithelial cells because of the gland's unique postnatal development and its distinct functional states. This review focuses on the contribution of the extracellular matrix (ECM) to stromal-epithelial interactions in the mammary gland. We describe how ECM physical properties, protein composition, and proteolytic state impact mammary gland architecture as well as provide instructive cues that influence the function of mammary epithelial cells during pubertal gland development and throughout adulthood. Further, based on recent proteomic analyses of mammary ECM, we describe known mammary ECM proteins and their potential functions, as well as describe several ECM proteins not previously recognized in this organ. ECM proteins are discussed in the context of the morphologically-distinct stromal subcompartments: the basal lamina, the intra- and interlobular stroma, and the fibrous connective tissue. Future studies aimed at in-depth qualitative and quantitative characterization of mammary ECM within these various subcompartments is required to better elucidate the function of ECM in normal as well as in pathological breast tissue.

O. Maller · H. Martinson · P. Schedin (✉)
Department of Medicine, Division of Medical Oncology,
University of Colorado Denver,
MS8117, RC-1S, 8401K, 12801 E 17th Ave,
Aurora, CO 80045, USA
e-mail: Pepper.Schedin@UCDenver.edu

O. Maller · H. Martinson · P. Schedin
Program in Cancer Biology, University of Colorado Denver,
MS8104, RC-1S, 5117, 12801 E 17th Ave,
Aurora, CO 80045, USA

P. Schedin
University of Colorado Cancer Center,
Bldg 500, Suite 6004C, 13001 E 17th Place,
Aurora, CO 80045, USA

P. Schedin
AMC Cancer Research Center,
Bldg 500, Suite 6004C, 13001 E 17th Place,
Aurora, CO 80045, USA

## Abbreviations

| | |
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| collagen IV | type IV collagen |
| DDR1 | discoidin domain receptor 1 |
| EM | electron microscopy |
| EGFR | epidermal growth factor receptor |
| ECM | extracellular matrix |
| ED | extra domain |
| FACIT | fibril associated collagens with interrupted triple helices |
| FGF | fibroblast growth factor |
| FN | fibronectin |
| GAG | glycosaminoglycan |
| H&E | hematoxylin and eosin stain |
| HGF | hepatocyte growth factor |
| LN | laminin |
| LAP | latency-associated peptide |
| LRR | leucine-rich repeats |
| LOX | lysyl oxidase |
| MMP | matrix metalloproteinase |
| MAGP | microfibril-associated glycoprotein |
| MCP-1 | monocyte chemoattractant protein-1 |
| SPARC | secreted protein acidic and rich in cysteine |
| SLRP | small leucine-rich proteoglycan |

| SD | Sprague Dawley |
| TN | tenascin |
| tTG2 | tissue transglutaminase 2 |
| TGF-β | transforming growth factor β |
| TLR | toll like receptor |

## Introduction

The mammary gland is an excellent model system to study the function of stromal-epithelial interactions because of the gland's unique development across its several distinct functional states, depending on reproductive state. Unlike most organs, which develop to morphological maturity during embryogenesis, the majority of mammary ductal morphogenesis occurs with the onset of ovarian function. Further, with each estrous (rodent) or menstrual (human) cycle, the alveoli undergo cyclic expansion and maturation, followed by a modest regression phase as ovarian hormone levels rise and fall, respectively [1–4]. The mammary gland is also unique in that terminal differentiation does not occur unless pregnancy and lactation ensue [5]. These unique attributes highlight the dynamic, non-homeostatic nature of the normal mammary gland.

Since mammary gland development is largely postnatal, organogenesis and differentiation are extended in time, occurring over a period of weeks in the rodent, rather than hours and days as observed in other organs. Further, in rodents, the development of mammary glands occurs along the entire flank of the animal with 5 pairs of glands in the mouse and 6 pairs in the rat. As a result, there is sufficient mammary tissue in these species to permit physical, functional, and biochemical characterizations. These features make the rodent mammary gland a highly suitable model to evaluate the interplay between stroma and epithelium throughout gland development and during distinct functional states such as pregnancy or lactation.

The focus of this review is on the extracellular matrix (ECM) component of the mammary stroma. ECM can be described as an interconnected meshwork of secreted proteins that interacts with cells to form a functional unit [6]. Cell-ECM interactions have been implicated in cell adhesion, survival, apoptosis, polarity, proliferation, and differentiation. ECM confers these cellular functions through structural properties such as physical support and boundary constraints, classical signal transduction pathways, and by providing mechanosensory cues [7]. Epithelial cells receive instructive cues from the ECM via integrin receptors and the non-integrin receptors such as discoidin domain receptor 1 (DDR1) [8], dystroglycan [9], and syndecan [10]. Integrins are transmembrane receptors composed of α and β subunits, with 18 α and 8 β subunits that heterodimerize to generate 24 canonical integrin receptors [11, 12]. A comprehensive description of ECM-integrin interactions is beyond the scope of this review; the reader is referred to reviews by Desgrosellier et al. and Larsen et al. [13, 14].

ECM synthesis and assembly in the mammary gland are the product of many cell types including epithelial cells, myoepithelial cells, fibroblasts, adipocytes, endothelial cells, and immune cells [15–17]. Bissell and colleagues describe the relationship between ECM and mammary epithelial cells as dynamic and reciprocal, leading to two-way communication, where ECM instructs and supports cells, while cells build, shape, and re-shape the ECM [18]. Mammary ECM can be separated into three broad structural categories: the highly-specialized basal lamina that directly abuts the basal side of mammary epithelial cells (Fig. 1), the intra- and interlobular stroma immediately adjacent to alveoli and lobules, respectively (Figs. 1 and 2), and the fibrous connective tissue that is devoid of epithelium (Fig. 2). In this review we explore the composition and function of each of these ECM categories using data obtained largely from rodent models. Although rodent models recapitulate many aspects of human mammary gland biology, there are interspecies variations in mammary stroma composition, organization, density, and function. Thus, while significant insight into mammary ECM is obtained through the study of rodents, future studies validating results in human breast tissue are essential.

## Rodent Mammary Gland as a Model System to Study Human Breast

The mouse and rat models recapitulate critical aspects of human mammary gland development and function. The rodent mammary gland and human breast have similar rudimentary mammary parenchyma, which consist of a layer of luminal epithelial cells, a basal epithelial cell layer that putatively contains mammary stem cells, and the outer contractile myoepithelial cells adjacent to the basal lamina ECM [19]. The parenchyma structure consists of a single elongated ductal tree (rodent) or multiple ductal networks (human) that develop by bifurcation of specialized end bud structures present during puberty [20]. While the overall epithelial structure is similar between humans, rats, and mice, the relative abundance of connective tissue varies considerably. Stroma surrounding the lobules and ducts (intra- and interlobular stroma) in mice is sparse and there is little acellular fibrous connective tissue between ducts. Instead, the mammary glands of mice are characterized by an abundance of white adipose tissue that is directly adjacent to the sparse interlobular stroma (Fig. 2c). In
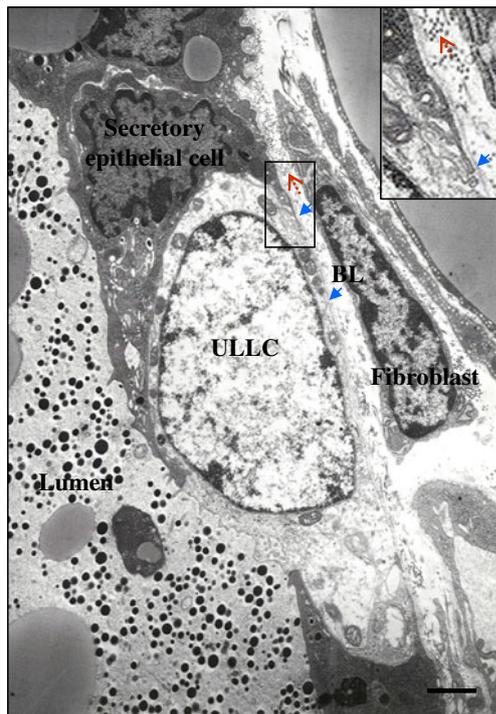
**Figure 1** Electron micrograph image of mammary epithelial cells from a lactating mouse. The solid blue arrow points to the basal lamina (BL) adjacent to a putative mammary progenitor cell known as undifferentiated large light cell (ULLC), and a secretory epithelial cell. The dashed red arrow points to intralobular fibrillar collagen adjacent to a fibroblast. Scale bar represents 5 microns

