Pubertal Mammary Gland

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The mammary gland is a compound, branched tubuloalveolar structure and a major characteristic of mammals. The mammary gland has evolved from epidermal apocrine glands, the skin glands as an accessory reproductive organ to support postnatal survival of offspring by producing milk as a source of nutrition. The mammary gland development begins during embryogenesis as a rudimentary structure that grows into an elementary branched ductal tree and is embedded in one end of a larger mammary fat pad at birth. At the onset of ovarian function at puberty, the rudimentary ductal system undergoes dramatic morphogenetic change with ductal elongation and branching. During pregnancy, the alveolar differentiation and tertiary branching are completed, and during lactation, the mature milk-producing glands eventually develop. The early stages of mammary development are hormonal independent, whereas during puberty and pregnancy, mammary gland development is hormonal dependent.

mammary gland development Pubertal

1. Introduction

The mammary gland is a compound, branched tubuloalveolar structure, and a major characteristic of mammals. The mammary gland evolved from the epidermal apocrine gland, the skin glands as a bilateral accessory reproductive organ located on the ventral surface of the body [1]. Mammary glands produce milk as a source of nutrition for supporting the postnatal survival of offspring for reproductive success in all mammals [2]. Morphologically, mammary glands are formed by several different types of cells. The epithelial cells elaborate the ductal network of the gland and maximize the surface area within a constrained volume, whereas a variety of stromal cells or connective tissues with extracellular matrix (ECM) protein supports the mammary glands. The major components of stromal connective tissues are adipocytes, which constitute the mammary fat pad and retain the embedded ductal network; fibroblasts which support the hematopoietic system; vascular endothelial cells which support the blood vessels; a variety of innate immune cells (both macrophages and mast cells); and nerves [3][4]. The fatty stroma is the supportive network for the epithelium bi-layered structure and provides nutrients, blood supply, and immune defenses besides the physical structure to the gland. Importantly, each specific stromal cell secretes instructive signals for specific aspects of the development and function of the epithelium [5]. There are two main types of epithelium in the mammary gland, namely, luminal and basal. The luminal epithelium forms the inner layer of the ducts as a laticiferous duct. It surrounds the hollow lumen that differentiates into the milk-producing secretory alveoli or lobules. In contrast, the basal epithelium consists of myoepithelial cells that form the outer layer of mature mammary ducts. It also harbors stem and progenitor cells, which form both luminal and myoepithelial cells/layer [6]. The epithelium ensheathes by one of the main types of ECM, the basement membrane (BM), which separates the epithelium from the stroma and influences the development of the mammary gland [7]. Thus, BMs

surround three cell types in the mammary gland, namely, the epithelium, the endothelium of the vasculature, and the adipocytes. In males, mammary glands are present as a rudimentary structure and generally nonfunctional form.

2. Journey of Pubertal Mammary Gland

After birth, the rudimentary mammary structure (ductal Anlagen) enters a phase of morphogenetic quiescence. However, the rudimentary mammary structure grows isometrically to the rest of the body and keeps up with normal body growth until puberty. At this stage, the female rudimentary mammary gland development is indistinguishable from the male breast [5]. In males, the onset of puberty androgen-mediated condensation of mesenchyme around the primary ducts results in the elimination of the ducts and the prevention of mammary epithelial growth [8]. In females, the onset of puberty (8–12 years) with the production of steroid hormones (mainly estrogen, E2) from ovaries and growth hormone (GH) from the pituitary along with other systemic factors promote a series of coordinated events that transform the rudimentary mammary structure into the mammary gland. The allometric growth of the mammary gland is due to ductal elongation and secondary branching with both stromal and epithelial growth. This development process is faster than general body growth. At puberty, the increase in breast size is mainly caused by the increased deposition of adipose tissue within the gland. However, progressive elongation and branching of the ducts create a more extensive ductal network [9]. An important aspect of ductal morphogenesis is the patterning, which involves four distinct mechanisms: (a) a bifurcator, which controls endbud splitting; (b) a periodic device, which determines how far apart the branches grow; (c) a restriction collar, which causes the growing epithelium to form a tube rather than a ball; and (d) negative feedback to prevent ducts from colliding [3]. The process of initial specification, formation, and in-growth is called anlage [5]. Thus, the ducts grow into a pre-existing stromal mammary “fat pad”, forming long, thin tubes that are extensively branched. New ducts largely develop from their tips, which are enlarged multicellular structures called “endbuds”. This distinct multilayered bulbous or club-shaped epithelial structure of the mammary gland is known as terminal end buds (TEBs). The tips of the ducts elongate and penetrate further into the fat pad, thus forming the complete branch epithelium tree as the main ductal system. Once the ducts reach the margins of the mammary fat pad, they regress [5][10], leaving behind blunt-ended ductal termini or smaller rounded buds. TEBs are described in primates, including humans and rodents (rats and mice) except ruminants. Ruminants have terminal ductal units (TDU), which direct the elongation and branching of ducts during puberty and resemble a multi-lobular TEB, with each ductule growing from a central chord of epithelial cells typically 4–5 cells thick [11].

Each mammary duct terminates in a single bulbous terminal end bud in the pubertal mouse at 4–5 weeks of age. The ducts develop and spread throughout the fat pad in the adult virgin mouse, and the end buds are entirely lost; although there is enough space present between the branched ducts. In contrast to human mammary glands, the mouse mammary gland has significant fat with small amounts of fibrous connective tissues. Interestingly, mouse mammary ducts contain epithelial cells that surround the lumen of the ducts. Like women, mouse luminal and myoepithelial cells express cytokeratins and actin. The myoepithelial cells form a basal layer as a laminin-containing basement membrane and separate the parenchymal and stromal compartments.

