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Chapter 5

# Stromal-Epithelial Interactions during Mammary Gland Development

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Additional information is available at the end of the chapter

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**Abstract**

Mammary gland is an organ, which undergoes the majority of its development in the postnatal life of mammals. The complex structure of the mammary gland comprises epithelial and myoepithelial cells forming the parenchymal tissue and adipocytes, fibroblasts, vascular endothelial cells, and infiltrating immune cell composing the stromal compartment. During puberty and in adulthood, circulating hormones released from the pituitary and ovaries regulate the rate of development and functional differentiation of the mammary epithelium. In addition, growing body of evidence shows that interactions between the stromal and parenchymal compartments of the mammary gland play a crucial role in mammogenesis. This regulation takes place on a paracrine level, by locally synthesized growth factors, adipokines, and cytokines, as well as via direct cell-cell interactions. This chapter summarizes the current knowledge about the complex nature of interactions between the mammary epithelium and stroma during mammary gland development in different mammalian species.

**Keywords:** mammary epithelial cells, mammogenesis, adipocytes, fibroblasts, immune cells, endothelial cells

## 1. Introduction

The origin of the mammary gland in the fossil record appeared about 220–300 million years ago in the Carboniferous geological period and was evolving for 130 million years to its current mammalian form [1]. In its earliest evolutionary form, the glandular structure ancestral to the mammary gland had functioned as a source of secretion that helped eggs withstand desiccation associated with incubation on land and appeared among tetrapods or among the

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basal amniotes-vertebrates. Comparison of mammary-expressed genes between mammalian taxa revealed the sheared presence and high degree of conservation of the genes. Mammary gland fully developed prior to emergence of diverse groups of mammals, and the milk compounds (fat globules, whey proteins, casein micelles, and sugars) are structurally similar across all mammalian species [2].

In contrast to most organs that achieve morphological maturity during prenatal development in the process defined as morphogenesis, the majority of mammary gland development leading to its complex morphological maturity occurs mostly during postnatal life of mammals [3]. During embryogenesis, the mammary gland development is driven mostly by mesenchymal cells. In postnatal life, subsequent stages of glandular development: mammogenesis (development of mammary epithelial tissue), lactogenesis (functional differentiation of the mammary epithelium leading to initiation of milk secretion), galactopoiesis (maintenance of milk secretion), and involution (regression of the glandular epithelium), take place under significant regulation of hormones. In parallel, the intraglandular milieu plays also an important role in controlling the progress of events related to mammary gland morphogenesis.

## 2. Stages of mammary gland development

At the embryonic period, the mammary gland is derived from ectoderm cell migration, followed by the formation of disk-shaped placodes. The mammary buds arise as a result of proliferation of the basal cells of the ventral epidermis due to factors secreted by mesenchymal cells present in the mammary bud in a process referred to as branching morphogenesis [4–6]. The mesenchyme is instructive and provides critical information to drive mammary gland development. Two different mesenchymal tissues with different properties are involved in this and, with other cells, become a part of stroma compartment. First type of mammary mesenchyme, termed the fibroblastic mesenchyme, is composed of fibroblastic cells surrounding the epithelial rudiment and the second comprise the fat pad cells, thus is known as the fat pad mesenchyme. A solid cord of epithelial cells extends from the mammary bud and grows through the fibroblastic mesenchymal tissue into the fat pad precursor mesenchyme, which at this stage is a small collection of preadipocytes. In rodents, a single epithelial sprout reaches the fat pad and begins to branch by equal division of the terminal bud. The terminal end buds (TEBs) are created as an outer layer of cap epithelial cells surrounding multilayered body epithelial cells located at the front of the branch that invades into the mammary mesenchyme. The body epithelial cells give rise to mammary epithelial cells and the cap cells are myoepithelial precursors. TEBs move forward through mesenchymal cells leading to formation of a rudimentary ductal system. In rodents, it is composed of 10–15 branches that are generated without hormonal input, and the rudimentary ducts remain largely quiescent until puberty [6, 7]. In humans, several sprouts form, creating multiple mammary trees that unite at the nipple, whereas in ruminants the rudimentary ductal network is connected to a small cisternal cavity that connects to the teat cistern and ultimately communicates with the teat meatus [6–8].

After birth, in the postnatal life until puberty, the gland remains quiescent and exhibits only minimal ductal growth. Interspecies differences occur in the extent of mammary gland development that occurs in neonates. In mice, the mammary tree consists of long, infrequently branching ducts and TEBs. Human mammary gland has a more complex structure composed of approximately 15–20 lobes of glandular tissue, each containing a lactiferous duct that opens onto the breast surface through the mammary pit [9]. In the case of ruminants, the mammary gland consists of terminal ductal units (TDU), which are formed during prenatal development accomplished through the coordinated growth, branching and extension of TDU, as well as growth of the loose connective tissue that surrounds the TDU as it invades the mammary fat pad [8].

With the onset of puberty, a combination of systemic and paracrine hormones induces TEBs to reappear at the ductal tips accompanied by a significant increase in the growth rate. Elongation and branching of the ducts, regulated by proliferation and migration of TEBs cells, rely on both endocrine and local growth regulatory signals, extracellular matrix (ECM) remodeling, and stromal influence. With the beginning of puberty, the epithelium bifurcates and invades into the surrounding stroma creating a tree-like structure of mammary ducts. The majority of mammary ductal morphogenesis occurs with onset of ovarian function because of the cyclic influence of reproductive hormones. Further, with each estrus cycle, the alveoli and ducts undergo cyclic expansion and maturation, followed by a modest regression phase as ovarian hormone levels rise and fall, respectively. These events are under the control of a complex interplay of circulating essential steroids (estrogen and progesterone), polypeptide systemic hormones (e.g., prolactin), metabolic hormones that are responsible for coordinating the body's response to metabolic homeostasis (e.g., growth hormone—GH, glucocorticoids, insulin, leptin), as well as locally acting paracrine hormones and growth factors (e.g., insulin-like growth factor I—IGF-I, hepatocyte growth hormone—HGF, transforming growth factor-β—TGF-β, epidermal growth factor—EGF) [10]. It is worth noting that the hormone acting network regulating the development of the mammary epithelium varies between different species.

The mammary gland is able to undergo its terminal differentiation only in female mammals during pregnancy and lactation. With the onset of gestation period and increased levels of progesterone, alveolar structures give rise to lobuloalveolar structures capable of milk production during lactation. After weaning of the offspring (or termination of milking), the gland undergoes post-lactating regression referred as involution, with loss of most of epithelial components gained during the preceding event. Early involution is evidenced by apoptotic death of alveolar secretory epithelial cells which subsequently are removed by efferocytosis (the process of engulfing and destroying apoptotic cells) [11]. Second phase of the mammary gland involution is defined by degradation of basement membrane and ECM proteins and reduction of lobuloalveolar structures.

## 3. Structure of fully developed mammary gland

Fully developed mammary gland is created by two compartments: epithelial and stromal. The epithelial compartment, termed parenchyma, is composed of the branching network of ducts

and lobuloalveolar structures comprised of mammary epithelial cells of two primary lineages: myoepithelial (basal) cells and epithelial (luminal) cells, forming a bilayered structure, which is embedded in the stroma [12]. Mammary ducts consist of apically orientated luminal epithelial cells that line ducts with alveolar structures at the ends and of basally orientated myoepithelial cells surrounded by a laminin and collagen-rich basement membrane (BM). Luminal epithelial cells are separated from all kinds of stromal cells, laying on top of myoepithelial cells. The functionally distinct basal layer contains myoepithelial cells with contractile properties and cells with demonstrated stem cell activity, referred as mammary repopulating units (MRUs). These cells have an ability to regenerate the bilayered glandular structure of inner luminal and basal outer epithelial cells [12]. The myoepithelial and stromal cells produce the basement membrane, which is a thin sheet composed of collagen IV, laminins, entactin, and proteoglycans, and forms physical barrier separating the epithelial and stromal compartments [3]. The stromal compartment is composed of two mesenchymal lineages: adipocytes and fibroblasts, as well as infiltrating immune and vascular endothelial cells [5]. These cells synthesize extracellular matrix (ECM) components essential for three-dimensional microstructure of the stroma. Stromal ECM components include collagens, which are the major structural proteins, as well as proteoglycans, hyaluronic acid, fibronectins, and tenascins [13, 14] (**Figure 1**).