humans the ratio of fibrous connective tissue to adipose tissue is opposite, with an abundance of stroma surrounding the alveoli and ducts, a predominance of fibrous connective tissue between ducts, and reduced adipose content (Fig. 2a). Organization of the stroma in outbred Sprague Dawley (SD) female rats is intermediate between mice and humans and thus is histologically more similar to humans than is the mouse (Fig. 2b) [21]. While the mechanisms driving the different ratios of fibrous connective to adipose tissues across species are currently unknown, we suggest a relationship to ovarian hormone exposure, particularly progesterone, may exist. Compared to mice, the SD rat has a robust luteal phase during the estrus cycle, resulting in cyclical progesterone exposure that is more similar to that for women [1]. Given that pituitary and ovarian hormone exposure drives most aspects of gland development and adult function, instructive cues from hormones are expected to also directly or indirectly influence composition and organization of the adjacent stroma [22–24]. Hence, studying the hormonal regulation of normal mammary gland biology may afford novel insights into how stromal organization is regulated under physiological conditions, which can be utilized to decipher this relationship in terms of tissue plasticity and breast disease. However, systemic/

host exposures are unlikely to completely explain variation in stromal density given that very distinct microenvironments can exist within individual adjacent lobules (Fig. 3).

## Pleiotropic Roles for ECM in the Mammary Gland

The ECM provides physical support that is essential for overall tissue architecture. Changes in ECM properties are communicated to epithelial cells via transmembrane receptors that provide a conduit between the ECM, the cytoskeleton and its transcriptional machinery [25]. Thus, ECM interactions ultimately determine epithelial cell phenotype. A role for ECM stiffness in the mammary gland has been increasingly explored including the role of stiffness requirements for establishing apical/basal cell polarity and lateral cell junctions [26], as well as for cell proliferation [27, 28]. Importantly, Discher and colleagues have determined that matrix stiffness and mechanotransduction are seminal factors in mesenchymal stem cell lineage specification [29]. By modulating matrix stiffness in vitro, this group demonstrated that mesenchymal stem cells acquired a neuronal gene profile on soft matrix, while acquiring osteoblast-like properties on rigid matrix [29]. In the mammary gland, $\beta 1$ integrin is a cellular "mechanosensor" that is imperative for maintaining basal progenitor cells, as demonstrated by inability of mammary epithelium lacking $\beta 1$ integrin to repopulate the gland upon serial transplantation [30]. These data suggest that physical properties and site-specific composition of adjacent basal lamina ECM may contribute to the "stemness" of mammary progenitor cells through $\beta 1$ integrin.

In order to understand how ECM stiffness influences the function and fate of mammary epithelial cells, it is vital to understand its composition as well as its architecture, which is largely defined by protein-protein interactions. While secreted ECM proteins can interact through numerous, weak non-covalent bonds, crosslinking these proteins via covalent chemical bonds is essential for ECM scaffold organization, tensional characteristics, and stabilization. Lysyl oxidase (LOX), a copper-dependent enzyme, catalyzes intra- and intermolecular crosslinking of fibrillar collagens and elastic fibers through oxidative deamination of lysine residues [28, 31, 32]. LOX activity has been shown to promote tissue stiffness and fibrosis as well as modulate elastic properties of elastic fibers, establishing LOX as a key regulator of tissue tension [28, 31–33]. Tissue transglutaminase 2 (tTG2) has also been implicated in crosslinking ECM proteins in the mammary stroma. The crosslinking activity of tTG2 depends on calcium and proceeds by catalyzing covalent bonds between glutamine and $\epsilon$-amino groups of lysine residues [34]. Future studies are needed to examine how LOX and tTG2 are regulated in

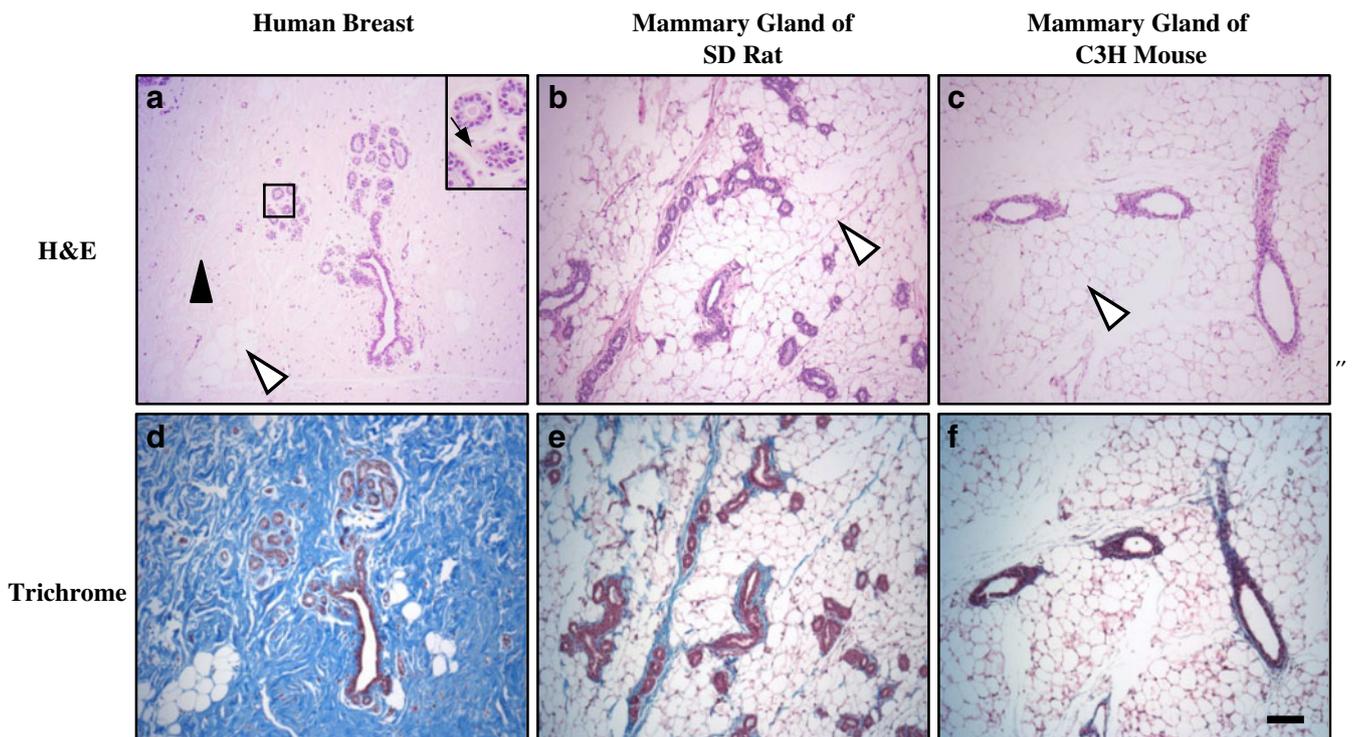|  Human Breast | Mammary Gland of SD Rat | Mammary Gland of C3H Mouse |



**Figure 2** Histological sections from human breast and rodent mammary gland from nulliparous females. **a–c** H&E-stained nulliparous human breast tissue (**a**), nulliparous SD rat mammary tissue (**b**), and nulliparous C3H mouse mammary tissue (**c**). In **a**, solid arrow head points to fibrous connective tissue (pink) that is dominant in human breast tissue. Arrow in the inset box points to intralobular stroma. Open arrow heads point to adipocytes, which are abundant in rat and mouse glands. **d–f** Serial sections stained with Masson's trichrome showing collagen as blue fibers in human nulliparous breast tissue (**d**), nulliparous SD rat mammary tissue (**e**), and nulliparous C3H mouse mammary tissue (**f**). Scale bar represents 100 microns

the mammary gland and to determine their unique contributions to ECM function.