3. Terminal End Buds (TEB)

The TEBs are bulbous and unique to peri-pubertal, highly proliferative, and hormone-dependent structures that grow at the end of growing ducts [12][13][14]. TEBs have two morphologically distinct cellular compartments. The outer compartment (distal surface) is a highly proliferative single-cell layer of “cap cells”, whereas the inner layer of cells surrounds the centrally located “body cells” [15]. The cap cells give rise to basal cells (basal lamina), interact with the surrounding stroma, give rise to the myoepithelial cells enveloping the mature ducts, and are a reservoir for regenerative mammary stem cells [16][17]. Cap cells express several markers for basal lineage, including keratin 5 and 14, smooth muscle actin, p63, and stem cell-specific isoform of SH2-containing inositol 5′-phosphatase (sSHIP) [16]. Body cells from the inner mass of the TEBs give rise to the luminal cells lining the duct’s interior and the presence of ductal and alveolar progenitors [18]. The innermost body cells are incompletely polarized, while body cells adjacent to the basal layer are polarized and form adhering-based adherens junctions. In contrast, cells in the interior are loosely held together with desmosomes [19]. The body cell layer has a high apoptotic index, supporting lumen formation [20]. Alveolar buds are present at the ends of TEBs during pregnancy and are responsible for milk production. Alveolar buds are the precursors of the secretory units called alveoli. Alveoli are a few millimeters in size, lined by one to two layers of cuboidal epithelium, surrounded by the myoepithelial cells, and have well-defined lumina.

During the periodic estrus (rodents) 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 [21][22]. With repeated menstrual cycles, the rudimentary gland transforms into a complex ductal network by bifurcation of the TEBs and secondary side branches that sprout laterally from the trailing ducts until the entire fat pad is filled with a network of branched ducts [5][23]. From the end of the tertiary branches, tubuloalveolar structures start forming. The lobules are relatively quiescent in the first half or follicular phase of the menstrual cycle. At this stage, lobules are small, with few alveoli, and there is low mitotic activity. During the luteal phase (after ovulation), the lobules and alveoli develop with open lumens and the highest mitotic activity [24]. Therefore, at the premenstrual phase, women commonly experience a sense of fullness due to the highest cell proliferation with vacuolization of epithelial cells that lead to edematous stroma [25]. The open architecture of the ductal network depends on the distance maintained from each other and epithelial cells in the ducts. Presumably, distinct mechanisms control the timing of endbud branching during the growth of major ducts and the periodicity of side branch eruption from pre-existing main ducts. Side branches appear intermittently along ducts, particularly during estrus/menstrual cycles and in pregnancy, and are the precursors of alveoli [26].

Ultimately, the development of TBE is regulated by several cell types orchestrated together with mechanical cues and cellular rearrangements, which finally established the pattern of the mammary gland [27]. The morphogenetic changes of the mammary gland during development are tightly regulated by the proliferation of progenitor cells, the differentiation programs, and the maintenance of tissue homeostasis, which controls turnover of cells (apoptosis) through steroid and peptide hormones, growth factors (GFs), receptor tyrosine kinases, extracellular matrix (ECM), and proteases [28].

4. Extracellular Matrix (ECM)

The mechanical properties of ECM support the stromal–epithelial interactions and pattern formation during the morphogenesis of the mammary gland. ECM determines the complex ductal network of TEBs and secondary side branching during ductal morphogenesis [29][30]. ECM is synthesized and secreted by epithelial, myoepithelial, endothelial, immune cells, fibroblasts, and adipocytes. ECM interacts through numerous weak non-covalent bonds and crosslinking via covalent bonds for scaffold organization, tensional characteristics, and stabilization [31]. ECM accumulates at the cleft of endbuds (bifurcation) and supports a wedge to split growth into a new direction. ECM density (stiffness) influences epithelial cell fate decisions through cell adhesion, survival or death, polarity, proliferation, differentiation, and lineage of stem cell progenitors [32].

Biochemical Composition of ECM

ECM is thin, ~100-nm thick sheets of glycoproteins and proteoglycans. The glycoproteins and proteoglycans are polymers of laminins (LM) and a cross-linked network of collagen IV fibrils [33]. LM is heterotrimeric (αβγ trimers) glycoprotein (~900 kDa) with 15 different combinations that are derived from five a, three β and three γ subunits, and coded from distinct genes [34]. In the mammary gland, four distinct LM isoforms [laminin-111 (LM-1; α1, β1, γ1), -322 (LM-5; α3, β3, γ2), -511 (LM-10) and -521 (LM-11)] are present [35][36]. Similarly, BM proteoglycans are complex glycosaminoglycans (GAG) chains that vary with developmental stages of the mammary gland [37]. However, the major component of BM is perlecans. BM interacts with mammary–epithelial cells (MEC) through integrins and transmembrane (TM) proteoglycans, dystroglycan, and syndecan, and is joined to the cytoskeleton for the signaling platform, and controls cell fate [38][39]. Epithelial cells receive instructive signals from the ECM through different receptors, including β1-integrin (collagen receptor) and non-integrin receptors discoidin domain receptor 1 (DDR1, recognize multiple ECM proteins) [40], dystroglycan, and syndecan [41]. Integrins are TM receptors and are well studied in MEC. Structurally, integrins are composed of 18α and 8β subunits as a heterodimer and form 24 canonical integrin receptors [42][43]. These canonical integrin receptors are for collagen (α1β1 and α2β1), LM-111, -511, -521 (α3β1, α6β1, and α6β4), LM-322 (α3β1 and α6β4), MEC fibronectin, and vitronectin (α5β1 and β3 integrins) [44]. The terms BM and basal lamina have been used interchangeably to refer to the highly specialized ECM, which organizes 20–100 nm thick structure directly underlying the epithelium [31]. The other major constituents of the basal lamina are LM, collagen IV, nidogens, and perlecan which act as adhesive contacts in epithelial cells. Collagen IV is a heterotrimer formed from six genetically distinct chains. Collagen IV provides an anchor for mammary epithelial cell viability. Nidogen (entactin) is a sulfated glycoprotein (150 kDa), synthesized by fibroblast, and helps in the formation of the basal lamina. Perlecan is a proteoglycan that consists of a core protein (470 kDa) with covalently linked heparin sulfate, a specific class of GAG, and increases the molecular weight of perlecan over 800 kDa [45]. Perlecan provides instructive cues and is dependent on the context and structural integrity of ECM. Therefore, ECM serves as a major reservoir for GFs and cytokines and alters according to tensional requirements and organization, which change throughout to generate the open architecture of the mammary gland development [27].