Stromal-epithelial interactions regulate mammary epithelial growth and differentiation during embryonic and postnatal development through soluble factors that are released into the environment, as well as through insoluble factors that are present in the stroma itself, referred as matrikines and matricryptins [14]. The stroma accounts for roughly 60% of the total tissue

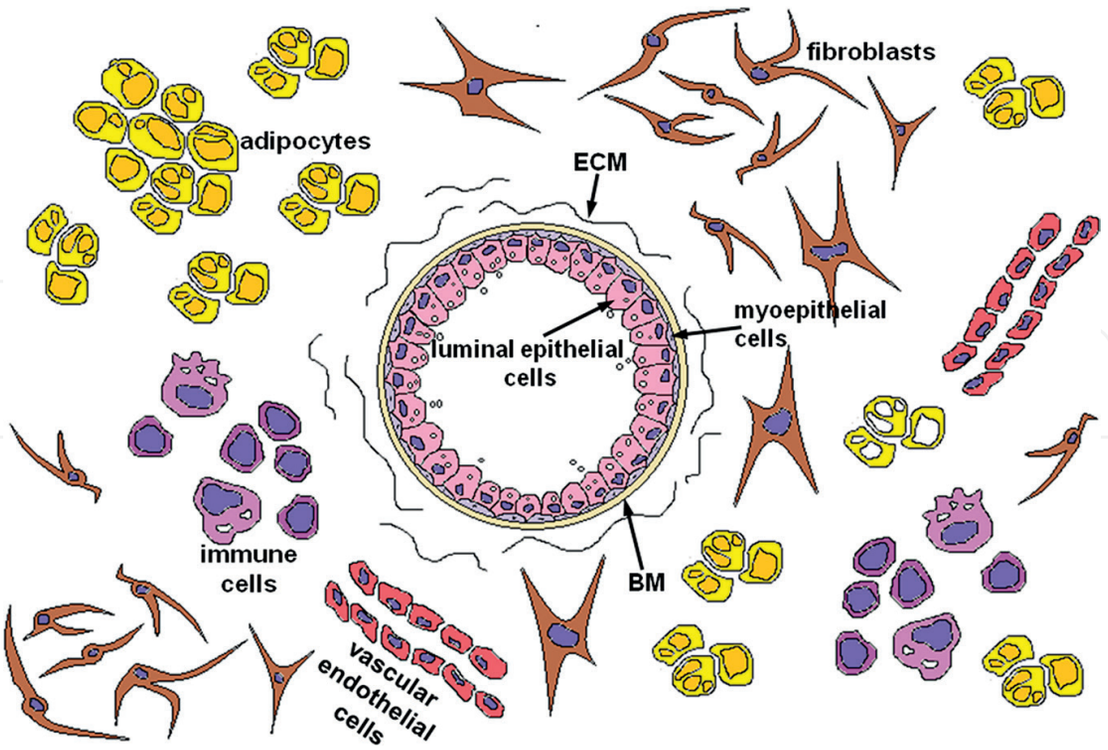


**Figure 1.** Schematic representation of cells found within the structure of fully developed mammary gland. Scheme presents cross section of mammary alveolus surrounded by stromal components (cells and extracellular matrix).

volume and exerts a dominant effect on tissue morphogenesis. Ratio between stromal and epithelial compartment changes at all stages of mammary gland development, still staying in its own harmony milieu. Stromal cells architecturally support the epithelium, providing structure, nutrients, blood, and immune defense. Large amount of data suggest that the mammary stroma not only provides a scaffold but also regulates mammary epithelial cells (MECs) function via paracrine, physical, and reciprocal signaling between MECs and underlying stromal cells, modifying proliferation, survival, polarity, differentiation, and invasive capacity of the mammary epithelium [4, 15]. The importance of stromal cells is reflected by the fact that signals emitted by embryonic mesenchyme dictate the differentiation of epithelial cells, and mammary epithelial cells form salivary gland-like structures when placed on top of salivary gland mesenchyme [16]. On the other hand, outgrowth of salivary epithelium in contact with mammary mesenchyme resembles a mammary gland ductal tree and responds to hormonal stimuli [16]. The following paragraphs of this chapter present the complex interactions between the mammary epithelium and different stromal cells that direct the progression of normal mammary gland morphogenesis.

## 4. Role of stromal cells in regulation of mammary gland development

### 4.1. Adipocytes

Adipocytes constitute the most abundant type of cells within the stroma of the mammary gland. Fat cells predominate in the stromal compartment of the mammary glands of rodents (mice and rats), whereas in the mammary glands of humans and ruminants adipocytes of white adipose tissue form the structure of a fibrous-adipose stroma along with fibroblasts. Adipocytes create a specific microenvironmental niche for MECs as the source of triglycerides and thus a source of energy, as well as a scaffold liable to invade, and a supply of various biologically active compounds.

Adipose tissue modulates epithelial development, remodeling, and function in a state-dependent manner. During embryonic morphogenesis, the fat pad together with the fibroblastic mesenchyme appears before ectoderm cell migration, creating environment and scaffold for mammary buds development. At this stage, each type of mesenchymal cells has different properties. It has been shown that fat pad mesenchyme induces elongation and branching of the mammary epithelium [5]. Lack of white adipose tissue in transgenic Z-ZIP/F1 female mice leads to compromised ductal growth during prenatal development, manifested by formation of only few underdeveloped ductal structures showing severe, abnormal distension [17]. Interestingly, these transgenic Z-ZIP/F1 mice produce a mass of lobuloalveolar structures in the mammary gland during pregnancy, which suggests that interactions between MECs and adipocytes are not essential for the functional differentiation of the mammary epithelium [17]. An alternative in vivo model of adipocytes depletion (FAT-ATTAC mice) allowed scientists to explore further the role of mammary-associated adipocytes. In FAT-ATTAC mice, elimination of adipocytes can be induced at any developmental

stage through induction of apoptotic cell death by administration of a FK1012 analog, which leads to the forced dimerization of a caspase-8 fusion protein uniquely expressed in adipose tissue [18]. This model allows for selective ablation of mammary adipocytes in female mice without affecting other fat pads. Under these conditions, Landskroner-Eiger and co-workers [18] demonstrated that the presence of adipocytes is necessary for proper formation of the extended ductal network in the mammary gland during puberty as well as for the maintenance of the normal alveolar structures that develop during adulthood. Ablation of adipocytes in mice starting from 2 weeks of age resulted in reduced ductal growth. Alterations in ductal features were caused by the loss of mechanical and physical support provided by adipocytes. However, when the loss of local adipocytes was initiated at 7 weeks of age in FAT-ATTAC mice model, an excessive lobulation was observed in the mammary gland. These observations indicate that adipocytes are critically involved in maintaining proper architecture and functionality of the mammary epithelium [18]. The important role of adipocytes in normal morphogenesis of the mammary epithelium was further confirmed in in vitro studies. MCF-10A human mammary epithelial cells co-cultured with human adipose-derived stem cells (hASCs) in Matrigel/collagen gels spread on silk scaffolds were able to create both alveolar- and duct-like structures. In contrast, monoculture of MCF-10A resulted in formation of only alveolar structures [19]. Consistently, EpH4 murine mammary epithelial cells cultured within adipose-rich collagen I formed branched mammary epithelial tubules within 24 h of culture [20]. It should be noted that the mammary-associated adipocytes also undergo massive morphological changes between the periods of lactation and involution. During lactation, adipose tissue serves as a major lipid store utilized as a source of energy for milk production. That is why in lactating mammary gland fat cells undergo lipid depletion and appear as long projections. At the time of involution, when milk synthesis ceases and mammary epithelium regresses, adipocytes regain their lipid stores, but some adipocytes undergo dedifferentiation into preadipocytes or are eliminated via apoptotic cell death [21].

#### *4.1.1. Adipokines*

Beyond the function of adipocytes as the energy storage depot, currently it is well accepted that these cells are actively producing and secreting a wide range of endocrine factors referred to as adipokines. Adipokines are signaling molecules that regulate various physiological processes in the body. In the context of the mammary gland, adipokines are thought to regulate normal development of this organ [22]. This group of compounds is also locally synthesized by adipocytes of the mammary stroma and act through juxtacrine or paracrine signals modulating epithelial cells proliferation. In vitro studies on normal human MECs (NMuMG cell line) elegantly demonstrated the effect of signaling molecules secreted by adipocytes. NMuMG cells were incubated for 24 or 48 h in the presence of conditioned medium derived from adipocytes (3T3-L1 cell line) at various degrees of differentiation: preadipocytes (preA), poorly differentiated adipocytes (pDA), and mature adipocytes (MA) [23]. After 24 h treatment human MECs showed significantly increased proliferative activity when cultured in conditioned media from pDA and MA, whereas after 48 h incubation the effect of increase proliferation was observed in the case of all conditioned media (preA, pDA, and MA) [23]. Another study revealed that 24 h treatment with conditioned medium from mature adipocytes

induced branching morphogenesis of mammary tubules, with sites localized to the ends of the tubules, without appreciable lumen formation, which indicates that the biologically active molecules produced by adipocytes influence mostly the ductal growth [20].