## ECM as a Reservoir and a Source for Growth Factors and Cytokines

Growth factors and cytokines can bind to ECM proteins, identifying another avenue by which ECM can regulate mammary morphogenesis and function. For example, transgluaminase crosslinks the large latent complex of transforming growth factor β (TGF-β), known as latent TGF-β binding protein, to ECM proteins [35]. Growth factors and cytokines bind to ECM through glycosaminoglycans (GAG), which are anionic linear polysaccharides consisting of repeating hexuronic acid and hexosamine. GAG can be thought of as "glue" that allows growth factors to "stick" to basal lamina and fibrillar ECM proteins. Interestingly, different classes of GAGs are associated with distinct aspects of mammary ductal elongation [20]. Silberstein et al. and Williams et al. used electron microscopy (EM) to show that the basal lamina surrounding the tips of end buds in mice is thin and rich in hyaluronic acid, while the basal lamina along the flank of end buds is thicker and associated with sulfated

GAGs [20, 36]. These observations are interesting because the terminal end bud tips are actively elongating structures while the flank of the end bud is not [37]. These observations raise questions on how GAG composition contributes to ECM thickness, rigidity, and the ability of ECM to serve as a reservoir for growth factors and cytokines, as well as the ability of epithelial cells to penetrate into the surrounding stroma and proliferate.

Release and activation of growth factors and cytokines from ECM can occur by altering matrix stiffness and/or inducing ECM proteolysis [38, 39]. For example, TGF-β activity can depend on fibrillar ECM proteolysis or altered matrix stiffness. TGF-β is secreted as a large latent complex (LLC) by multiple cell types and associates with fibrillar ECM proteins [40]. TGF-β is activated by proteolytic cleavage of the latency-associated peptide (LAP) or by conformational change in LAP through binding to the αvβ6 integrin receptor [41, 42]. However, Hinz and colleagues recently published evidence suggesting that mechanical tension is also involved in the release of TGF-β1 from the ECM [43]. They suggested that high contractile activity of myofibroblasts on a stiff matrix may lead to a conformational change in LAP, resulting in TGF-β1 release, while on soft matrix the latent complex stays
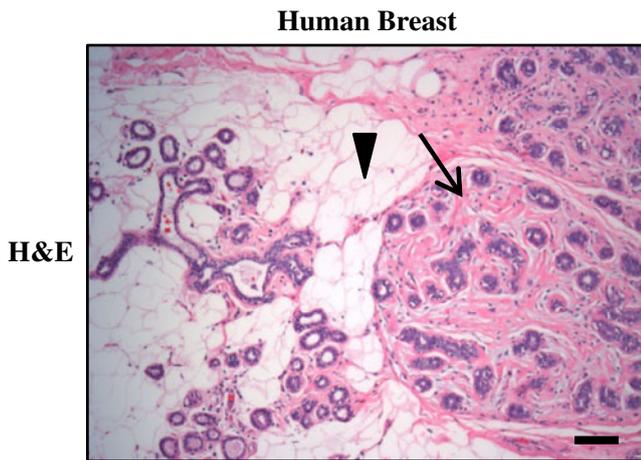
**Human Breast**



**H&E**

Figure 3 H&E-stained nulliparous human breast tissue. This histological section demonstrates how adjacent lobules can exist in very distinct microenvironments. The arrow points to fibrous intralobular stroma and the arrow head points to adipocyte-rich stroma. Scale bar represents 100 microns

intact due to lack of mechanical stretch [43]. Additional support for mechanical tension regulating ECM function is presented in studies demonstrating how fibroblast traction forces lead to large-scale directional patterning of fibrillar ECM proteins such as collagen I [44, 45]. Given that ECM tensional requirements and organization are expected to change throughout mammary gland development, it is likely that the ability of ECM to serve as a reservoir for growth factors and cytokines would be altered accordingly.

During expansion of the ductal network, Daniel and colleagues postulated that TGF-β1 regulates branching morphogenesis by inhibiting ductal growth through direct suppression of epithelial cell proliferation and by stimulating production of collagen I, GAG, and chondroitin sulfate within the periductal stroma at puberty [46]. Evidence for this proposal was obtained by administering TGF-β1 directly into the mammary gland of young female mice, which resulted in premature inhibition of ductal elongation concurrent with aberrant and increased deposition of ECM proteins [46, 47]. Moreover, as discussed above, these researchers observed high expression of TGF-β3 in the flank region of end buds, suggesting it may positively regulate ECM production at this site [48]. Given the growth suppressive role of TGF-β during pubertal gland development, it was surprising that expression of TGF-β2 and TGF-β3 transcripts was elevated during pregnancy [48]. As mentioned by Robinson et al. [48], this observation is not consistent with the growth inhibitory function that TGF-β(s) has on mammary epithelial cells during ductal elongation. A later study suggested that TGF-β1 only impedes epithelial cell growth in ducts, but not in alveoli, although the mechanism of this differential effect is unknown [49]. During lactation, the expression of TGF-β(s) is significantly downregulated [48], which may prevent TGF-β(s) from negatively regulating expression of milk proteins such as β-casein [48, 50, 51]. Upon the onset of involution when the gland remodels toward its pre-pregnant state, there is an upregulation of TGF-β transcripts, particularly for TGF-β3 [52]. TGF-β signaling may further contribute to the remodeling of the involuting gland by inducing ECM production, upregulating MMP expression, and by recruiting immune cells [53–55]. Taken together, TGF-β(s) expression and activation are tightly regulated in mammary gland development where they mediate stromal-epithelial interactions, in part by orchestrating ECM deposition and remodeling.

Instructive cues are also provided to cells from the ECM by the controlled release of protease-cleaved ECM fragments, termed 'matricryptins' [56, 57]. These fragments exhibit altered biological functions compared to intact ECM proteins. For example, fibronectin (FN) fragments initiate apoptosis in epithelial cells [58], while intact FN promotes cell adhesion and proliferation [59, 60]. A subclass of matricryptins, "matrikines," are short peptides from ECM proteins that function in a similar manner as cytokines [56, 57]. Instructive cues from ECM fragments will be further discussed below in the context of immune cells and ECM remodeling.