5. Stroma

The mammary stroma supports proper ductal elongation and branching morphogenesis. The stroma is adjacent to the basal lamina (BM), a 10–100 μM thick band of an organized collagen-rich structure known as the intralobular stroma. The stroma surrounds individual alveoli or endbuds, which remodel as the cells collectively invade the intralobular stroma [31]. Bands of stroma surrounds a cluster of alveoli that forms a single lobule, referred to as the interlobular stroma. The mammary stromal cell type includes adipocytes, fibroblasts, macrophages, eosinophils, neutrophils, and endothelial cells, primarily solitary and embedded within the fibrous ECM [46]. Thus, the stromal ECM influences cell-matrix interactions within the mammary epithelium and controls ductal development and alveolar functions. Stromal ECM components include fibrillar collagens, proteoglycans, hyaluronic acid, fibronectin (FN), and tenacins. However, the composition varies with the developmental stages of the mammary gland during pregnancy [47]. During duct formation and alveolar expansion, the dominant structural components are fibrillar collagens type I, III, and IV with other ECM, which regulate intra- and inter-lobular stroma composition and functions [31]. The TEBs directly contact stromal cells during ductal elongation.

FN is a dimeric glycoprotein (~500 kDa) encoded by a highly conserved gene and supports regulatory protein for branching ductal outgrowth and increases the formation of TEBs. The fibronectin fibrils accumulate at the clefts between new buds and support branching [48][49].

There are several ECM proteins, including tenascins (TN), secreted protein acidic and rich in cysteine (SPARC/osteonectin), small leucine-rich proteoglycan (SLRP), decorin, and biglycan, along with fibrous connective tissues and elastic fibers that support the cleft formation between new buds and branching [31]. SPARC is a 32 kDa glycoprotein, secretes in ECM, and supports cell–matrix interactions through FN assembly and integrin-linked kinase activity [50][51]. SLRP is an N-terminal cysteine-rich motif with a tandem leucine-rich repeats (LRR) core protein attached with one or more GAG chains. SLRPs bind to cell surface receptors and GFs and support signal transduction pathways [52]. Decorin has a core protein (~38 kDa) linked covalently with single chondroitin sulfate (CS) or dermatan sulfate (DS) chain resulting in 90–140 kDa secreted protein and is expressed in fibroblasts and astrocytes. Decorin has a pivotal role in the spatial alignment of stromal collagen fibers and inhibits EGFR signaling by initiating EGFR internalization and degradation by caveolar endocytosis [53]. Biglycan is a member of the SLRP class I family with a core protein (~38 kDa) and covalently attached to two GAG chains (chondroitin sulfate and/or dermatan sulfate) with an overall MW of 150–240 kDa [54]. Decorin and biglycan have distinct, non-overlapping roles [55]. Biglycan can induce elastic fiber-associated protein fibrillin-1 expressions that subsequently retain elastic properties of the tissue [56].

Other structurally distinct stromal components are acellular fibrous connective tissue, found in large volumes in both rodents and the human mammary gland. These tissues are characterized by the presence of elastic fibers, fibroblasts, immune cells, and high fibrillar collagen content except the epithelium [31]. The fibrous connective tissue is separated from the intra- and inter-lobular stroma and shares ECM proteins, including fibrillar collagens, FN, TN, SLRPs, and SPARC. Elastic fibers comprise a 31 kDa secreted protein called microfibril-associated glycoprotein (MAGP) and a 350 kDa fibrillin glycoprotein assembled into 10–12-nm microfibril that provides elasticity structural support to the mammary gland [57]. These microfibrils are associated with other elastic fibers, including elastin, fibulins, and proteoglycans, forming MAGP/fibrillin microfibrils that align parallel to fibroblasts. Ultimately,

microfibrils support tropoelastin deposition (70 kDa) as a soluble precursor of elastin. Tropoelastin is rich in hydrophobic amino acids and contains a low amount of polar amino acids, including several lysine derivatives. The lysine derivatives are imperative for covalent crosslinking between monomeric elastin via lysyl oxidase (LOX) [31][57]. An additional constituent of elastic fibers is the 50–200 kDa fibulin family protein. Fibulin proteins have calcium-binding sites and consist of I, II, III domains with EGF-like fragments in their central segment. Fibulin 5, a 66 kDa, has a strong calcium-dependent binding to tropoelastin and weak binding to the carboxyl-terminal of fibrillin 1. The proteoglycans, mainly biglycan and decorin, have been implicated in elastogenesis through binding to tropoelastin, fibrillin-1, and MAGP.