Adipokines detected in the mammary gland include hormones (leptin and adiponectin), growth factors (HGF, IGF), cytokines (interleukin 6—IL 6, tumor necrosis factor alpha—TNFα), as well as ECM components (collagen VI). It has been proven that HGF is especially important stimulator of branching morphogenesis [20]. HGF secreted by human pre-differentiated hASCs affected the duct-like structure formation by mammary epithelial cells (MCF10A) in co-culture [19]. Moreover, systemic hormones (prolactin, GH) not only exert their action directly in epithelial cells but also can act indirectly via the stromal compartment of the mammary gland. Studies have shown that GH stimulates the mammary gland adipocytes to produce IGF-I [17]. It is also evident that pubertal branching morphogenesis in vivo is stimulated by steroid hormones, including estrogen, which act on receptors located in the stroma to induce production of mitogens including HGF [20].

Leptin and adiponectin are the most extensively studied hormones synthesized by adipose tissue. They are found in higher concentrations in the mammary tissue than in blood and thus may be a part of an important paracrine or juxtacrine signaling system between adipose-rich stroma and epithelial cells [24]. Leptin, which was the first known adipokine discovered by Friedman and Coleman in 1994, is a 16 kDa nonglycosylated protein encoded by the *Ob* gene. This protein hormone is secreted mainly by adipose tissue to regulate body energy balance, suppressing food intake and thereby inducing weight loss. In the context of mammary gland physiology leptin actions are associated with regulation of the metabolic changes occurring during pregnancy and lactation, due to the fact that it is the key hormone regulating the metabolic adaptation of nutrient partitioning during the energy consuming processes [25]. MECs express leptin receptors (OB-Rb) and therefore may undergo direct regulation by leptin, whereas local production of leptin by mammary adipose tissue is under control of several hormones: insulin, glucocorticoids, and prolactin. Prolactin, the main lactogenic factor, was shown to regulate leptin and leptin receptor gene expression in the bovine mammary gland [26]. It is believed that prolactin may be the key signaling factor stimulating the mammary gland to interact with leptin in the regulation of milk synthesis during lactation [27]. In the presence of prolactin, leptin was shown to enhance the expression of α-casein gene (milk protein gene) in bovine mammary gland, indicating that leptin and prolactin interact to alter milk synthesis during lactation [27]. Estradiol, which is known to regulate ductal morphogenesis in the mammary gland, also plays an important role in the regulation of the extracellular levels of leptin, as well as adiponectin in normal human mammary gland [28].

In contrast to leptin, circulating levels of adiponectin are inversely correlated with the body mass index (BMI). Adiponectin is a 240 amino acid protein of approximately 28–30 kDa existing as a monomer, although it forms dimmers and multimers, circulating as low, medium, and high molecular weight isoforms. Two types of receptors, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2), have distinct distribution patterns in different tissues. Both receptors were shown to be expressed in normal mammary epithelial cells [29, 30]. Binding of adiponectin to its receptor activates adenosine monophosphate-activated protein

kinase (AMPK), a nutrient-sensing enzyme, which regulates several key pathways involved in protein synthesis and cellular energy metabolism. One of the few researches on bovine mammary gland disclosed that adiponectin expression in the mammary gland decreases in the peak and late-lactation period, although adiponectin receptor 1 (AdipoR1) expression increases in the same period [30]. Moreover, leptin/adiponectin ratio is directly proportional to the size of stem cell population in vivo. It was evidenced that leptin alone is sufficient to stimulate mammary stem cell self-renewal, leading to significant increase in the stem cell population. In contrast, unopposed adiponectin decreases the size of the mammary stem cell pool in vitro. It is believed that leptin and adiponectin may function as both endocrine and paracrine/juxtacrine factors to modulate the size of the normal stem cell pool [24].

Recent studies have shown chemerin as a novel adipokine, which may actively take part in regulation of the mammary gland lactogenesis. Chemerin, also called retinoic acid receptor responder protein 2 (RARRES2), is a 16 kDa chemoattractant cytokine (chemokine) mainly expressed in and secreted from white adipose tissue. Chemerin is secreted as a 143-amino acid inactive precursor, pro-chemerin, and is activated by proteolytic removal of six to seven amino acids from its C-terminus by proteases such as elastase or cathepsin G. Three G protein-coupled receptors are able to bind chemerin with high affinity, namely chemokine receptor-like 1 (CMKLR1), G protein-coupled receptor 1 (GPR1), and C-C chemokine receptor-like 2 (CCRL2). Chemerin inhibits cAMP production and promotes phospholipase C activation, IP3 release, calcium mobilization as well as activation of PI3K and MAPK pathways [31]. In bovine mammary gland, the expression of chemerin was greater in adipose tissue of postpartum dairy cows versus pregnant cows, and two out of three chemerin receptors (CMKLR1 and CCRL2) were expressed in bovine MECs [31]. Studies with immortalized bovine MECs treated with chemerin revealed upregulated expression of genes associated with fatty acid synthesis, glucose uptake, and casein synthesis; thus, it is postulated that chemerin may play a role of lactogenesis regulator in bovine mammary epithelium. Surprisingly, adiponectin reduced the expression of CMKLR1 receptor, without altering CCRL2 expression [30]. These results imply that adiponectin is not only able to counteract the effects of leptin but also able to regulate the influence of chemerin on mammary epithelial cells.

#### *4.1.2. Other adipocyte-related molecular regulators of mammogenesis*

Adipocytes of the mammary stroma also express retinoids (RARs), which are potent transcription regulators [32]. Co-cultures of primary adipocytes, or in vitro differentiated adipocyte cell line, with mammary epithelium showed that when activated, adipocyte-RARs contribute to generation of secreted proliferative and pro-migratory factors affecting branching morphogenesis [33]. RARs expressed by adipocytes were shown to be important regulators of secreted growth factor—pleiotrophin (PTN), involved in paracrine regulation of epithelial ductal tree development [33]. Adipocyte-RARs induced parathyroid hormone receptor (PTHR) expression leading to increased expression of PTN, which in turn regulated mammary epithelial migration.

Adipocytes also express vitamin D receptor (VDR), which is expressed in both epithelial and stromal compartment of the mammary gland and is known to participate in regulation of hormone-induced growth and differentiation throughout development [34]. VDR complexes with the active ligand, 1a,25-dihydroxyvitamin D3 (1,25D3), to induce cell cycle arrest,

differentiation, and apoptosis in human MECs, regulating growth in normal and transformed cells [34–36]. The ability of human MECs to synthesize 1,25D3 locally within the mammary epithelium to regulate cellular growth and differentiation may constitute a potential mechanism by which elevated serum 25D3 is associated with a decreased risk of developing breast cancer or metastatic progression [37]. Ching and co-workers [38] investigated the hypothesis that adipocytes from the mammary stroma express the signaling components necessary to participate in vitamin D3 synthesis and act via VDR, potentially modulating ductal epithelial cell growth and differentiation. Mammary adipocytes expressing VDR were shown to participate in bioactivating 25-hydroxyvitamin D3 (25D3) to the active ligand, 1,25D3, and secrete it to the surrounding microenvironment. Active vitamin D3 in turn was able to inhibit the ductal epithelial cell growth [38]. Similar results were obtained by Matthews and co-workers, who used a different animal model in their studies [39]. This group generated CVF transgenic mice with adipose-specific *Vdr* gene deletion and noted that adipose deletion of *Vdr* significantly enhanced mammary epithelial density and branching, supporting the hypothesis that vitamin D receptor in mature adipocytes exerts anti-proliferative effects on the mammary epithelium [39].

#### *4.1.3. Influence of mammary epithelium on stromal adipocytes*

In terms of investigating interactions between epithelial and stromal compartments of the mammary gland, it is important to expand our knowledge about reciprocal cell-cell interactions within the gland. There are still relatively few studies focused on the influence of MECs on the adipocytes population. A vivid example is an in vitro model of three-dimensional (3D) collagen gels containing differentiated adipocytes, which were used to investigate the mutual interactions between adipocytes and MECs during branching morphogenesis [20]. In this research, 3T3-L1 mouse preadipocytes were embedded in collagen, differentiated, and then treated with MECs-derived conditioned medium. Samples treated with conditioned media formed fewer and smaller fatty clusters and showed lower expression of lipoprotein lipase (LPL) and adipogenic transcription factor PPARγ2. These data suggest that MECs either inhibited or delayed differentiation of the preadipocytes [20]. In vivo, during embryonic mammary gland development, the fat pad is present before the epithelium invades, and epithelial compartment invades the stroma causing its reduction [20]. Similar conclusion was made by investigators who demonstrated that MECs produce the enzyme galactose 3-*O*-sulfotransferase 2 (GAL3STS2), which was able to inhibit the expression of adipogenic transcription factor C/EBPb and fatty acid-binding protein 4 (FABP4)—a marker of adipocytes differentiation [40]. In addition, accumulation of triglycerides was also inhibited under the influence of GAL3STS2. The authors postulate that GAL3ST2 may generate multiple signals related to integrin activation, including its effect on preadipocyte differentiation [40]. Taken together, it seems that epithelial compartment reduces the adipose tissue during mammary gland morphogenesis and works as negative feedback creating an appropriate/ favorable microenvironment for itself.

