Seminars in Cell and Developmental Biology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Seminars in Cell and Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

# Integrin-mediated adhesion and mechanosensing in the mammary gland☆

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ARTICLE INFO

*Keywords:*
Mammary gland
Integrin
Extracellular matrix
Contractility
Stiffness
Mechanotransduction

ABSTRACT

The mammary gland is dynamically remodelled during its postnatal development and the reproductive cycles. This inherent plasticity has been suggested to increase the susceptibility of the organ to carcinogenesis. Morphological changes in the mammary epithelium involve cell proliferation, differentiation, apoptosis, and migration which, in turn, are affected by cell adhesion to the extracellular matrix (ECM). Integrin adhesion receptors function in the sensing of the biochemical composition, patterning and mechanical properties of the ECM surrounding the cells, and strongly influence cell fate. This review aims to summarize the existing literature on how different aspects of integrin-mediated adhesion and mechanosensing, including ECM composition; stiffness and topography; integrin expression patterns; focal adhesion assembly; dynamic regulation of the actin cytoskeleton; and nuclear mechanotransduction affect mammary gland development, function and homeostasis. As the mechanical properties of a complex tissue environment are challenging to replicate *in vitro*, emphasis has been placed on studies conducted *in vivo* or using organoid models. Outright, these studies indicate that mechanosensing also contributes to the regulation of mammary gland morphogenesis in multiple ways.

## 1. Introduction

Mammary gland, the milk-producing organ in mammals, continues to develop after birth and fully differentiates only upon pregnancy and lactation. The cyclic morphological changes in the mammary gland epithelium (parenchyma) are orchestrated with systemic hormonal regulation and involve a diverse interplay with the stromal (mesenchymal) cells and changes in extracellular matrix (ECM) composition, mechanical tension, and tissue architecture [1]. Altogether, the cellular plasticity and extensive remodelling of the mammary gland in the adulthood in response to hormones have been suggested to increase the susceptibility of the organ to carcinogenesis.

The mammary gland rudiment that forms during embryonic development from ectodermal mammary placode picks up a new growth phase in response to pubertal hormones, oestrogen in particular, and begins to invade into the surrounding collagenous adipose tissue in a process called branching morphogenesis [2]. The ends of the invading ducts, termed terminal end buds (TEB) (Fig. 1A), are enriched with lineage-restricted progenitor cells that divide and differentiate into their cognate basal and luminal mammary epithelial lineages along the growing mammary ducts [3,4]. The luminal epithelium, composed of distinct ductal and secretory alveolar luminal cells, is surrounded by the basal epithelium, formed of contractile myoepithelial cells [2]. Interestingly, basal epithelial cells were recently demonstrated to regain bipotency upon luminal cell injury in adult mice to regenerate both cell lineages [5]. The entire tubulo-alveolar network is enveloped by a basement membrane (BM), an ECM layer that separates the epithelium from the surrounding stroma (Fig. 1B-D). Overall, ECM is an integral

*Abbreviations:* 3D, 3-dimensional; Adamts18, adam metallopeptidase with thrombospondin type 1 motif 18; BM, basement membrane; Cdc42, cell division control protein 42 homolog; Src, proto-oncogene tyrosine-protein kinase Src; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; ERα, oestrogen receptor alpha; FA, focal adhesion; FAK, focal adhesion kinase; Fgfr2, fibroblast growth factor receptor 2; FN, fibronectin; GAP, GTPase activating protein; GEF, guanyl exchange factor; ILK, integrin-linked kinase; LATS1, large tumor suppressor kinase 1; MaSC, mammary stem cell; MEC, mammary epithelial cell; MLCK, myosin-light chain kinase; MMTV, mouse mammary tumor virus; MRTF, myocardin-related transcription factor; RGD, Arg-Gly-Asp tripeptide; RIAM, Rap1-interacting adaptor molecule; ROCK, rho-associated protein kinase; SHARPIN, Shank associated RH-domain interactor; SRF, serum response factor; STAT5, signal transducer and activator of transcription 5; TACS, tumor-associated collagen signatures; TAZ, transcriptional coactivator with PDZ-binding motif; TDLU, terminal ductal lobular unit; TEB, terminal end bud; WAP, whey acidic protein; YAP, yes-associated protein.

☆ Review article for Special Issue in *Seminars in Cell and Developmental Biology* dedicated to "Cell and Developmental Biology of the Mammary Gland"