5.1. Glycosaminoglycans (GAG)

Various classes of GAGs are associated with a distinct role in ductal elongation [31]. GAGs are anionic linear polysaccharides with repeating hexuronic acid. The hexosamine binds to GFs and cytokines in ECM [31]. GAG act as “glue” that allows GFs to “stick” to the basal lamina and fibrillar ECM proteins. Electron microscopy has shown that the BM that surrounds the tips of end buds in mice are thin and rich in hyaluronic acid, while the BM along the flank of end buds is thicker and associated with sulfated GAGs [58]. Release and activation of GFs and cytokines from ECM can alter matrix stiffness and induce ECM proteolysis [59][60].

5.2. Actin and Tubulin

The network of actin and tubulin supports cytoskeletal polymerization at the peripheral end buds and promotes protrusive force in ducts [61][62]. Actin is a ~42-kDa globular protein with three isoforms (α, β, and γ); whereas tubulin is a ~55 kDa globular protein with two isoforms (α- and β-). Both actin and tubulin support the trafficking of integrins through early endosomes and the formation of new matrix adhesions and controls cell migration [63].

5.3. Lysyl Oxidase (LOX)

LOX, a copper-dependent enzyme, catalyzes intra- and inter-molecular crosslinkers of fibrillar collagens and elastic fibers through oxidative deamination of lysine residues [31][64]. LOX activity promotes mammary tissue stiffness, fibrosis, and modulates elastic properties of elastic fibers to establish tissue tension [31][64]. 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 is supported by catalyzing covalent bonds between glutamine and ε-amino groups of lysine residues [65].

5.4. Cadherins

Cadherins are calcium-dependent type-1 transmembrane adhesion proteins. Epithelia are strongly cohesive via cadherin contacts, whereas individual cells that migrate into the stroma are deleted by apoptosis via matrix interaction changes from BM to collagen [66]. The spatial orientation of luminal and myoepithelial cells is controlled by differential adhesivity among the cells. Both luminal and myoepithelial cells express the desmosomal cadherins (Dsg2/Dsc2), whereas the myoepithelial cells express desmosomal cadherins Dsg3/Dsc3. The luminal cells are

intrinsically more adhesive and thereby restrict the myoepithelial cells to more external and basal locations as an organization. The adhesive properties are inhibited without the Dsg3/Dsc3 function [67]. The BM matrix supports the bilayered organization along with laminin-111 produced by myoepithelial cells. Interestingly, the myoepithelial cells contain hemidesmosomes, which rivet the cells to BM [68][69].

5.5. Integrins

Integrins are receptors that support and coordinate cell signaling through the ECM. Integrin-associated scaffold proteins, including filamin in mammary tissue, detect mechanical cues within the ECM, regulate cell shape, motility, and cell cycle, and control morphogenesis [70][71]. β1-integrins are required to maintain mammary stem cells and help mammary ductal cells to proliferate endbuds [68][72]. The β1-integrins activation influences the expression of FGF receptors (FGFR) [73]. In contrast, FGFR is required for LM-a5 expression and supports a positive feedback loop for the maintenance of FGFR [73]. The BM proteoglycans regulate the delivery of GFs and cytokines and act as a reservoir for GF receptors to control the transfer of GFs across the BM and epithelium [74]. Integrins also regulate c-met (MET or MNNG HOS Transforming gene) signaling by sensing laminin molecules within the BM and allow MET signaling to control morphogenesis [75]. Netrin-1, a laminin-related protein, helps in mammary morphogenesis by controlling cell survival and migration of cells.

5.6. Adipocytes and Fibroblasts

Adipocytes are the largest population of cells within the fat pad in the mammary gland. In addition to ECM, adipocytes provide the structural environment for the epithelium branching and function and physical support to the immune, lymphatic, and vascular systems. In women, the fibrous connective tissue ratio to adipose tissue is inversed [31]. There is a predominance of fibrous connective tissue between ducts and decreased adipose content [31]. During puberty, mammary adipocytes support formation and branching of TEBs. Adipocytes secrete adipokines, promote the expression of ERα, IGF1, and HGF within the stroma, and promote secondary and tertiary mammary duct branching. At puberty, adipocytes synthesize and secrete vascular endothelial growth factors (VEGF) as an inducer of vascular growth and support mammary branching [1].

Fibroblasts are spindle-shaped cells distributed around the TEBs. Fibroblasts are biosynthetically quiescent. Fibroblasts secrete ECM macromolecules, proteolytic enzymes, fibroblast growth factors (FGFs), hepatocyte growth factors (HGFs), insulin-like growth factors (IGF-1), cytokines, and chemokines under the influence of estrogen (E2), growth hormones, and extracellular cues to support contraction of connective tissue. Moreover, fibroblasts maintain ECM synthesis and degradation by producing laminin, collagen, fibronectin, proteoglycans, MMPs, and TIMPS [76].

5.7. Macrophages and Eosinophils

In the stroma, macrophages are localized to collagen fibers and support the synthesis of long collagen fibers in ECM around the neck region of the TEBs and promote ductal elongation and branching [77]. Macrophages are recruited by producing colony-stimulating factor 1 (CSF1) by epithelial cells around the neck region of TEBs.

Macrophages are also present within the body cell layer and support lumen formation by clearing apoptotic cells via phagocytosis [77][78]. Interestingly, macrophages in the stromal compartment of the mammary gland maintain epithelial stem cells as epithelial progenitor cells in an undifferentiated state and maintain TEBs numbers [79]. Mast cells, which are effectors of the innate immune system, also surround the TEBs during puberty [80][81] and induce branching by secreting serine proteases [81][82].