#### *4.1.4. Summary*

Stromal adipocytes play a profound role in regulation of mammogenesis during both embryonic and postnatal development of the mammary gland. These cells are necessary for proper ductal elongation and branching and are critically involved in maintaining proper architecture and

function of the mammary epithelium. This effect is exerted through direct cell-cell contact with the mammary epithelial cells as well as through paracrine signals induced by secreted adipokines. This group of biologically active molecules includes HGF supporting ductal morphogenesis, leptin and adiponectin that may modulate the size of the mammary stem cell pool within the glandular tissue, as well as chemerin, which may be a novel, local regulator of lactogenesis, as it is involved in regulation of fatty acids and milk protein synthesis and glucose uptake (**Figure 2**).

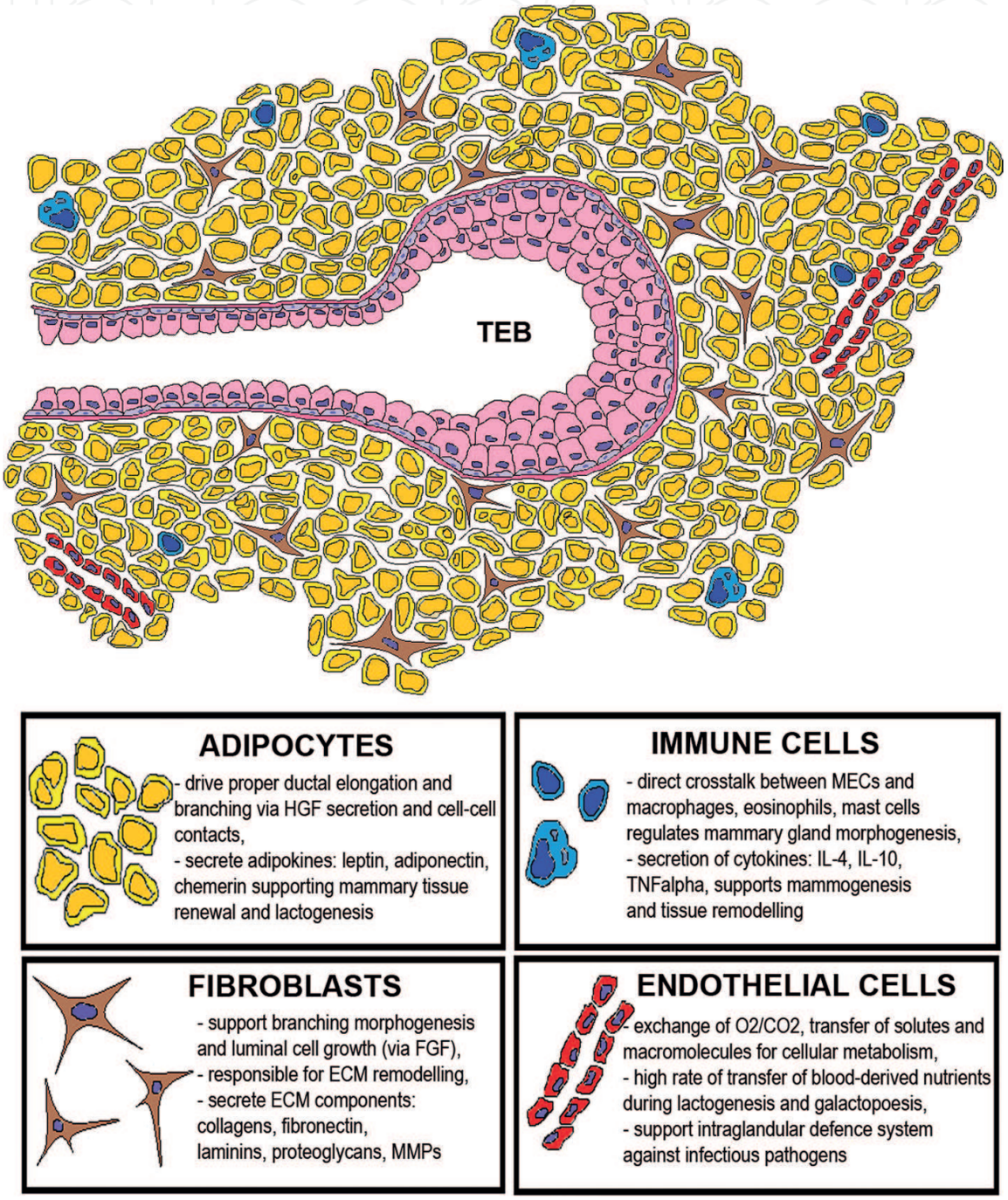


**Figure 2.** Schematic representation of different types of interactions between mammary epithelial cells [forming terminal end bud (TEB)] and stromal cells during mammogenesis.

### 4.2. Fibroblasts

Fibroblasts, together with adipocytes, are the major cellular components of the mammary stroma and play an integral role in regulating mammary gland development. As mentioned previously, during prenatal period of mammogenesis, the fat pad and fibroblastic mesenchyme appear before ectoderm cell migration, creating the environment and scaffold for emerging mammary buds [4]. Fibroblastic cells of the mesenchyme are in direct contact with the developing epithelial rudiment, and their signals first determine the identity of MECs [41]. In parallel, the epithelium also influences mesenchymal maturation. Research done on murine model of mammary gland morphogenesis revealed that by day 14 of mouse embryonic development the mammary mesenchyme condenses to form a few layers of fibroblast-rich cells closely surrounding the epithelial rudiment, and it is distinct from the fat pad precorsor tissue, which develops from more deeply located subcutaneous mesenchymal cells [41].

Moreover, it has been shown that more than one phenotype of normal fibroblasts can be distinguished within the stromal compartment of the mammary gland, and each has the potential for various epigenetic effects on normal epithelial cells depending on their proximity to the parenchyma [42]. Intralobular fibroblasts can be distinguished from interlobular fibroblast as they differ in the expression patterns of several proteins such as collagen type XIV and CD13 [43]. Morsing and co-workers conducted a study using fluorescence-activated cell sorting analysis, by which they were able to isolate and characterize two lineages of stromal fibroblasts from human mammary gland, and showed their different impact on the mammary epithelium [44]. Lobular fibroblasts were characterized by high expression of a surface glycoprotein CD105 (which is a part of the TGF beta receptor complex) and low expression of CD26 surface marker, also known as dipeptidyl peptidase-4. In terms of biological properties, $CD105^{high}/CD26^{low}$ lobular fibroblasts resembled mesenchymal stem cells and supported luminal epithelial growth and branching morphogenesis. On the other hand, the second identified fibroblastic cells subpopulation, termed interlobular fibroblasts, showed low expression of CD105 and high expression of CD26 and did not exert such impact on the branching morphogenesis of epithelial progenitors [44]. It has been suggested that the interstitial stroma serves mainly to form a barrier between capillaries and epithelium, across which epitheliotropic stimuli from the blood supply must pass [44].

It is worth noting that contrary to the overall structure of the mammary parenchyma, which is similar among mammalian species being composed of bilayered luminal and basal epithelial cells, the relative abundance of connective tissue is more species-specific. Stroma surrounding the lobules and ducts (intra and interlobular stroma) in mice is sparse, and there is little non-cellular fibrous connective tissue between ducts, whereas the white adipose tissue is abundant. In humans, the ratio of fibrous connective tissue to adipose is opposite, with an abundance of stroma surrounding the alveoli and ducts, predominance of fibrous connective tissue between ducts, and reduced adipose content [14]. Interestingly, in the case of outbred Sprague Dawley female rats, the organization of the mammary stroma is intermediate between mice and humans, and it is thought that its histological pattern is more similar to the one observed in humans than mice. The mammary stroma in cattle is also more fibrous

and contains less adipose tissue than the fatty mouse mammary stroma. The morphology of the bovine mammary gland resembles that of the human breast, because the mammary epithelium is generally closely associated with fibrous connective tissue, which in this case is extensively developed [45].