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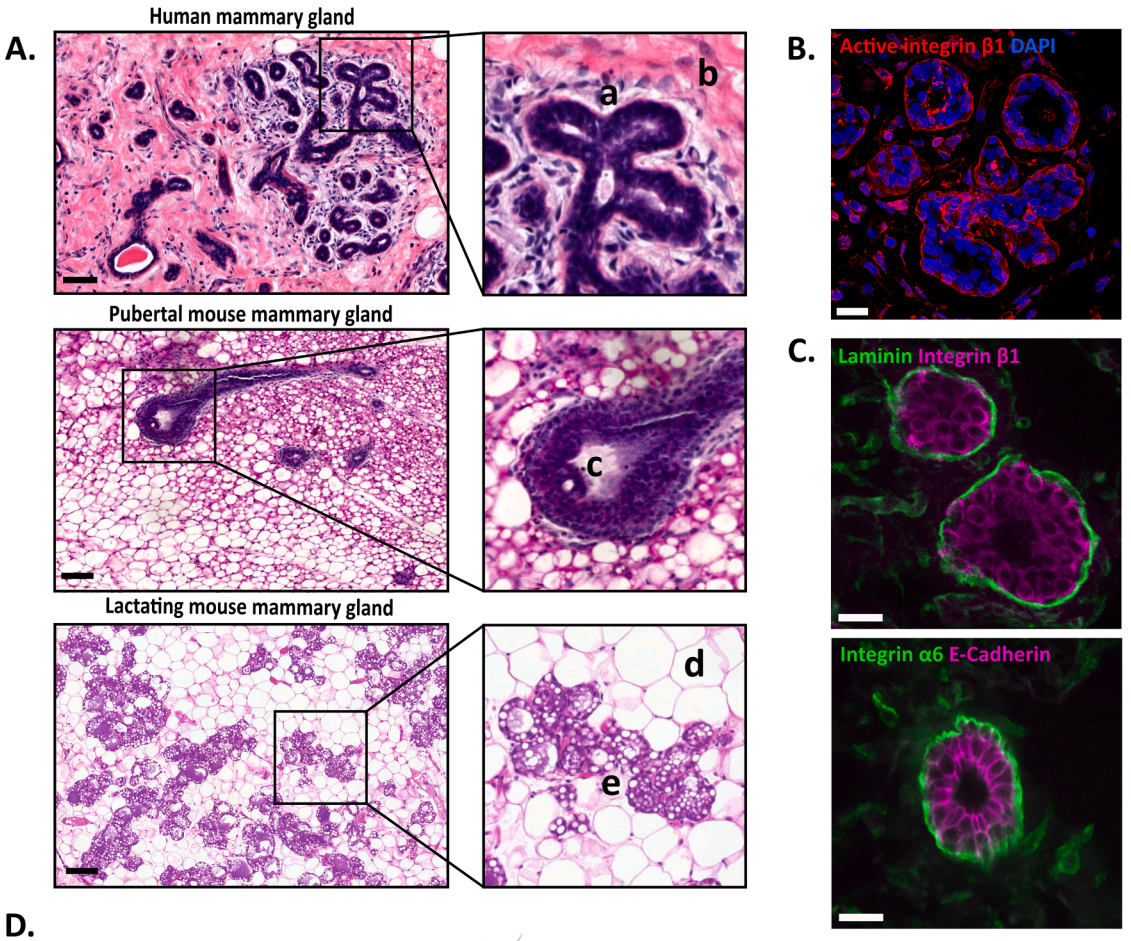
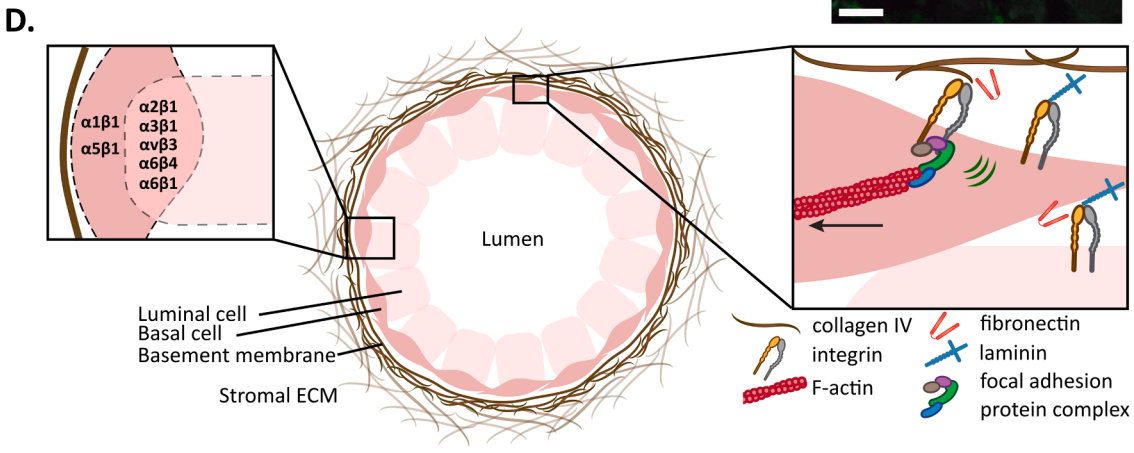
https://doi.org/10.1016/j.semcdb.2020.10.010
Received 5 July 2020; Received in revised form 17 October 2020; Accepted 23 October 2020


Please cite this article as: Oona Paavolainen, Emilia Peuhu, *Seminars in Cell and Developmental Biology*, https://doi.org/10.1016/j.semcdb.2020.10.010