## ECM Composition in the Mammary Gland

In order to understand the myriad of functions mediated by the ECM, one must have an in-depth understanding of the ECM proteins that contribute to each of the distinct ECM subcompartments. Our current understanding of ECM composition is rudimentary and largely derives from studies of rodent mammary gland tissue (mouse and rat). Until recently, the standard method for identifying ECM proteins was by biochemical isolation and Edman sequencing and/or by antibody-based detection of candidate proteins. Mass spectrometry-based proteomic analysis provides an unbiased, high-throughput comprehensive overview of ECM composition. Unfortunately, the traditional digestion approaches required to prepare proteins for proteomic analysis have proven ineffective for ECM proteins due to their resistance to proteolysis. In the past few years, Liang and others have used media conditioned by mammary epithelial cells in vitro to analyze their secretomes and have identified peptides originating from ECM proteins such as FN, laminin (LN), and type IV collagen (collagen IV) [61]. An in-solution digestion approach for trypsin-resistant ECM proteins was recently developed by Hansen et al., permitting the identification of known and previously unknown ECM proteins within the rat mammary gland [62]. In the next section of this review, we describe many of the well-known ECM proteins in the

mammary gland and highlight several ECM proteins not previously recognized. We discuss these ECM proteins in the context of the three distinct subcompartments depicted in Figs. 1 and 2.

Basal Lamina

The terms basal lamina and basement membrane have been used interchangeably to refer to the highly specialized ECM directly underlying the epithelium. While basement membrane refers to the ECM adjacent to the epithelium that is visible by light microscopy, the basal lamina is a sub-light microscope structure visualized only by EM. The basal lamina lies immediately beneath the epithelium and is a highly organized 20–100 nm thick structure (Fig. 1). Basal lamina in the mammary gland is thicker along the flanks of the terminal end bud compared to the growing bud tip, which correlates with compositional and possibly functional differences, as mentioned above [36]. The basal lamina consists of three layers: the middle electron dense layer termed the lamina densa, and the inner and outer less-dense electron layers referred to as the lamina lucida externa and interna, respectively [63]. The lamina lucida layers contain high levels of proteoglycans (hence they appear as clear or 'lucid' areas) that decorate a meshwork of small filaments that attach the lamina densa to the epithelial cell surface, and to the collagen fibrils within the stroma on the basal side. This creates a highly organized ECM layer between the epithelial cell and its underlying stroma. The major constituents of basal lamina are LN, collagen IV, nidogens, and perlecan.

*Laminins* LNs are large heterotrimeric glycoproteins (~900 kDa) found in at least 15 different combinations that are derived from five $\alpha$, three $\beta$, and three $\gamma$ subunits, coded from distinct genes [64]. Each LN chain includes globular, coiled coil, and rodlike regions [65]. The three distinct chains are intertwined at the coiled coil regions by disulfide bonds to form a cross-shape structure [65]. LN deposition appears to be restricted to the region of the basal lamina, is in direct physical contact with epithelial cells, and thus is likely critical mediators of epithelial cell-ECM interactions in vivo. Different LNs can be associated with specific tissue compartments or organs. For example, LN8 ($\alpha$4, $\beta$1, $\gamma$1) is expressed by endothelial cells and contributes to the vascular basal lamina [15, 66], while LN10 ($\alpha$5, $\beta$1, $\gamma$1) is associated with villus cells of the small intestine [67]. These and other examples suggest that particular LNs may be coupled with tissue-specific differentiation or function. In this review, we will focus on LN111 ($\alpha$1, $\beta$1, $\gamma$1; known as LN1) and LN332 ($\alpha$3, $\beta$3, $\gamma$2; previously known as LN5) due to their established presence in the mammary gland.

LN111 is one of the major constituents of the basal lamina and was the first LN characterized. LN111 was isolated from a poorly-differentiated murine sarcoma (Engelbreth-Holm-Swarm (EHS) sarcoma), and is a principal component of Matrigel, a commercially-available basement membrane substitute [68, 69]. LN111 is necessary for cell differentiation as documented in murine embryogenesis, where loss of any LN111 chain ($\alpha$1, $\beta$1, or $\gamma$1) causes embryonic lethality due to improper basal lamina assembly and function [70, 71]. An insightful example was obtained after deletion of specific domains in the C-terminus of the LN $\alpha$1 chain [9]. The C-terminus of the LN $\alpha$1 chain consists of 5 globular domains with known cell binding motifs [72]. Deletion of the $\alpha$1 globular 4–5 domains, which are binding sites for the ECM receptor dystroglycan, impeded pluripotent epiblast differentiation [9]. Interactions between $\alpha$1 globular 4–5 domains and dystroglycan are crucial for gastrulation and for the formation of the primary germ layers, likely because LN is needed for epithelial cell polarization [9]. Using a 3D culture model, Bissell and colleagues showed that LN111-rich reconstituted basement membrane is pivotal for apicobasal polarization of mammary epithelial cells and for lumen formation of acini [73–75]. Cultured luminal mammary epithelial cells did not form acini in collagen I gels unless the cells were mixed with myoepithelial cells [76]. Petersen and colleagues identified myoepithelial cells as the primary source of LN111 via in vitro assays and through staining of human breast tissue [76]. The in vitro studies have demonstrated that LN111 instructs acinar formation, in part by facilitating deposition of a mammary-specific basal lamina, given that newly-synthesized LN332 and collagen IV are incorporated into a basal lamina-like structure in a 3D culture model in the presence of LN111 [77, 78].

LN332 is expressed by mammary epithelial cells and can induce adhesive contacts in epithelial cells via $\alpha$6$\beta$4 or $\alpha$3$\beta$1 integrins [79, 80]. Moreover, MMP2-cleaved LN332 fragments (from the $\gamma$2 chain) have been shown to bind the epidermal growth factor receptor (EGFR) [81]. LN332 fragment-EGFR interaction was associated with pro-migratory effects on mammary epithelial cells as well as downstream activation of EGFR signaling, although the results from the cell growth assay were inconclusive [81]. Further, by culturing freshly-isolated murine mammary epithelial organoids that included luminal epithelial and myoepithelial cells in a 3D culture model, Werb and colleagues demonstrated that the front edges of actively-elongating ducts were devoid of intact LN332 [80]. Moreover, LN332 fragments are present in the mammary gland during mid-pregnancy and involution, suggesting that LN332 contributes to the tissue remodeling that occurs during these stages [81, 82]. Using a pan anti-LN antibody,

LN fragments were apparent in mammary tissue during early involution in the rat [23]. Nevertheless, the functional consequences of LN turnover during involution are still unknown. While LNs experience turnover during early involution of the gland [23], LN transcript and total protein levels change only modestly with pregnancy, lactation and involution, suggesting a fundamental requirement for LNs in mammary epithelial maintenance, rather than for stage-specific functions.

*Collagen IV* Collagen IV is also a major component of the basal lamina and is thought to be its primary scaffold protein [16, 83]. Unlike fibrillar collagen, collagen IV is a network-forming collagen. It is a heterotrimer and can be comprised from six possible genetically distinct $\alpha$ chains. Collagen IV $\alpha$ chains trimerize to form three distinct 'protomers' that can be distributed in a tissue-specific manner [84]. Two collagen IV protomers bind through the C-terminal non-collagenous 1 (NC1) domain to form a hexamer [16]. Hexamers interact with one another through N-terminal 7 S domains to generate a tetramer [16]. Yurchenco and colleagues demonstrated that the lateral interactions among tetramers created a polygonal network of collagen IV and a 3D mesh [16, 85]. The $\alpha1.\alpha1.\alpha2$ protomer was suggested to form the initial sheet-like basal lamina during embryogenesis [84]. Disruption of the COL4 $\alpha1$ or $\alpha2$ locus in mice is embryonic lethal [84]. Surprisingly, in collagen IV-deficient mice, LN111 and nidogen 1 were deposited and organized into a basal lamina-like structure, during early embryogenesis (prior to E10.5). The basal lamina in these mice had a discontinuous and altered structure, suggesting that collagen IV is not required for LN111 and nidogen assembly but is required to stabilize the nascent basal lamina [84]. Furthermore, other collagen IV $\alpha$ chains did not compensate for loss of the $\alpha1.\alpha1.\alpha2$ protomer [84]. Later in development, the $\alpha1.\alpha1.\alpha2$ protomer is replaced with different collagen IV protomers such as $\alpha3.\alpha4.\alpha5$ in the glomerular basal lamina [86]. Evidence for the importance of protomer tissue specificity has been obtained from patients with Alports syndrome who have a mutation in the COL$\alpha5$ chain. This mutation leads to absence of the $\alpha3.\alpha4.\alpha5$ protomer (IV) based network in the glomerular basal lamina, impeding the required switch from the fetal $\alpha1.\alpha1.\alpha2$ protomer to the $\alpha3.\alpha4.\alpha5$ protomer (IV) [86]. Alports syndrome leads to progressive renal failure among other conditions [87].