*4.2.1. Fibroblast-mammary epithelial cell interactions during mammogenesis*

The composition of the mammary stroma largely determines the progression of glandular epithelium development. Attempts to recapitulate human breast epithelial morphogenesis by introducing human MECs into the cleared mammary fat pads of mice were unsuccessful for a long time, due to improper composition of murine stroma comprising mainly adipocytes. Kuperwasser and co-workers used a different approach, creating a model of humanized mouse mammary gland by injecting immortalized human mammary stromal fibroblasts labeled with green fluorescence protein (GFP) into the cleared mice mammary fat pad prior to injection of human breast organoids. Addition of human fibroblasts to the murine fat pad effectively stimulated human MECs proliferation and promoted organization of differentiated acini structures [46]. This experiment pointed to tight stromal-epithelial species affinity [46]. A follow-up study was made, in which human macrophages were also injected. This procedure intensified humanization of the murine fat pad by enhancing fibroblast proliferation and engraftment of the mammary fat pad, thereby providing a larger stromal scaffold for breast epithelial growth and acini formation [47].

*4.2.2. Paracrine factors produced by stromal fibroblasts*

Stromal fibroblasts play a significant role in the development of the mammary gland, not only by creating a complex scaffolding network but also being a source of bioactive compounds. Fibroblasts may also take part in transmission and modulation of signals from superior hypothalamic-pituitary-gonadal axis (HPG). During puberty, mammary fibroblasts surrounding the branching TEBs become activated in response to estrogen and GH released by the ovaries and pituitary gland, respectively [48]. Stromal fibroblasts express growth hormone receptor (GHR) and through secretion of IGF-I may modulate epithelial compartment growth especially in pubertal state [6].

In general, fibroblasts exist in a relatively quiescent state, proliferating slowly and synthesizing only low levels of ECM proteins and matrix metalloproteinases (MMPs) to maintain ECM integrity [48]. During branching morphogenesis, fibroblasts actively synthesize growth factors and proteases. For example, signaling pathways induced by fibroblast growth factors (FGFs) play a major role in the process of mammary placode development [49]. FGFs family contains 18 secreted proteins that can interact with four FGF receptors (FGFRs) having tyrosine kinase activity. These secreted FGFs function as auto- or paracrine factors, but some also show an endocrine function. In addition, there are intracellular FGFs (iFGFs), which are non-signaling proteins serving as cofactors for voltage-gated sodium channels and other molecules [50]. Interaction of FGF ligands with their receptors is regulated by protein or proteoglycan cofactors and by extracellular-binding proteins. The first line of evidence confirming the role of FGF signaling in embryonic stage of mammogenesis came after it was demonstrated that mice

lacking either FGF10 or FGFR2b fail to form mammary placodes 1, 2, 3, and 5 [51]. In mouse embryos lacking *Fgf10* gene, an epithelial sprout derived from placode 4 failed to branch, which completely inhibited the formation of a primitive epithelial network in the neonatal mice after birth [51]. In humans, a birth defect known as Poland syndrome, which is characterized by the underdevelopment of the somite-derived pectoral muscle on one side of the body and a corresponding hypoplasia of the overlying breast on the same side, arises from disruption in FGF10 signaling, because *Fgf10*+/− glands show reduced thickening of the ectoderm along the mammary line and subsequent loss of buds 3 and 5 [6]. Furthermore, secreted FGFs are known to stimulate TEBs promoting luminal epithelial cell expansion, ductal branching, and their differentiation into myoepithelial cells. The majority of FGFs is involved in branching process and involution, both of which require ECM rearrangement. In the case of pregnancy, signals through FGFR2-IIIb are essential to stimulate normal lobuloalveolar development [48]. Recent studies revealed that *Spry2* gene, which encodes an inhibitor of signaling via receptor tyrosine kinases, is essential for regulation of both FGF2-based ductal elongation and FGF10-mediated epithelial invasion during normal mammary gland development. For example, loss of *Spry*2 expression results in increased FGF signaling activities, causing more rapid ductal elongation and epithelial invasion, which leads to accelerated epithelial invasion during pubertal branching. Conversely, a decrease of FGF signaling leads to slower than normal ductal elongation and invasion, resulting in stunted epithelial invasion during postnatal branching of the mammary gland [52]. It was also revealed that basal epithelial cells lacking *Fgfr2* gene did not generate an epithelial network due to failure in luminal differentiation, and *Fgfr2*−/− epithelium was unable to undergo ductal branching initiation, which depends on directional epithelial migration [53]. The results of the abovementioned studies demonstrated that distinct types of FGFs stimulate epithelial cells on different levels. FGF2 controls the ductal elongation process, which depends on cell proliferation and expansion, while FGF10 regulates the branch initiation process depended on directional epithelial migration.

Other fibroblast-derived bioactive compounds like TGF-β1, HGF, or stroma cell-derived factor-1 (SDF-1) also known as CXCL12, were shown to influence mammary parenchyma development in a paracrine manner [54, 55]. HGF is a multi-functional cytokine stimulating invasion, motility, and morphogenesis. Its presence was found in conditioned media from human mammary fibroblasts [56, 57]. Fibroblast-derived conditioned media containing HGF were shown to induce tubulogenesis and branching morphogenesis of TAC-2 mouse mammary epithelial cell line [20]. In addition, it is well documented that fibroblastic HGF mediates the proliferation of estrogen receptor positive (ER+) mammary epithelial cells [43]. HGF was identified as one of the major mediators of this effect, because in in vitro experiments the proliferative activity of MECs cultured in fibroblast-derived conditioned medium was completely abolished by a neutralizing antibody against HGF [41].

Another important growth factor—TGF-β1, secreted by the mammary stroma, acts in an auto/paracrine manner to regulate glandular morphogenesis and remodeling by preventing inappropriate side branching. The presence of TGF-β1 was detected in mature periductal ECM in mice, and it was specifically downregulated at sites where side branches were being initiated [58]. Furthermore, TGF-β1 plays an important role in regulation of growth and activity of fibroblasts. This growth factor functions by signaling to cell surface type II receptors, which recruit

type I receptors, resulting in activation of downstream signaling cascades, including canonical Smad pathways that modulate gene transcription [59]. TGF-β signaling in fibroblasts functions to modulate expression of tissue remodeling factors, including ECM proteins, proteases, and angiogenic factors. During lactation, the expression of TGF-β1 is significantly downregulated, which may prevent TGF-β1 from negatively regulating the expression of milk proteins. Upon the onset of involution, when the gland remodels toward its pre-pregnant state, there is an upregulation of TGF-β1 transcripts. TGF-β1 signaling may further contribute to the remodeling of the involuting gland by inducing ECM production, upregulating MMPs expression, and by recruiting immune cells [14, 19]. Recent studies revealed that TGF-β1 promotes mammary fibroblast proliferation and may cause severe side effect in mammary gland structure and function in dairy cows [60]. TGF-β1 not only affects the development of the epithelial compartment by inhibiting formation and differentiation of mammary ducts and induction of apoptosis. Treatment of bovine mammary fibroblasts with TGF-β1 significantly promoted their proliferation and accelerated the cell cycle. Further research using a mouse model showed that TGF-β1 significantly increased the proportion of fibroblasts and accelerated the cell transition from the G1 to G2/M phases. Thus, TGF-β1 is a cytokine which may also cause negative effect in the mammary gland by contributing to the development of mammary gland fibrosis [60].

#### *4.2.3. Fibroblast-derived extracellular matrix components*

As mentioned earlier, fibroblasts together with other stromal cells synthesize the main amount of ECM components, such as collagens (collagen I, III, and V), proteoglycans, elastin, integrins, and fibronectin; thus, these stromal cells are responsible for mammary tissue architecture and stiffness [5, 48]. ECM can be described as an interconnected meshwork of secreted proteins interacting with cells to form a functional unit [14]. Additionally, mammary gland fibroblasts synthesize many matrix metalloproteinases (MMPs), like MMP2, MMP3, MMP14, that are able to remodel the ECM and release growth factors and cytokines harbored or embedded within the ECM [19]. MMPs consist of a family of over 20 zinc-dependent proteinases synthesized as latent enzymes, in a zymogen form, activated post-translationally and regulated by endogenous inhibitors referred to as tissue inhibitors of metalloproteinases (TIMPs) [5, 56, 57]. MMPs are secreted by stromal cells, but MMP2 and MPP3 exclusively by fibroblasts [61]. MMPs are important for ECM remodeling as well as for the microenvironmental signaling necessary to carry out morphogenic programs within the mammary gland [5]. Increased level of the active MMP3 leads to excessive side branching, and advanced alveolar morphogenesis but as a side effect is responsible for causing production of reactive oxygen species (ROS) leading to genomic instability [5]. MMP3, described also as stromelysin 1 (Str1), is expressed by mammary fibroblasts in vivo at elevated levels in the glands of virgin animals during ductal elongation. The highest level of MMP3 is found around the end buds and rear branch points, where mammary epithelial cells display the highest mitotic activity [57]. Overexpression of another matrix metalloproteinase—MMP14 in the mammary gland was demonstrated to cause excessive side branching and advanced alveolar morphogenesis [56]. The hemopexin domain of MMP14 is important for sorting mammary epithelial cells to points of branching. It has also been shown that only the short intracellular domain of MMP14, which does not contain kinase activity, is needed to resource branching morphogenesis in MMP14-deficient

cells [5]. MMP14 intracellular domain interacts with β1-integrin on the basal surface of cells, and this interaction is required for transducing the extracellular signals needed for epithelial cells to invade [5].