Eosinophils are recruited by the secretion of eotaxin by TEBs and surround the TEBs [76][78]. E2 and P4 dependent amphiregulin secretion by TEBs promotes eotaxin secretion and promotes branching [76][78][83]. Eosinophils secrete different cytokines and growth factors, including VEGF [84]. Ultimately, both macrophages and eosinophils spread throughout TEBs and support pubertal remodeling of the mammary gland through cellular renewal and formation of lumen of the ducts.

6. Basement Membrane (BM)

The cap and myoepithelial cells deposit the BM during ductal elongation, which results in both polarization of the luminal layer and geometric confinement of the subtending duct. Laminin-1 is expressed by myoepithelial cells and supports the polarization of luminal cells [58][85][86]. The tip of TEB covers in hyaluronic acid and laminin. In contrast, BM in the neck of TEB is a meshwork of collagen IV, laminin-1 and 5, and heparan sulfate proteoglycan [30].

7. Role of Hormones and Growth Factors in Regulation of Pubertal Mammary Gland

The morphogenesis of the female rudimentary mammary gland up to puberty is a hormone-independent process. Although various hormone receptors are expressed before puberty, the fetus is exposed to high maternal and placental hormones [1][87][88]. At the beginning of puberty, the rudimentary mammary epithelium has asymmetric branched geometry and patterning information encoded in the pre-existing non-spherical structure. The onset of puberty, the ductal elongation and branching of the mammary gland are regulated by many hypothalamic–pituitary–ovarian hormones [5][89][90]. During pubertal mammary development, various hormonal signals are at nano- or pico-molar concentration, amplifying through temporal and spatial autocrine-paracrine signaling molecules and transcriptional coactivators that express and diffuse as a morphogen differentially in TEBs and integrated between the epithelium and stroma. Ultimately, these signaling molecules trigger TEBs proliferation and bifurcation in a controlled manner [27].

7.1. Estrogen (E2)

Estrogen is the critical regulator of branching in the pubertal mammary gland. E2, the female sex steroid hormone, is synthesized and secreted primarily by granulosa cells of developing follicles in the ovary during the estrous and menstrual cycle. E2 is also synthesized locally by adipose tissue in the mammary gland. E2 signaling is mediated by two receptors, ERα and ERβ, in 30% of mammary luminal epithelium, while basal cells do not express ER-receptors. Both humans and rodents have ERα and ERβ have a similar sequence homology and binding affinity for

E2. Studies have demonstrated that loss of ERα signaling causes a deleterious mammary phenotype and impaired function, whereas ERβ loss does not. The multiple regulatory elements regulate the ERα gene (ESR1) expression, including transcription factors, chromatin environment, autocrine, paracrine, and endocrine secreted factors and multiple environmental factors (cell–cell and matrix interactions, mechanical forces) [91]. The ERα receptor has six structural domains along with two sub-domains, namely, a ligand-independent (AF-1) and a ligand-dependent (AF-2) subdomain. The AF-1 domain is transactivated and phosphorylated at serine 104/106, 118, or 167 by kinases in response to growth factors including epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), tumor growth factor (TGFα).

The presence of ERα in the stroma is not required for mammary gland development [92]. In contrast, ERα is required for TEBs and ducts development inthe fat pad [92], including prepubertal growth, alveologenesis, and lactation during late pregnancy [93]. Ultimately, E2 through ER supports TEBs proliferation with ductal elongation and clefting (bifurcation) of the ducts to generate branches. E2 is a dominant regulator of epithelial cell proliferation. However, E2 has synergistic effects on epithelial and stromal cells along with local growth factors. E2, upon binding with ERα in luminal cells, promotes multiple signaling pathways including genomic action through binding to DNA directly or indirectly (tethering) by physically interacting with other transcription factors including stimulating protein 1 (SP1), activator protein 1 (AP-1), signal transducer, and activator of transcription 3 (STAT3) and Nuclear factor–κβ (NF-κβ), and by a generation of second-messenger molecules (cAMP-dependent signal transduction pathway in the cytoplasm. The nongenomic action of ER-signaling includes crosstalk with growth factor receptors and G-protein coupled receptors in the cytosol. ERα can activate Src-kinase, leading to epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3-kinase signaling. E2 through ERα in luminal cells promote amphiregulin (Areg) expression as a transmembrane precursor and are cleaved by ADAM17 to stromal cells. Areg signals as a paracrine factor to the stroma through EGFR and promotes additional growth factors, including stromal FGF [94]. FGF binds with epithelial FGFR2 and promotes epithelial cell proliferation. E2 also supports FN expression in the mammary gland [95]. FN protein level is increased up to three folds in the mammary epithelium in pre-puberty and sexual maturity [95]. Moreover, mouse models revealed that the transcriptional activity of ERα depends on its interaction with coregulators. In this process, GATA3 regulates FOXA1, which in turn regulates ERα, while GATA-3 and ERα regulate each other positively in the mammary epithelium, the balance between the basal and the luminal lineages and altered mammary development [91].

The mammary fat pad is highly vascularized during lactogenesis and lactation for transporting fluids and nutrients. Interestingly, the VEGF promoter contains an E2 response element [96] that permits transcription of the VEGF gene in cells expressing ERα upon binding of E2 [97]. Thus, during puberty, ovarian E2 also induces communication between endothelial cells within blood vessels, epithelium, and adipocytes.