Although it is unclear which $\alpha$ chains comprise collagen IV in the mammary gland, collagen IV deposition has been found necessary for proper formation of the mammary lamina densa. Disruption of collagen deposition in the basal lamina in vivo resulted in collapsed alveolar structures in proliferative mammary epithelium, resulting in a phenotype similar to that seen during early involution [88]. In another study, Kidwell and colleagues demonstrated that mammary epithelial cells preferentially attached to collagen IV verses collagen I in vitro [89]. Hence, collagen IV is essential for supporting basal lamina structure and provides an anchor for mammary epithelial cells, indicating its specific role in maintaining cell viability. On a different note, cryptic domains of type IV collagen fragments were demonstrated to function as anti-angiogenic factors. Tumstatin is a fragment from the C-terminus of collagen IV $\alpha3$ chain and inhibits angiogenesis by blocking $\alpha V\beta3$ integrin signaling [90, 91]. Further, during early to mid-involution, alveolar loss is associated with both basal lamina remodeling and a decrease in vascular support needed for lactation [90, 92]. Thus, there may be a link between basal lamina turnover and vascular regression that occurs during early to mid-involution.

*Nidogen* Nidogens (also known as entactins) are sulfated glycoproteins (150 kDa) synthesized by fibroblasts as in various tissues such as skin, lung, central nervous system, and limb [93–95]. There are two genes in the nidogen family that encode nidogen 1 and nidogen 2. Nidogens are incorporated into the basal lamina upon secretion [93, 96]. Germ layer cooperation in the assembly of basal lamina is highlighted by the fact that mesodermal-derived fibroblasts make nidogen and ectoderm/endoderm-derived epithelial cells produce LNs. Nidogen is found in a 1:1 stoichiometric ratio with LN and can be viewed as a stabilizing component of the basal lamina by serving as a bridge between LN111 and collagen IV [97]. For example, the globular domain G3 of nidogen 1 binds to the C-terminal short arm of a LN $\gamma1$ chain and the G2 domain of nidogen binds collagen IV [98, 99]. Nevertheless, deletion of nidogen 1 in mice demonstrated that it was not essential for basal lamina assembly [100], as nidogen 2 appeared to compensate [101]. Furthermore, mice lacking both nidogen 1 and nidogen 2 do not die during embryogenesis, indicating that nidogens are not necessary for basal lamina assembly in most organs. However, lungs from newborn mice deficient in both nidogens had small alveolar spaces, thicker septa, and a prenatal decrease in expression of basal lamina constituents such as LN $\gamma1$ chain, which recovered to control levels by the perinatal period [102]. In addition, the ability of type II pneumocytes to produce pulmonary surfactant may be impaired given that the expression of surfactant protein B was reduced in double knockout newborn mice [102]. Pulmonary surfactant is important in lowering the alveolar surface tension as well as maintaining alveolar structure. Taken together, these data suggest that nidogen 1 and nidogen 2 are needed for proper function of alveolar epithelial cells in the lung. Their absence in the lung may be the reason mice lacking both nidogens die shortly after birth [102]. Nidogens also support tissue-specific differentiation as nidogen 1 promotes the ability of

LN111 to induce β-casein expression by mammary epithelial cells in vitro [103]. Collectively, these and other data suggest a functional relevance for nidogens in a tissue-specific manner, but surprisingly, also indicate that nidogens are not essential for basal lamina formation during embryogenesis.

*Perlecan* Perlecan is a proteoglycan that consists of a 470 kDa core protein with covalently-linked heparan sulfate, a specific-class of GAG, which increases the molecular weight (MW) of perlecan to over 800 kDa [104]. Analysis of perlecan distribution during development revealed its expression in the basal lamina of liver, lung, the vascular system, and other tissues as well as in interstitial stroma such as the interterritorial ECM of articular cartilage [105]. Further, mammary epithelial cells have been shown to secrete heparan sulfate proteoglycan, suggesting epithelial cells as a possible source of perlecan [17]. Disruption of the gene encoding perlecan (Hspg2) caused embryonic lethality in a large portion of mice. Lethality occurs at E10.5 due to defective cephalic development and effusion of blood to the pericardium, which is thought to result from basal lamina deterioration [106, 107]. The remaining perlecan-null mice died perinatally with severe skeletal anomalies, presumably due to the role of perlecan in bone matrix (as described above) [106, 107]. Moreover, Fässler and colleagues observed discontinuous areas in the basal lamina in vivo that separated the neuroepithelium from the underlying mesenchyme [106]. All together, the phenotypes of the null mice suggest that perlecan is essential for basal lamina assembly in several organs and for bone matrix function. Additionally, perlecan's soluble C-terminal fragment, endorepellin, disrupted capillary morphogenesis in vitro by inhibiting endothelial cell migration, and angiogenesis [108]. Moreover, perlecan mediates the effects of several fibroblast growth factor (FGF) family members. Perlecan was suggested to interact with FGF2 through its core protein and GAG group [109]. These interactions can enhance the potency of FGF2 signaling through FGFR1 and FGFR3 in vitro and consequently have a pro-angiogenic effect [109, 110]. As with most ECM proteins, perlecan provides instructive cues dependent on context and structural integrity.

Intra- and Interlobular Stroma

Immediately adjacent to the basal lamina are 10–100 μm thick bands of organized, collagen-rich stroma, known as intralobular stroma, that surround individual alveoli (Fig. 2a, arrow in the insert box). Clusters of alveoli that compose a single lobule are further surrounded by bands of stroma referred to as interlobular stroma. Fibrillar collagens are the dominant structural constituents, with several other

ECM dictating intra- and interlobular stroma organization and functions.

*Fibrillar Collagens* Collagen I, III and V are the major fiber-forming collagens, where their individual proteins organize into bundles of various thicknesses and lengths to form the pink or blue fibrous material observed in H&E (Fig. 2a–c) and trichrome-stained (Fig. 2d–f) mammary sections, respectively. Collagen I has been demonstrated to be synthesized by fibroblasts, chondroblasts, osteoclasts, and odontoblasts during normal development and by activated fibroblasts during wound healing. Thus, mesenchymal cells are thought to be the primary source of collagen I. The collagen I precursor, procollagen (500–600 kDa) consists of two identical α1 (I)-chains and one α2 (I)-chain, each derived from a unique gene. Each α chain is made up of approximately 300 Gly-X-Y repeats where X is frequently proline, and Y is hydroxyproline [32, 111]. The Gly-X-Y repeats are essential for 'self-assembly' of collagen chains into a triple helix procollagen structure, where all the glycines face inward while the larger amino acids such as proline cover the outer positions [111, 112]. After assembly into the triple helix, peptidases remove non-helical registration peptides from the secreted procollagen [111] and subsequently it is transformed into insoluble 300 nm tropocollagen [111]. At the end of each triple helix tropocollagen molecule there are short non-helical telopeptides. These allow further stabilization and aggregation by covalent intra- and intermolecular crosslinking via LOX resulting in the basic fibrillar structure of collagen observed in histological sections [32, 111]. Based on its ubiquitous and abundant presence, fibrillar collagen I is considered the backbone of stroma, including that in the mammary gland. As such, the physical and biochemical properties of collagen I likely contribute significantly to the functional attributes of the mammary stroma.