The role of fibroblasts should also be described in the context of the mammary gland remodeling observed extensively during post-lactating involution. Mammary involution is analogous to a wound healing response, involving complex epithelial-stromal cell interactions, degradation of basement membrane driven by protease production originating from fibroblasts. Stromal fibroblasts contain elevated fibronectin, laminins, and higher level of fibrillar collagens to remodel mammary tissue during involution [48]. Fibrillar collagen-epithelial interactions, especially collagen I, III, and V, are crucial during this process [14]. Studies revealed that the epithelial compartment is highly malleable and that cell fate and tissue function are strongly influenced by the stromal compartment of the gland [48].

#### *4.2.4. Different properties of fibroblasts derived from normal and cancerous stroma*

When discussing the role of stromal fibroblasts in mammary gland biology, one needs to mention about epithelial-stromal interactions in the context of breast cancer development. Fibroblasts arising from tumor stroma, described as cancer-associated fibroblasts (CAFs), compared to normal fibroblasts, have acquired distinct properties mainly leading to the promotion of cancer cell proliferation and invasion. CAFs, which are characterized by their high expression of alpha smooth muscle actin, are detected in large numbers in malignant breast cancers and their presence is correlated with poor clinical outcome [62]. Particularly in breast cancer, the progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) is believed to be actively driven by complex interactions with the surrounding microenvironment including interactions with various activated stromal fibroblasts [63]. It is believed that CAFs contribute to cancer cell survival and progression not only through enhanced secretion of cytokines, growth factors, and proteases such as TGFβ1, HGF, SDF-1, and MMP2, respectively, but also by secreting high levels of nutrient-rich ECM, promoting persistent chronic inflammation within the tumor microenvironment and inducing epithelial-to-mesenchymal transition (EMT) of tumor cells [48]. During EMT, downregulation or loss of the epithelial adhesion molecule E-cadherin and upregulation of N-cadherin represent a key step in the acquisition of the phenotype for many tumors. Interestingly, normal fibroblasts induce a strong E-cadherin enhancement even in cancer mammary epithelial cells; thus, these fibroblasts appear to favor the maintenance of the normal tissue architecture [64]. In vitro studies investigating the relationship between mammary carcinoma cells and stromal cells revealed that normal mammary fibroblasts function to suppress tumor progression by negatively regulating expression of oncogenic signaling factors [65]. Furthermore, co-culture of cancerous cells with stromal fibroblasts has been shown to induce significant changes in tumor development and progression [56].

#### *4.2.5. Summary*

Fibroblasts are the principal component of the stromal connective tissue. These cells are responsible for ECM remodeling and secrete FGFs and ECM components, such as collagens,

fibronectin, laminins, elastin, proteoglycans, and MMPs. Due to their properties, fibroblasts support the luminal epithelial growth and branching morphogenesis as well as participate in the mammary gland tissue remodeling during involution (**Figure 2**).

### 4.3. Ivmmune cells

Regulation of the mammary gland morphogenesis also pertains to the involvement of immune cells and the utilization of immune-related signaling molecules [67]. The immune system may contribute to mammary development at each stage via cytokine secretion and recruitment of macrophages, eosinophils, neutrophils, mast cells, and lymphocytes (T and B cells) [48, 66]. The gland is intercalated with extensive vascular and lymphatic networks present throughout the fat pad. During pubertal mammary gland development, the lymphatic network develops in close association with the mammary epithelial tree and blood vasculature. The presence of immune cells within the surrounding stroma was shown to be important for ductal branching as these cells are recruited to the branching tips of the epithelium to mediate invasion into the fat pad [67].

#### *4.3.1. Regulation of immune cells present within the mammary stroma*

Immune microenvironment of the mammary gland is also driven by the hypothalamic-pituitary-gonadal axis. Hormones act directly on epithelial cells and may modulate immune impact on tissue remodeling. Estrogen, progesterone, and prolactin each regulate immune cell functions, which in turn support the morphogenic processes occurring in the pubertal and adult mammary gland [68]. The effects of these hormones on immune cells can be either direct or indirect. The direct effects are mediated when the immune cells express receptors for estrogen, progesterone, and prolactin, which are activated by their respective ligands. The indirect effects of these hormones on immune cells are mediated by paracrine signals derived from MECs and the surrounding stroma [66]. It has been shown that mice lacking the expression of estrogen receptor alpha (ERα) and amphiregulin (a member of EGF family) and showing deficient signaling driven by EGF receptor (ERBB1) fail to develop mature ductal trees and have inhibited recruitment of macrophages and eosinophils to the site of tissue remodeling [66]. In vitro experiments demonstrated that estrogen-stimulated macrophages significantly enhanced fibroblast proliferation and invasion by tumor necrosis factor (TNFα) and MMP9 secretion, thus modifying stromal tissue compartment for epithelial expansion [47].

The profile of immune cells within the microenvironment of the mammary gland varies depending on the changes in hormonal stimuli occurring during the estrus/menstrual cycle. In humans, during the luteal phase of the menstrual cycle, the dominant subpopulation of Th lymphocytes is the Th2 cells secreting IL-4 and IL-10. Increasing concentrations of estrogen increase the abundance of regulatory T cells (Treg) in blood and enhance their immunosuppressive functions [66]. It is speculated that since estrogen and progesterone regulate the number of Treg cells in blood, the abundance of these cells in the mammary gland may also be hormone-dependent and fluctuate over the menstrual cycle. In addition, progesterone during pregnancy and prolactin during lactation were shown to stimulate the recruitment of Th2 cells. These hormones induce MECs to produce Th2-like cytokines, such as IL4, IL5, IL10, and IL13 [69, 70].

*4.3.2. Role of eosinophils in mammary gland morphogenesis*

Eosinophils belong to immune cells found around the growing TEBs. These cells are attracted by eotaxin, another chemokine produced by mammary gland [43]. Eosinophil knockdown mice show altered elongation and branching during mammary gland development as well as insufficient milk productions at the time of lactation [48]. Similar abnormalities can be noted in knockout mice with deficiency of interleukin 5 (IL-5), a cytokine to which eosinophils are particularly responsive [71]. Mammary tissues from IL-5-deficient females had fewer TEBs, less well-branched mammary ducts, and lower overall density of the mammary gland structures. Furthermore, IL-5-deficient pups nursed by IL-5-deficient mothers were notably underweight, with a high percentage of pre-weaning mortality, in contrast to well-developed IL-5-deficient mice which were nursed by IL-5-sufficient foster mothers [71]. Interestingly, overabundance of eosinophils during puberty results in retarded morphogenesis of the mammary epithelium, suggesting the existence of mechanisms controlling the number of these cells that reside in the gland and are involved in MECs expansion during morphogenesis [66]. In addition to eosinophils, mast cells were also shown to be important for normal mammary gland development. Mice deficient in mast cells have defective mammary branching during puberty. It may be associated with the lack of vascular endothelial growth factor (VEGF) released by these cells that assist in mast cell degranulation [48]. Through activation of their serine proteases and degranulation, mast cells are involved in normal branching during puberty, and they accumulate and possibly activate plasma kallikrein, thus activating the plasminogen [5]. Furthermore, it was 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 regaining the pre-pregnant state [14].