7.2. Growth Hormone (GH) and Insulin like Growth Factor-1 (IGF-1)

Growth Hormone (GH, somatotropin) is a single chain polypeptide (191-amino acid) that promotes the development of TEBs and ductal branching [98]. GH is synthesized, stored, and secreted by somatotropic cells of

the anterior pituitary gland. GH acts on mammary development through GH-receptor (GHR). GHR homodimerizes upon GH binding and activates the cytoplasmic tyrosine kinase, JAK2 [99][100]. Subsequently, JAK2 activates three major signaling systems in response to GH, namely, transducers and activators of transcription (STATs, mainly STAT5b), phosphatidylinositol 3-kinase (PI3K), and extracellular signal-regulated kinase (ERK) [101][102][103]. STAT5b activation triggers the transcriptional activity of GH targeted genes [102]. Several molecular signaling pathways coupling GH to ERK are activated, including the SHC-Grb2-Sos-Ras-Raf pathway, EGFR, insulin receptor substrate-1 (IRS-1), Gab-1 tyrosine phosphorylation, and SHP-2 tyrosine phosphatase activity (Frank, 2008). ERK is critical for GH-induced c-fos transcriptional regulation, promoting proliferation and crosstalk with EGF signaling [102].

IGF-I plays an essential role for the GH/IGF axis as a paracrine factor for the growth and development of the mammary gland. In response to GH, IGF-I is mainly synthesized in the liver and acts on the mammary fat pad, although IGF-I is synthesized in mammary stromal fibroblasts. IGF-I acts through IGF-IR, which are predominantly epithelium cells. The complete action of GH on mammary development is mediated by IGF-I [89]. Interestingly, both E2 and P are dependent upon IGF-I for their actions, as with several other growth factors [89]. E2 enhances the action of IGF-I through a stromal epithelial interaction and pubertal mammary development. The IGF-I-PKB axis is an important survival path for the proliferation and differentiation of TEBs [104]. Thus GH, IGF-I, ovarian E2, and P4 and their respective receptors are important in the post-pubertal branching morphogenesis of TEBs.

7.3. Wnt, Hedgehog (Hh), and Fibroblast Growth Factor (FGF) Signaling

Wnt and Hh signaling are coordinated by primary cilia present on mammary epithelial cells during puberty and epithelial plasticity. Hh signaling is involved in tissue homeostasis, regeneration, and stem cell maintenance of the pubertal mammary gland [105]. Upon Hh activation, the primary cilia serve as the processing sites for Gli transcription factors. They are involved in a multi-protein complex consisting of a subset of intraflagellar transporter proteins, protein kinase A (PKA), glycogen synthase kinase 3β (GSK3β), casein kinase (CK), etc. [106][107]. Studies have shown that the suppressor of fused (Sufu) plays the role of a negative regulator of Hh/Gli signaling [108].

Both canonical and non-canonical Wnt pathways are activated during mammary cell fate determination, maintenance of mammary progenitor cell populations, side branching morphogenesis, and alveogenesis. The ligands (Wnt4 and Wnt5a) and non-canonical receptors are localized to the luminal compartment, whereas Wnt6 is localized to the basal layer. Primary cilia are regulated by FGFs in epithelial tissues and interact with growth factors (GFs), WNT, and Hh signaling during mammary morphogenesis. Another component of Wnt signaling is a transcription factor LEF1, requiring the development of mammary rudiments [109]. GH and IGF1 induce local Wnt expression and Frizzled family of Wnt receptors in the TEBs to support the proliferation of the mammary gland. Interestingly, the serine/threonine phosphorylation of the N-terminal domain of β-catenin by GSK3β and CK1 kinases play a significant role in the compartmentalization of β-catenin [31]. In the normal mammary gland, the majority of β-catenin localizes to cell–cell adherent junctions through association with cadherins. The cadherin/catenin complexes are critical to mammary integrity. The cytosolic and nuclear β-catenin transduces

signals from multiple pathways into cell-context-specific gene expressions essential for mammary development [110]. However, the detailed Wnt signaling pathway is not well studied in pubertal TEBs development.

The FGF1, FGF2, FGF4, FGF7, and FGF10 are highly expressed during mammary ductal elongation [49][111]. FGF7 has an inhibitory role in branching and inverse relation with TGFα signaling [49]. FGF10 produces by adipose tissues. FGF10 is localized in mesenchyme near ducts and alveoli and acts on TEBs epithelium through FGFR2b in a paracrine manner. Recent studies suggest that FGF and its receptors maintain the basal compartment for regeneration [112][113].

7.4. Epidermal Growth Factors Signaling

Epidermal growth factors (EGF) and epidermal growth factor receptors (EGFRs) act as an integrated paracrine factor in the epithelium and stroma during pubertal ductal growth and branching of TEBs. EGF family ligands are expressed as transmembrane precursors, cleave enzymatically (ADAM and MMPs), and bind with the receptors. Subsequently, receptors dimerized and activated intracellular kinases through phosphorylation. The ligand neuregulin (NRG1–4) and ErbB receptors (EGFR/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4) contribute to the proliferation and survival of mammary epithelial cells. ErbB2/HER2 and ErbB3/HER3 are highly expressed in the epithelium, but EGFR or ErbB4/HER4 is required only in the stroma . Interestingly, EGFR and ErbB2 appear most prominently involved in ductal morphogenesis in puberty. Activating the tyrosine kinase in ErbB receptors results in the phosphorylation of multiple tyrosine residues within their intracellular domains (210). Phosphorylation at ten tyrosine residues of EGFR leads to the recruitment of many docking and signaling proteins, including Grb-2, SHC, PTP-1B, SHP-1, SHP-2, Cbl, and PLC-γ that influences several downstream signaling pathways. Moreover, cholinergic stimulation through parasympathetic innervation triggers epithelial cells to release heparin-binding EGF and promotes branching morphogenesis by EGFR. Ultimately, downstream signaling pathways of ErbB receptors regulate gene expression and cell behavior. The ductal development proceeds only when EGFR phosphorylation occurs through the transmembrane zinc-dependent cell surface disintegrin and metalloproteinase domain-containing protein 17 (ADAM17, TNFa converting enzyme, TACE) with AREG expression on mammary epithelial cells and EGFR expression in stromal cells. ADAM17 is a family of zinc-dependent cell-surface enzymes and is responsible for releasing all EGFR ligands, including epithelial AREG. AREG activates stromal EGFR, elicits reciprocal response to orchestrate mammary epithelial development [114], and acts as a paracrine regulator of E2-induce ductal morphogenesis. AREG is also present during post-pubertal mammary development. AREG transcripts are substantially enriched in TEBs, and ducts' growth compared to the epithelium-free stroma. Thus, ADAM17–EGFR axis acts as an essential paracrine pathway in mammary gland development.