Work from the Taylor-Papadimitriou and Sonnenschein labs demonstrated that collagen I supported mammary ductal formation in 3D culture [113, 114]. Further, when the collagen-specific α2 integrin subunit was knocked out in vivo, branching complexity in mammary glands was reduced [115]. Nonetheless, the epithelial layer in vivo is separated from the intralobular stromal ECM by the basal lamina. Therefore, how do these ECM subcompartments and stromal cells such as fibroblasts cooperate to influence mammary epithelial morphogenesis? Krause et al., embedded mammary epithelial cells with and without fibroblasts in collagen I gels and in Matrigel-collagen I gels [114]. Epithelial cells alone could only form ductal structures in collagen I gels in comparison to Matrigel-collagen I gels, however when co-cultured with fibroblasts, the epithelial cells formed ductal structures in both gel conditions [114]. Moreover, fibroblast-secreted factors such as hepatocyte

growth factor (HGF) can enhance the ability of mammary epithelial cells to form duct-like structures in collagen I gels [113]. Collectively, these studies demonstrate that inter-plays between fibrillar collagens, integrins, and growth factors are required for mammary duct development.

Fibrillar collagen-epithelial cell interactions are also critical for supporting the mammary epithelium during pregnancy and lactation. Collagen-associated receptor, DDR1 may control the tremendous increase in epithelial proliferation that occurs during pregnancy [8]. DDR1-null mice demonstaraed hyperproliferation of the mammary epithelium during pregnancy and were unable to lactate, despite the ability of luminal epithelial cells to express normal transcriptional levels of milk proteins [8]. Hence, interrupting the collagen-DDR1 interaction causes misguid-ed instructive cues that are imperative for proper alveolar expansion and secretory function [8].

With cessation of milk secretion and weaning, the mammary epithelium involutes and returns toward its pre-pregnant, non-secretory state. The stroma in the involuting gland shares attributes with fibrotic, desmo-plastic stroma seen during wound healing. During involution, fibrillar collagens are upregulated in the mammary gland and an influx of innate immune cells such as macrophages are observed [116–118]. Based on gene array data, the onset of involution is characterized by elevated expression of several fibrillar collagens including collagen I, III, and V as well as an increase in the expression of genes involved in ECM turnover [23, 52]. We and others have demonstrated that non-fibrillar denatured collagen can be a positive chemoattractant for macrophages in vitro [118]. Although it is unclear whether macrophages are necessary for collagen accumulation or remodeling of the mammary ECM during involution, monocyte chemoattractant protein 1 (MCP-1) increases in early to mid-involution [118]. The presence of MCP-1 was shown to be important for collagen fiber formation in a chemical-induced skin fibrosis model, as MCP-1 null mice were resistant to chemical-induced skin fibrosis, including fibrillar collagen deposition [119]. Further, the loss of MCP-1 was associated with a decrease in macro-phages and other innate immune cells, in addition to an increase in the small leucine-rich proteoglycan (SLRP) decorin. The relationship between SLRP and collagen fibrillogenesis will be discussed later in this review. Collectively, the data suggest that fibrillar collagen-macrophage interactions may, in part, drive the tissue remodeling programs within the involuting mammary gland.

Another open question is whether unique functions are imparted by distinct fibrillar collagens with respect to collagen bundle formation, ECM turnover, and cell-collagen interac-tions. Although collagens III and V form oriented super-structures characteristic of fibrillar collagen, these collagens have features distinct from collagen I. For example, localized helix instability in collagen III can be caused by the higher content of glycine in $\alpha 1$ (III) chains [32]. This structural feature may cause an increase in the turnover rate of collagen III and subsequently the collagen III matricryptins may serve as potent chemoattractants for innate immune cells. Further-more, collagen III is a homotrimer consisting of a single $\alpha$ chain, while collagen V is a heterotrimer composed of three different $\alpha$ chains [111]. These variations in primary structure likely cause additional changes in biochemical properties, as fibrillar collagen I, III, and V interact with one another to assemble into supramolecular collagen bundles [111]. The assembly of these bundles is further supported by short triple-helical fibril associated collagens with interrupted triple helices (FACIT) collagens such as collagen IX, which can function as crosslinking bridges [32, 111]. Interactions between fibrillar collagens and FACIT collagens may be important for mammary gland remodeling by dictating collagen organization and turnover.

In summary, the function and expression levels of fibrillar collagens are altered during the various stages of mammary gland development. These studies suggest unique roles during duct formation, alveolar expansion during pregnancy, as well as to the demoplastic-like microenvironment during involution, highlighting the dy-namic role of fibrillar collagens in the mammary gland. However, even for ECM proteins as ubiquitous as fibrillar collagens, our basic understanding of both function and mechanism of action remains largely unknown.

*Fibronectin* FN is a large dimeric glycoprotein (~500 kDa) that mediates cell adhesion, migration, proliferation, and branching morphogenesis. FN is encoded by a highly-conserved single gene and its deletion causes embryonic lethality during gastrulation, emphasizing its essential function [120]. FN consists of three distinct repeating modules known as type I, II, and III domains [121]. Although FN protein is encoded by a single gene, there are several variant FN forms that arise due to alternative splicing [121, 122]. Specific examples are the spliced sites in the type III domain, termed extra domain (ED) EDA, EDB, and IIICS. Extracellular stimuli such as HGF have been shown to regulate splicing in vitro [122]. EDA and EDB have been implicated in wound healing, as their expression is undetectable in normal liver but is rapidly upregulated during injury [123]. In addition to its essential role in early embryogenesis, FN is an essential regulatory protein for salivary gland branching morphogenesis [124]. Yamada and colleagues blocked cleft formation and branching in the salivary gland ex vivo by downregulating FN expression in salivary epithelial cells via siRNA or by inhibiting FN-epithelial cell interaction via anti-FN anti-

body or antibodies against β1 integrin subunit [124]. Further, branching morphogenesis was rescued by adding exogenous FN to siRNA-treated glands [124]. These studies have clearly demonstrated that FN is required for salivary gland branching morphogenesis, although, the question of whether this requirement is FN variant-specific is unknown [124].

Ovarian steroids are suggested to regulate FN expression in the mammary gland [24]. Haslam and colleagues measured a three-fold increase in FN protein levels around mammary ducts between pre-puberty and sexual maturity [24]. Moreover, ovariectomy of sexually-mature mice resulted in a 70% decrease in the FN concentration in the mammary epithelium [24]. FN and the fibronectin receptor α5β1 integrin are dramatically downregulated during late-pregnancy and lactation, and then upregulated during mid-involution [23, 24]. Interestingly, the EDA isoform associated with pathological conditions in the liver is developmentally regulated in the rat mammary gland similar to total FN, suggesting a role for EDA within the normal mammary gland [23]. During the early stages of involution, elevated matrix metalloproteinase (MMP) activity correlated with higher levels of cleaved FN [23]. Additionally, FN fragments can induce MMP9 activity in mammary epithelial cells in vitro, suggesting a positive feedback loop between FN fragments and MMP activity [23]. This has implications for clearance of the secretory epithelium in involuting glands because FN fragments can induce apoptosis in epithelial cells [58].