*4.3.3. Role of macrophages in mammary gland development and remodeling*

The role of macrophages at different stages of glandular morphogenesis as well as remodeling are better recognized. In the pubertal mammary gland, macrophages are recruited to the highly mitotic terminal end buds from which ducts elongate and branch to give rise to a mature ductal tree [48]. Macrophage colony stimulating factor-1 (CSF1) secreted by myoepithelial cells is a key cytokine that regulates the recruitment, proliferation, and survival of macrophages [48, 72]. Estrogen-regulated CSF1synthesis is essential for expanding of epithelial ducts and buds and alters structural alignment of collagen fibers around the expanding TEBs [70]. Macrophage abundance changes over the estrous cycle, peaking at metestrus and diestrus phases, and being the lowest at proestrus and estrus [66]. Studies on $Csf1^{op/op}$ mice, which are homozygous for a null mutation in *Csf1* gene, revealed that these animals exhibited multiple defects and had reduced macrophage numbers in most tissues including the mammary gland [72]. Depletion of mammary gland macrophages observed in $Csf1^{op/op}$ mice altered the mammary stem/progenitor cell activity, which was reflected in a substantially reduced outgrowth potential of the mammary epithelium. The mammary glands of $Csf1^{op/op}$ mice displayed lower number of TEBs as well as reduced ductal branching and elongation. During pregnancy, $Csf1^{op/op}$ glands developed precocious alveolar units but failed to switch to the lactational state resulting in impaired lactation [72]. These observations prove a continued requirement for normal macrophages during ductal morphogenesis and their stimulatory role on the putative basal progenitor cells. Macrophages also mediate the switch from

pregnancy to lactation through regulation of tight junction permeability. In mice, activation of NF-κB by toll-like receptor 4 (TLR4) signaling pathway increases permeability of the milk-blood barrier [66].

Post-lactating involution, which is analogous to a wound healing response, involves complex stromal-epithelial interactions, activation of elements of both innate and adaptive immune system, as well as stimulation of inflammatory cytokines and proteinases expression. This process is mediated in part through Jak/Stat signaling pathway and is characterized by the apoptotic death of MECs and their removal and engulfing by phagocytic cells: macrophages and epithelial cells by process of efferocytosis [11]. Tissue resident and infiltrating macrophages have special role in that process. Specific depletion of these cells in the involuting mammary gland leads to a reduction in both lysosomal-mediated and apoptotic cell death [73]. Involution is associated with the polarization of macrophages away from proinflammatory (M1) phenotype to an alternatively activated state (M2) [74]. This phenotypic switch is STAT3-dependent and occurs within an infiltrating macrophage population from day 3 of involution [75]. Re-emergence of adipose tissue is an important feature of involution associated with infiltration of macrophages into the gland form [14]. In the mouse mammary gland, gene expression profiling during postlactational tissue regression showed an increase in genes linked to the immune system, which coincides with increasing levels of interleukins: IL-4 and IL-13 acting as macrophage chemoattractants [76]. Furthermore, ECM can fragment into matrikines and matricryptins that also serve as attractants for the peripheral immune cells [14]. Fragments of collagen I, collagen IV, laminins, and nidogen-1 have all been shown to promote chemotaxis of monocytes and neutrophils within the interstitial tissue. Once in the mammary gland, macrophages and neutrophils secrete proteases such as MMP9 and elastase that are involved in further ECM breakdown [73]. Thus, without the influx of macrophages or neutrophils, the remodeling of the mammary tissue during involution, that serves to return the gland to the non-secretory postpartum state, could be delayed or incomplete [14].

ECM fragments not only aid the immune cells infiltration into the mammary gland but also may act as ligands to receptors present on leukocytes residing in the mammary gland. Fragments of biglycan, heparan sulfate, and hyaluronan have been shown to act as ligands for toll-like receptor 4 (TLR4) [14]. Toll-like receptors (TLRs) are part of the pattern recognition receptor family expressed on the cell surface of innate immune cells and dendritic cells. Binding the ligand to its TLR activates the immune cell or induces secretion of cytokines by these cells, resulting in further activation of cells of the adaptive immune system. For example, binding of soluble biglycan TLR 2/4 on macrophages stimulates them to synthesize and release a proinflammatory cytokine interleukin-1β [77]. Other ECM components, such as heparan sulfate and hyaluronan, have been shown to bind to the TLR4 on dendritic cells, causing their maturation [78, 79]. In turn, mature dendritic cells are able to activate cells of the adaptive immune system, which migrate to the site of ECM remodeling [14]. Also the presence of B lymphocytes in involuting mammary gland may be connected with the chemoattractive properties of ECM fragments. In vitro studies revealed that interleukin-4 and fibronectin stimulated B cells motility, and both compounds are known to be upregulated during involution. In fact, the presence of B cells during early to mid involution has been confirmed, prior to the peak in macrophage recruitment [35].

#### *4.3.4. Summary*

The intricate interactions between immune and epithelial cells are an inherent part of the mammary gland physiology. Paracrine factors secreted by Th2 lymphocytes and macrophages (IL-4, IL-10, and TNFα) as well as direct crosstalk between MECs and macrophages, eosinophils, mast cells are involved in regulation of all stages of mammary gland morphogenesis, from early embryogenesis, puberty, through pregnancy, lactation and involution (**Figure 2**).

### 4.4. Vascular endothelial cells

Mammary gland development, occurring during pre- and postnatal life of female mammals, serves to create a highly branched network of ducts and alveoli made of secretory epithelium that actively synthesizes and secretes milk at the time of lactation. To fulfill its function, the mammary gland also requires an expanded network of vascular endothelium. Currently, it is thought that the vasculature not only provides nutrients to the developing and functionally active mammary parenchyma, but also it is important for maintaining homoeostasis of the mammary epithelium.

#### *4.4.1. Development of mammary blood vasculature*

Vasculature in the mammary gland undergoes repeated cycles of expansion and regression concomitantly with the cycles of growth, differentiation, and regression of the mammary epithelium [80]. The development of blood vessels occurs in parallel with mammogenesis. In the course of vascularization, first the process of de novo blood vessel formation takes place in the embryonic life, followed by angiogenesis which serves to form new vessels from pre-existing ones [80]. Angiogenesis is driven in main part by epithelial and stromal cells through secretion of the vascular endothelial growth factor (VEGF) and matrix metalloproteinases, especially MMP-9. Furthermore, studies have shown that development of the vessels in the mammary gland is driven by the same hormones that stimulate growth of the glandular parenchyma, that is the metabolic and sex hormones and the growth factors [81].

Before pregnancy, the mammary vasculature is composed of a thin layer of simple squamous endothelial cells forming a complex vascular network along with myoepithelial cells and connective tissue [82]. The structure of the glandular vasculature has been the best characterized in the mouse mammary gland. It is described as the basket-like capillary beds surrounding the alveoli clusters [83]. The capillary vessels run in parallel or encircle the mammary parenchyma and branch throughout the adipose tissue [82]. In humans, a high number of small capillaries are surrounding the ductal structures, whereas the acini of the lobular structures are interspersed by fewer, but significantly larger capillaries, which are sinusoidal in shape [80]. Such morphology provides a slower blood flow, thus a prolonged contact of the lobuloalveolar epithelium with circulating hormones and nutrients. During pregnancy, the growth of the mammary vessels intensifies along with expanded development of the parenchyma in order to increase the cell number and surface area to provide a maximal interface for nutrient transfer and milk secretion after parturition. Furthermore, increased surface area of the luminal endothelium is also accomplished by formation of microvilli and marginal folds

on individual endothelial cells [82]. Studies on bovine model of mammogenesis showed that the blood volume expands in the pregnant animal, and about 15% of the cardiac output is directed to the fetoplacental unit toward the end of pregnancy, but at parturition most of the blood flow is redirected from the uterus to the mammary glands [81].

Functional differentiation of the mammary gland during lactogenesis is also tightly connected with further changes in morphology and properties of the endothelial cells, which occur in order to support the efficient milk synthesis and secretion. The vasculature of the lactating gland is composed of a well-developed capillary meshwork enveloping the secretory alveoli with basket-like honeycomb structures [84]. The mammary endothelial cells show elevated number of mitochondria supporting their increasing demands for energy during milk production period. A higher number of pinocytotic vesicles is also observed in the endothelial cells, providing efficient transportation of plasma solutes and molecules, such as glucose [85]. In addition, increased capillary permeability occurs during early lactation. Capillaries have thinner walls and are in closer contact with the mammary alveoli, which also aids the enhanced transfer of nutrients and fluids in the functionally active gland [80, 82]. Studies done on rodents have shown that the development of the mammary vasculature, measured as the number of capillaries per individual lobular ductile, surpasses the development of the parenchymal network during lactation [82, 86]. This underlines the important role of the glandular vascular system supporting the optimal function of the mammary gland during the milk production period.