AREG activity downstream of the EGFR family triggers intracellular Ras/mitogen-activated protein kinase (MAPK) and PI3-K/Akt signaling pathways for its mitogenic action [115]. The branching or morphogenetic activity of MAPK is downstream of TNFα and EGFR, whereas transient proliferation action of MAPK is downstream of FGF7 and FGFR2 [49]. The mammary may use the temporal responses to integrate and interpret distinct signals [27]. Several other receptor tyrosine kinases have profound effects on pubertal mammary development, including Ron receptor

tyrosine kinase (RON, also known as MSPR) [116], ephrin type-A receptor 2 [117], and fibroblast growth factor receptors (FGFRs).

7.5. Hormonal Regulation of Transcription Factors

Various hormones and growth factors regulate gene expression through receptors ligand-activated transcription factors and converting the hormonal and growth factors stimulus into a transcription response. Multiple transcription factors are involved in the process of mammary ductal morphogenesis, including AP-2γ (TFAP2G/TFAP2C), Gata-3, E-cadherin repressors Snail, Slug, and Twist, six families of homeodomain transcription factors, Sim2, CCAAT/enhancer-binding protein, C/EBPβ, Lef1, and DNA-binding protein inhibitor Id1. AP-2gamma (AP-2γ) is expressed in the cap cell layer and a subset of body cells of TEBs and regulates genetic processes downstream of ovarian hormones. Gata-3 is a GATA family member and contains two GATA-type zinc fingers that regulate T cell development supports endothelial cells and ductal outgrowth. Gata-3 binds directly to the promoter of the forkhead transcription factor FOXA1, which is required to bind ERa to chromatin and supports E2 signaling in the mammary gland. FOXA1 mediates crosstalk between GATA3 and ERα signaling [4].

The E-cadherin repressors Snail and Slug, and the basic helix–loop–helix transcription factor Twist, are critical regulators of mammary gland development. A significant upregulation of the Snail and Twist transcripts in TEBs compared to mature ducts suggests that EMT regulators may regulate epithelial plasticity in the mammary gland. Snail and Slug also regulate tight junction stability , gap junctional protein expression, desmosome disassembly, and protease expressions. Indeed, Snail and Slug are expressed in response to numerous EMT stimuli, including FGF and Wnt signaling, Lbx1, TGF-β signaling, loss of Sim2 expression, and hypoxia. Similarly, Twist represses E-cadherin, induces mesenchymal gene expression, and initiates invasion. Like Snail and Slug, Twist targets numerous EMT-inducing stimuli, including hypoxia and Msx2. The convergence of multiple EMT pathways on Snail, Slug, and Twist are critical nodes in the networking of EMT signaling.

The Six families of homeodomain transcription factors, mainly, Six1, Six2, and Six4, regulate EMT. Six1 is commonly expressed in the embryonic, neonatal, and pubertal mammary glands; thereafter, its expression significantly decreased. However, the functions of the Six family proteins in mammary gland development remain unclear.

Sim2 is a basic helix loop helix/Per-Amt-Sim (bHLH/PAS) transcription factor repressor. Sim2 is involved in mammary gland development by regulating epithelial plasticity, duct hollowing, and apicobasal polarity.

The CCAAT/enhancer-binding protein, C/EBPβ, has a critical function in promoting proper lobuloalveolar development in the mammary gland. These factors are necessary for the proper development of ductal trees in the virgin mammary gland and subsequent development during pregnancy.

7.6. Matrix Metalloproteinases (MMPs)

Matrix remodeling is required for cells to sprout from the main ducts and form branches for endbud progression In addition to GF receptors, MMPs as epithelial and stromal proteases are expressed differentially in mammary tissues as a local regulator of mammary branching by creating a path for TEBs in the surrounding ECM and fat pad. MMP2 (gelatinase A) expresses in the epithelia near lateral branching and promotes cell survival. In contrast, MMP3 (stromelysin 1) expresses throughout the stroma and induces the local degradation of BM collagen IV and laminin at the site of lateral branching. Interestingly, MMP1 is present in both stroma and epithelial cells. MMP2 cleaves laminin-332 (LN332) fragments from the γ2 chain, which binds to the EGFR. LN332 is an essential fragment in the mammary gland during mammary morphogenesis, which promotes proliferation by activating integrin signaling directly.

MMP9 expresses homogeneously with low levels in both the epithelium and the stroma. MMP9 has an inhibitory role in pubertal mammary development. MMP14 expresses in and around the TEBs. An inhibitor of MMP14, the tissue inhibitor of metalloproteinases 3 (TIMP3), is downregulated in and around the TEBs; whereas TIMP1 does not inhibit MMP14 being upregulated at these sites. There is a crosstalk between MMP14 and GFs during mammary development through cell motility. A high level of MMP14 signaling through the CD44 surface receptor and the RHO pathway promotes motility and directional persistence of migrating cells.