*Tenascins* The tenascin (TN) family consists of 5 members: TN-C, TN-R, TN-W, TN-X, and TN-Y. TN-C was the first member of the TN family to be discovered, and is a six-armed glycoprotein with a 3D structure known as hexabrachion. Each arm contains N-terminal structural domains for self-assembly, followed by EGF-like repeats, alternative-spliced FN type III domains, and a C-terminal globular fibrinogen-homology domain [125]. The MW of TNs ranges between 180 and 300 kDa, which may depend on alternative splicing [125, 126]. TN is suggested to display anti-adhesive properties via disruption of the interactions between the transmembrane heparan sulfate receptor sydecan-4, α5β1 integrin receptor, and fibronectin [127, 128]. An anti-adhesive effect of TN-C on cells such as satellite cells has been shown to be context-dependent because other cells, for example, embryonic dorsal root ganglion explants, were able to spread, migrate, and grow on TN-C ex vivo [129]. Disruption of the TN-C gene in mice did not produce any visible abnormalities [130]. Although, Kusakabe and colleagues demonstrated neurological defects in TN-C null mice [131, 132]. Nonetheless, TN-C expression is associated with multiple tissues during embryogenesis and pathological conditions. In general, TN-

C expression is transient and largely restricted to embryogenesis, yet it is upregulated in breast cancer [125, 133]. Hence, TN-C has been described as an oncofetal protein. TN-C was also demonstrated to be overexpressed in skin during wound healing, particularly in the dermis–epithelial junction [134]. In the mammary gland, TN-C is transiently expressed in dense stroma surrounding the budding epithelium during rodent embryogenesis, with little to no expression observed in the quiescent adult gland [135]. TN-C expression is again upregulated during lactation and early postpartum involution [23, 136]. TN-C was found both in the milk of lactating mice and women [137, 138]. The function of TN during lactation is unclear, especially in light of fact that β-casein expression was inhibited in mammary epithelial cells cultured on reconstituted basement membrane containing exogenous TN-C [136].

Another TN family member that was identified in the mammary gland is TN-X [62]. TN-X has only a tribrachion structure and is prominently expressed in testis and muscle [139]. Burch and colleagues identified a connective tissue defect in TN-X-deficient patients that involved hyperextensible skin and joints, vascular fragility, and improper wound healing similar to Ehlers-Danlos syndrome [140]. These observations suggest that TN-X maintains tissue elasticity. During lactation the mammary epithelial cells can experience considerable stretch forces, and it is of interest to determine whether TN-X provides such a function at this time.

*SPARC* SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin) has been identified in the mammary gland by gene array and mass spectrometry based proteomics [52, 62]. SPARC is a small, 32 kDa secreted glycoprotein that mediates cell-matrix interactions [141]. SPARC contains a calcium-binding C-terminal extracellular module, follistatin-like module, and N-terminal acidic module [142, 143]. SPARC-null mice exhibit abnormal collagen fibril morphology [144]. Using picrosirius red staining, the effect of SPARC on collagen fibrillogenesis was evaluated in the dermis [144]. Compared to wild type mice, the dermis in SPARC-null mice contained less striated collagen fibers [144]. Sage and colleagues found that SPARC preferentially binds to ECM proteins such as collagen I and LN111, but not to collagen III [145]. They also demonstrated that SPARC displays an epitope-specific anti-adhesive function that causes cell rounding [145]. In contrast, SPARC was shown to positively regulate FN assembly and integrin-linked kinase activity, which resulted in FN-induced stress fiber formation in lung fibroblasts [146]. SPARC has been reported to be developmentally upregulated within the mammary gland during the transition between lactation and postpartum involution, correlating with increased levels of collagens

and FN [23, 118]. Future research focused on SPARC function in collagen fibrillogenesis and FN assembly in a dynamic organ such as the mammary gland is warranted.

## Small Leucine-Rich Proteoglycan (SLRP)

SLRP family members are characterized by N-terminal cysteine-rich motifs and tandem leucine-rich repeats (LRR) in their core protein. The core protein of SLRP is 'decorated' with one or more GAG chains. The SLRP gene family was categorized into five different classes based on several factors such as gene homology, distinctive N-terminal cyteine-rich motifs, and characteristic GAG side chains [147]. SLRPs were initially thought to only provide structural support and participate in collagen fiber formation. However, additional biological functions for SLRPs were recently identified [147]. SLRPs can directly bind to cell surface receptors and to growth factors, implicating them in various signal transduction pathways and cellular processes [147]. We will focus below on decorin and biglycan from class I, as these SLRPs have been determined to be present in the mammary gland via gene array and proteomics in multiple studies [52, 62].

*Decorin* Decorin core protein (~38 kDa) is covalently linked with a single chondroitin sulfate (CS) or dermatan sulfate (DS) chain resulting in an overall MW of 90-140 kDa [148]. Decorin is a secreted protein and is expressed in various cell types such as fibroblasts and astrocytes [149, 150]. Although decorin-deficient mice experienced skin and lung fragility, possibly due to aberrant organization of fibrillar collagen, they are viable. Variability in the shape and size of collagen fibrils in these mice was observed compared to wild-type mice, as determined by EM of dermal collagen [151]. Based on these studies, decorin has been suggested to be pivotal for proper spatial alignment of stromal collagen fibers. Another function of decorin was demonstrated by Iozzo and colleagues, where decorin suppressed signaling through ErbB2 in breast cancer cells in vitro and in xenograft models [152]. They suggested that decorin may inhibit EGFR signaling by initiating EGFR internalization and degradation by caveolar endocytosis [153]. Angiogenesis was also negatively affected by decorin as it downregulated vascular endothelial growth factor expression in tumor cells [154]. When endothelial cells were cultured in media from decorin-expressing tumor cells, they failed to migrate or form capillary-like structures in vitro [154]. An additional role of decorin is to bind TGF-β1 and sequester its activity in vitro [155]. Given that decorin is crucial for proper fibrillar collagen organization, one question concerns the role of decorin in mediating tensional changes required for TGF-β1 activation as

discussed in previous sections [26]. Array data demonstrate that decorin is upregulated during mammary gland involution [52], where it likely contributes to the transient increase in collagen fibers [118].

*Biglycan* Biglycan is also a member of the SLRP class I family and is comprised of a core protein (~38 kDa) that is covalently attached to two GAG chains (chondroitin sulfate and/or dermatan sulfate) with an overall MW of 150–240 kDa [148]. Young and colleagues observed a reduction in bone formation and mass in biglycan-deficient mice [156]. Disruption of biglycan gene expression did not cause upregulation of decorin expression and vice versa [151, 156, 157], although both biglycan and decorin belong to the same SLRP subfamily [157]. These results suggest that decorin and biglycan have distinct, non-overlapping roles [156]. Schaefer et al. have tested the function of biglycan in maintaining renal tissue elasticity under pressure-induced injury caused by unilateral ureteral ligation [158]. They concluded that biglycan can induce elastic fiber-associated protein fibrillin 1 expression in renal fibroblasts and mesangial cells, which subsequently help to retain elastic properties of the damaged tissue [158]. Their data is especially intriguing when it is taken together with work from Salgado et al. who demonstrated dynamic expression and localization of decorin and biglycan in the mouse uterus during the estrous cycle and pregnancy, suggesting that it is hormonally-regulated [159, 160]. These studies raise the question of whether biglycan and decorin are also hormonally-regulated in the mammary gland and whether they influence the elastic properties of the gland during periods of expansion. Moreover, biglycan transcript abundance in the mammary gland increased four- to five-fold during the transition from lactation to involution [52, 118].