After weaning or termination of milking, when mammary gland involution takes place, the endothelium undergoes regression similarly to the mammary epithelium. Although the mechanisms controlling endothelial regression have not been well recognized so far, it seems that apoptotic cell death at least partially accounts for the remodeling of the vasculature [84]. It is worth noting that the timing of endothelial and epithelial regression is not equal, and MECs apoptosis precedes the death of the endothelial cells [84]. This indicates that the changes in the structure of the mammary gland are initiated in the parenchymal compartment and the altered microenvironment of the gland induces the changes in the vasculature. It is possible that the vascular regression is induced mechanically by disruption of the contact and anchoring between the endothelium and the collapsing mammary epithelial cells. The signals could be mediated by integrins and their cognate intracellular signal transducers, such as members of the Src family and the focal adhesion kinase (FAK); however, further studies are needed to confirm this hypothesis. Djonov and co-workers [84] also suggested that the massive endothelial regression cannot be exclusively due to apoptotic cell death since apoptotic endothelial cells were observed only occasionally in the involuting gland [87]. The authors proposed another mechanism involving regressive remodeling of the endothelium, which they termed angiomeiosis, taken from the Greek words angio (vessel) and meiosis (dwindling, retraction).

#### *4.4.2. Function of endothelial cells in immune response to infections in the mammary gland*

One of the most important functions of the endothelial cells is the ability of these cells to regulate the immune response of the host to protect the mammary gland during pathogen

exposure. This function is especially relevant in regard to bovine mammary gland which is highly prone to infections due to extended period of lactation connected with intensive milk production. Exposure to pathogens initially triggers a response from MECs and resident immune cells which produce and secrete a variety of inflammatory mediators, such as cytokines. These inflammatory mediators also activate the endothelial cells, increasing vascular permeability which is necessary for the influx of neutrophils to ingest pathogens and limit extravascular tissue damage [82]. Endothelial cells produce a variety of vasoactive mediators, such as nitric oxide (NO), prostacyclin ($PGI_2$), endothelin-1, and histamine. At the onset of inflammation, endothelial nitric oxide synthase (eNOS) becomes activated by increased intracellular calcium levels, leading to conversion of arginine to citrulline and NO. Subsequently, NO activates cellular pathways that result in inhibition of calcium influx into the endothelial cells, thus relaxation of the actin cytoskeleton. In addition to NO biosynthesis, constitutive cyclooxygenase-1 (COX-1) is activated by increased intracellular calcium and facilitates the synthesis of $PGI_2$ and oxylipid. By releasing the vasoactive mediators, endothelial cells modulate the vascular tone in order to provide an optimal endothelial surface to facilitate rolling, attachment, and migration of leukocytes that serve to regulate an appropriate immune response to infection [82]. However, during very early stages of infection and inflammation, an opposing process of vasoconstriction is also very important to protect the host's organism in the event of mechanical injury and bleeding. Interestingly, production of vasoconstrictors, such as platelet-activating factor (PAF), by endothelial cells may in turn induce increased production of NO, to prevent sustained vasoconstriction [88]. This suggests that modulation of vascular tone during the initial inflammatory response is tightly regulated to prevent unnecessary damage to blood vessels and interstitial tissue [82].

Endothelial cells, lining the extensive vascular network of the mammary gland, may also contribute to the production of inflammatory mediators, especially IL-1, IL-6, IL-8, and granulocyte colony-stimulating factor (GM-CSF), during inflammation of the mammary gland (mastitis). IL-8 directly stimulates bovine neutrophil migration, phagocytosis, priming, and enzyme degranulation. Both epithelial and endothelial cells contribute to the production of IL-8 during *Escherichia coli* infection. In cows experimentally infected with *E. coli* via injection in the teat canal, MECs showed increased levels of IL-8 mRNA until 24 h post infection, whereas endothelial cells showed increased levels of IL-8 mRNA 24 h after infection, resulting in sustained IL-8 level in tissue [89]. Studies on bovine mammary endothelial cells demonstrated that in early reaction to *E. coli* infection vascular-derived PAF seems to play a prominent role [90]. PAF is a potent phospholipid mediator and endothelial cells work as a target and a source of this molecule. In bovine mammary endothelial cells stimulated in vitro with endotoxin obtained from *E. coli,* PAF biosynthesis began as early as 30 min after the endotoxin challenge and peaked at 1 h following the challenge. The biosynthesis of PAF preceded the endotoxin-induced IL-1β and IL-8 mRNA expression that reached peak expression between 4 and 12 h following stimulation. These results suggest that vascular-derived PAF is an early proinflammatory mediator during pathogen invasion in bovine mammary gland [90]. Therefore, the endothelium enables the progression of a self-limiting inflammatory response to milk-producing tissue through modulation of vascular tone and blood fluidity, vascular permeability, endothelial adhesiveness, and production of inflammatory mediators.

*4.4.3. Lymphatic vasculature in the mammary gland*

When describing the vasculature present within the structure of the mammary gland, one needs to mention also the lymphatic vasculature, which plays a distinct role in the gland's function. Lymphatic vessels serve to return the interstitial protein-rich fluid to the bloodstream, absorb dietary fats and fat-soluble vitamins from the digestive tract, and traffic the immune cells to the site of their physiological destination, as well as at the time of infection [91]. Very little is known about the course of lymphatic vessel formation during mammogenesis. Betterman and co-workers described the process of lymphangiogenesis during the postnatal development of the mouse mammary gland [91]. The authors showed that lymphatic vessels share an intimate spatial association with epithelial ducts and large blood vessels. Lymphatic vessels were observed to encircle epithelial ducts in the mammary glands of virgin and pregnant mice; however, these vessels were not dispersed throughout the stroma and were excluded from alveoli during pregnancy [91]. In contrast, lymphatic vessels in the rat mammary gland were found throughout the interlobular connective tissue and in close association with the alveoli during pregnancy, pointing at substantial interspecies differences [92]. The results of the study performed by Betterman and co-workers [91] have indicated that myoepithelial cells are the source of prolymphangiogenic growth factors, such as VEGF-C and VEGF-D, that drive the expansion of lymphatic vasculature. Interestingly, the lymphatic vessels were not observed in close proximity to alveoli in the pregnant and lactating murine mammary glands. This phenomenon could be caused by insufficient prolymphangiogenic stimuli originating from myoepithelial cells which form a discontinuous sheath around the secretory MECs of the alveoli. Alternatively, the absence of lymphatic vessels could result from repulsive bioactive compounds secreted by the alveolar epithelium [91]. Among the considered molecules showing possible properties of repelling the lymphatic vascular growth is soluble VEGF receptor 2 (sVEGFR-2), which was shown to maintain the lymphatic state of cornea by sequestering endogenous VEGF-C [93].

*4.4.4. Summary*

Mammary vasculature supports three aspects of mammary gland physiology: (1) capillary endothelial cells form a semipermeable barrier that facilitates the exchange of serum compounds to provide oxygen, remove $CO_2$, and transfer solutes and macromolecules for cellular energy metabolism; (2) vascular endothelium provides a high rate of transfer of blood-derived components, such as glucose and amino acids for efficient synthesis of milk; (3) it also plays a significant role in orchestrating host defense to infectious pathogens, which is especially important in extensively active bovine mammary gland producing milk volumes that exceed the nutritional requirements of the offspring. Still, the intricacy of the epithelial-endothelial interactions and their impact on mammary gland development remain largely undiscovered. Further research is needed to gain more knowledge about the role of endothelial cells in the complex interactions between the stromal and epithelial compartments of the mammary gland (**Figure 2**).

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## Conflict of interest

The authors declare no conflict of interest.

## Nomenclature

| | |
|---|---|
| BM | basement membrane |
| CAFs | cancer-associated fibroblasts |
| CSF1 | colony stimulating factor-1 |
| ECM | extracellular matrix |
| EGF | epidermal growth factor |
| EMT | epithelial-to-mesenchymal transition |
| ERBB1 | epidermal growth factor receptor |
| ERα | estrogen receptor alpha |
| FGF | fibroblast growth factor |
| FGFRs | fibroblast growth factor receptors |
| GH | growth hormone |
| GHR | growth hormone receptor |
| HGF | hepatocytes growth factor |
| IGF-I | insulin-like growth factor-I |
| IL | interleukins |
| MECs | mammary epithelial cells |

| | |
|---|---|
| MMPs | matrix metalloproteinases |
| MRUs | mammary repopulating units |
| TDU | terminal ductal units |
| TEBs | terminal end buds |
| TGF-β | transforming growth factor-beta |
| TIMPs | tissue inhibitors of metalloproteinases |
| TLRs | toll-like receptors |
| TNFα | tumor necrosis factor alpha |
| VEGF | vascular endothelial growth factor |
| VEGFR-2 | vascular endothelial growth factor receptor-2 |

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