7.7. Transforming Growth Factor β (TGFβ)

Tissue geometry and side branches are regulated through morphogenesis created by the autocrine–paracrine signaling morphogen and coordinated events of ECM turnover. TGFβ is a ubiquitously expressed cytokine and determines the mammary gland’s tissue geometry as a morphogen. TGFβ (TGFβ1, TGFβ2, and TGFβ3) is secreted within epithelium as a large latent complex (LLC) in all the stages of the mammary development. TGFβ is associated with fibrillar ECM proteins deposition and inhibition of proliferation. TGFβ1 and TGFβ3 have an overlapping expression pattern in the epithelium during ductal elongation. TGFβ3 is only expressed within cap cell layers of TEBs. TGFβ2 expresses very low levels in TEBs and is upregulated during pregnancy. TGFβ is activated by proteolytic cleavage of the latency-associated peptide (LAP) through the conformational change in LAP through binding to the αvβ6 integrin receptor or by MMP-9 thrombospondin or plasmin. TGFβ activity is dependent on fibrillar ECM proteolysis or altered matrix stiffness. The mechanical tension is also involved in the release of TGFβ from the ECM. The secreted TGFβ binds to target cells through TGFβ receptor type I/II and initiates multiple signaling cascades, including the canonical Smad signaling pathway, promoting branch initiation and maintaining proper ductal spacing within the duct-ensheathing ECM. During puberty, high levels of E2 inhibit TGFβ expression, and low levels of TGFβ stimulate branching morphogenesis, whereas high levels of TGFβ inhibit ductal growth. Importantly, Wnt5a is an essential mediator of TGFβ, suggesting that low thresholds of β-catenin signaling are maintained during pubertal ductal morphogenesis through TGFβ and Wnt5a antagonism. Highly localized expression of TGFβ in the TEBs control bifurcation by increasing ECM deposition within the cleft. TGFβ provides additional support for mechanical tension regulating ECM function through fibroblast traction force that leads to large-scale directional patterning of fibrillar ECM proteins, including collagen I.

7.8. Axonal Guidance Molecules

Several other signaling molecules are expressed during mammary gland development and support tissues morphogenesis and TEBs elongation and branching similar to axonal growth and migrations. These molecules include SLIT2 and its receptor ROBO, Netrin1/Neogenin, brain acid-soluble protein 1 (BASP), small proline-rich protein 1A (sprr1A), and semaphoring 3B, which are expressed differentially in cap cells of TEBs.

8. Pattern Formation during Pubertal Mammary Morphogenesis

Mammary morphogenesis is regulated through epithelial–mesenchymal transition (EMT), and mesenchymal–epithelial transition (MET) generates mechanical stresses by individual cells. EMT and MET transmit and concentrate into stress gradients spanning many cell lengths and ultimately affect the cell behavior (proliferation and apoptosis) pattern and state (differentiation) near to the future branch sites from quiescent ducts [27]. Studies using microfabrication-based culture models combined with computational approaches have demonstrated that within epithelial cells, endogenous mechanical stress gradient arises owing to contraction of the actin cytoskeleton by myosin motors that transmit between adjacent cells in epithelial tissues through cadherin-mediated adhesions and lead to regulate mammary branching [27]. This mechanotransduction in mammary epithelial tissues senses mechanical stress through integrins and other mechanosensory proteins, including focal adhesion kinase (FAK), and activates specific ductal branch sites. The migration of individual epithelial/stromal cells follows an orchestrated choreography through actin-rich protrusions, and cell–ECM adhesions promote forward propulsion like amoeba movement. EMT is used to increase cells' collective motility during this morphogenesis while maintaining their connectivity. A wide range of other extracellular signals induces cell motility and polarity, including GFs, chemokines, and ECM proteins along with spatially localized activation of intracellular signaling components including PI3K, MAPKS, SRC, and Rho GTPases. Moreover, many signaling pathways involved in the regulation of EMT regulation are also present in the TEB, including Wnt, FGF, TGFb, slug, and snail.

A genome-wide transcript analysis has shown that the EMT-related transcription factors SNAI1 (SNAIL1), TWIST1, and TWIST2 are expressed in the TEB microenvironment [118]. Advances in real-time imaging have identified large-scale coordinated movements of epithelial cells as a critical aspect of pubertal mammary development through cellular migration. Time-lapse confocal imagings of primary organoids have shown that the advanced TEBs consist of multi-layered luminal epithelial cells that rearrange dynamically and exhibit reduced apicobasal polarity. Ultimately, the molecular symphony in the EMT through ECM drives mammary morphogenesis.

The patterning of the mammary gland is characterized by long and thin ducts with branch spacing and depending on the periodic location of progenitor cells. The progenitors are maintained within a stem cell niche, which depends on integrin–matrix interactions, and controlled by lateral inhibition signals, including notch and planar cell polarity. These intracellular forces are provided by localized cadherin expression and cytoskeleton contraction with cell expansion in the ducts longitudinally. Branching morphogenesis is a complex process regulated by various factors expressed in the epithelium and stroma, including hormones and growth factors, ECM, MMPs, morphogens, and immune cells [6]. These molecules ultimately involve stromal–epithelial interaction and provide positional and orientation cues for ductal morphogenesis as collagen fibers radiate out from the TEB. Ductal development follows

topographical cues in the ECM,. Ultimately, an orchestrated interactive complex network of intra- and inter-cellular microenvironment in the epithelial, luminal, and basal cells and the stroma develop mammary gland. In women, the mammary epithelial ductal structures proliferate and undergo increased branching during the luteal phase and then regress during the follicular phase [119].

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