Importantly, several members of the class II SLRP family including lumican, mimecan, and prolargin have been identified in the mammary gland, as well as evidence for their differential expression across the reproductive cycle [52]. Future studies are required to investigate the function of the class II SLRP family in the mammary gland.

## Fibrous Connective Tissue

The third structurally-distinct stroma in the mammary gland is the relatively acellular fibrous connective tissue, which is often found in large swaths (mm to cm range in the rat and human) and is characterized by the absence of epithelium and presence of fibroblasts, immune cells, and high fibrillar collagen content (Fig. 2a, solid arrow head). While the fibrous connective tissue is considered to be separate from the intra- and interlobular stroma, these

compartments share many of the same ECM proteins such as fibrillar collagens, FN, TN, SLRPs, and SPARC. However, a unique feature of fibrous connective tissue is the presence of elastic fibers.

*Elastic Fibers* Elastic fibers provide structural support and elasticity to various tissues. Elastic fibers are comprised of a 31 kDa secreted protein called microfibril-associated glycoprotein (MAGP) and another glycoprotein, the 350 kDa fibrillin protein that assembles into 10- to 12-nm microfibrils [31]. Microfibrils then associate with other elastic fiber constituents including elastin, fibulins, and proteoglycans. The process of organizing elastic fibers in the connective tissue begins with the formation of the MAGP/fibrillin microfibrils that align parallel to fibroblasts and serve as tracks for the deposition of tropoelastin (70 kDa), which is a soluble precursor of elastin. Tropoelastin is rich in hydrophobic amino acids and contains low amount of polar amino acids including several lysine derivatives. The lysine derivatives are imperative for covalent crosslinking between monomeric elastin via LOX [31]. Microfibrils are described as a scaffold that facilitates the alignment of soluble monomeric elastin to coalesce and organize into amorphous elastin [31]. As elastic fibers mature postnatally, elastin becomes the dominant component, with microfibrils being gradually displaced to the outer layer. The resultant fibers have physical properties that can withstand changes in the mechanical environment [161]. An additional constituent of elastic fibers is the fibulin family (50–200 kDa), which have calcium-binding sites and consist of I, II, III domains with a central segment that is composed of EGF-like modules. Fibulin 5 (66 kDa) displays strong calcium-dependent binding to tropoelastin, while it weakly binds to carboxyl-terminal of fibrillin 1 [162]. Further, fibulin 5 knockout mice were generated to analyze fibulin 5 function in elastic fiber formation in the dermis. These mice demonstrated a requirement for fibulin 5 in tropoelastin deposition and potentially for optimal LOX activity in vivo [162, 163]. Moreover, proteoglycans such as biglycan and decorin were implicated in elastogenesis by binding to tropoelastin, fibrillin 1, and MAGP [158, 164, 165]. While much is known about the functions of elastic fibers in other systems such as lung, skin, and vascular system, their function in the mammary gland is still elusive. Elastic fibers are anticipated to be particularly important in the lactating mammary gland as the tensile forces continuously change due to suckling, milk accumulation between nursing, and myoepithelial contraction on the secretory alveolar epithelial cells. Further research on the function and regulation of the fibrous connective tissue, including elastic fibers, is necessary given the correlations between higher mammographic density, tissue tension, and increased breast cancer risk [28, 166, 167].

## Future Directions

### Immune Cells and ECM Remodeling

The ECM of the mammary gland plays a role in the infiltration and activation of immune cells. We have previously characterized involution as a wound healing microenvironment with high levels of alternatively activated or M2 type macrophages [118]. How the immune cells are recruited and 'activated' still remains unclear. However, many of the proteins that make up the ECM can fragment into matrikines and matricryptins, which have been demonstrated to influence leukocyte infiltration in other systems. Fragments of collagen I, collagen IV, LN and nidogen-1 have all been shown to promote chemotaxis of monocytes and neutrophils within the interstitial tissue, but it remains unknown whether tissue-derived matrix fragments enter the blood stream to recruit immune cells or augment their motility upon arrival to the local tissue [118, 168–171]. Once in the mammary gland, macrophages and neutrophils secrete proteases including MMP9 and elastase that are able to breakdown the ECM [118, 172, 173]. Without the influx of macrophages or neutrophils and their crucial proteases, remodeling of the mammary tissue to its non-secretory postpartum state could potentially be delayed or incomplete. Werb and colleagues have evaluated the importance of secreted proteases during involution in the mammary gland by inhibiting a serine protease, plasma kallikrein. They demonstrated that inhibition of this mast cell-associated protease during involution caused an accumulation of fibrillar collagen and delayed repopulation of adipocytes, thus preventing the gland from returning to its pre-pregnant state [174]. Combined, these studies indicate that ECM turnover and immune cell function are highly linked, creating a feedback loop that is necessary to complete gland remodeling after lactation.

In addition to facilitating immune cell infiltration, ECM fragments may act as ligands to receptors present on leukocytes in the mammary gland. Fragments of biglycan, heparan sulfate, and hyaluronan have been shown to act as ligands for toll like receptor 4 (TLR4). TLRs are part of the pattern recognition receptor family expressed on the cell surface of innate immune cells and dendritic cells. When a ligand binds to a toll like receptor the immune cell becomes activated and/or secretes cytokines that can further activate cells of the adaptive immune system. Babelova et al. recently demonstrated that soluble biglycan binds to the TLR 2/4 on macrophages, stimulating them to synthesize and release interleukin-1β, a proinflammatory cytokine [175]. Additionally, heparan sulfate and hyaluronan have been shown to bind to the TLR4 on dendritic cells, resulting in dendritic cell maturation [176, 177]. Once mature, dendritic cells are able to activate the adaptive immune system, where adaptive

immune cells respond and migrate to the site of ECM remodeling. Consistent with a similar mechanism of adaptive immune cell activation due to ECM fragmentation in the mammary gland, the presence of B cells in the mammary gland during involution has been confirmed; however their role remains elusive [172]. The adaptive immune system is typically activated after the innate response, although B cells have been shown to infiltrate the gland during early to mid-involution, prior to the peak in macrophage recruitment [172]. The presence of B cells may indicate that cells of the adaptive immune system can be attracted to the gland, similar to the innate immune cells, by the presence of proteolytic ECM fragments. Interleukin-4 and fibronectin, which are both upregulated during involution, have been shown to stimulate motility of B cells in vitro, and may account for the increased presence of B cells in the mammary gland during involution [118, 178]. The infiltration of immune cells such as macrophages, neutrophils, mast cells, and B cells into the postpartum mammary gland during involution suggests both the innate and adaptive immune systems play an important role in returning the gland toward its pre-pregnant state. However, with limited research on the function of immune cells in the mammary gland, additional studies are necessary to determine the role of the ECM in immune cell activation, as well as the role for immune cells in restructuring the mammary gland in vivo.

## Summary

The in situ analysis of mammary stroma by EM, cytological stains, and immunohistochemistry have provided snapshot views depicting the mammary stroma as diverse and highly complex. Cell culture studies using purified ECM components clearly demonstrate distinct, non-overlapping roles for ECM proteins with functions that range from induction of cell death to induction of a secretory phenotype. Studies using ECM preparations derived from tissues [EHS tumor and rat mammary glands] further demonstrate the complex and dynamic nature of the endogenous ECM. To further our understanding of the composition and function of ECM in the mammary gland, it is necessary to increase our ability to obtain better qualitative and quantitative ECM proteomes. Progress in proteomics is beginning to permit non-biased, high throughput, quantitative assessments of ECM proteomes. Such results will likely transform future in vitro experiments by permitting a systems approach to the study of mammary ECM-epithelial cell interactions.

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