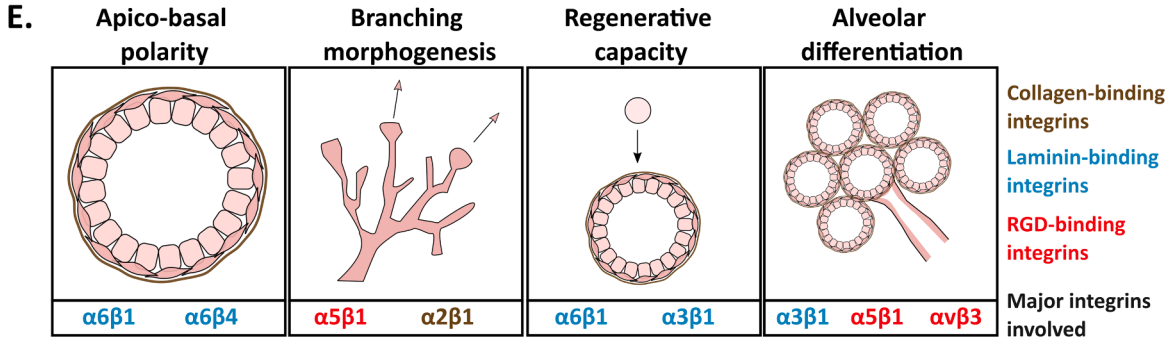




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**Fig. 1.** Integrin-mediated adhesion in mammary gland development. A. Hematoxylin-eosin staining of frozen human breast tissue sections (top), and pubertal (middle) or lactating (day 1; bottom) mouse mammary gland paraffin tissue sections demonstrate the histological differences in the mammary gland between the species and developmental stages. Magnified images of regions of interest are shown on the right. a, ductule; b, collagenous stroma; c, TEB (terminal end bud); d, adipose stroma; e, alveoli. B. Immunofluorescence labelling of active integrin β1 (antibody clone 9EG7, red) and nuclei (blue) at the edge of a lobulus in a human mammary gland frozen tissue section demonstrating higher integrin β1 activity adjacent to the basement membrane (BM). C. Immunofluorescence labelling of laminin (BM, green) and integrin β1 (mammary epithelium, magenta) (top), or integrin α6 (basal, green) and E-cadherin (luminal, magenta) (bottom) in frozen sections of a pubertal mouse mammary gland duct. Laminin and the laminin-binding integrin α6 are localized at the basal cell-BM interface. D. A schematic representation demonstrating the mammary ductal bilayer organization, and BM location. The expression of integrin heterodimers by luminal and/or basal mammary epithelial cells (left), and components of ECM-integrin adhesion and mechanosensing in the mammary epithelial bilayer (right) are also depicted. E. Integrins are involved in all the main processes of mammary gland development. The integrin heterodimers with high demonstrated influence (colors indicate their preferred ECM ligands; RGD, Arg-Gly-Asp -motif) are listed below each process. Scale bars represent A. 100 μm B-C. 20 μm.

part of the microenvironment, *i.e.* the niche that maintains cell identity and tissue homeostasis also in the mammary gland.

Upon pregnancy and lactation, oestrogen, progesterone and prolactin induce extensive branching and the formation of milk-producing structures, alveoli, at the branch ends [2] (Fig. 1A). This process is termed lobulo-alveolar development or alveologenesis, and it requires the activity of alveolar progenitor cells that contribute to the development of secretory lobules upon successive pregnancies [6]. Alveolar differentiation is characterized by the expression of milk proteins, such as β-casein and whey acidic protein.

Most of our current knowledge of mammary gland development comes from studies on rodents. Unlike the mouse mammary gland that is composed of a ductal network, human breast is formed of 17–30 lobes in which larger ducts ultimately side-branch to form lobules, structures also known as the terminal ductal lobular units (TDLU) [7]. The tips of the lobular branches are termed ductules or acini, which resemble alveoli rather than TEBs (Fig. 1A). Human breast tissue also has a higher fibrillar collagen content than mouse mammary gland (Fig. 1A). However, despite their differences, similar fundamental mechanisms are anticipated to function in both human and mouse mammary glands [8].

The capacity of cells to adhere to their surrounding ECM, and thereby support survival, proliferation, and migration, is predominantly mediated by integrin adhesion receptors, although syndecans, discoidin domain receptors and CD44 also form contacts with ECM [9]. The diverse integrin family of heterodimeric transmembrane proteins contains receptors for collagens, laminins, fibronectin (FN), vitronectin, and a number of leukocyte-specific ligands [10]. Each αβ integrin heterodimer exhibits time and cell-type specific expression patterns, and demonstrates variable affinity to different ECM ligands (Fig. 1B-E) [10]. Furthermore, integrin activity ie. the portion of inactive and active integrin conformations, is subject to regulation by both intracellular and extracellular factors [11]. Overall, the presence of specific ECM ligands, expression and activity of integrin receptors, and the cytoplasmic proteins that form the focal adhesion (FA) complexes at intracellular integrin tails collectively contribute to the appropriate binding of cells to their surrounding matrix in tissues. In return, integrin-mediated adhesion activates multiple intracellular signalling pathways, including those that promote cell survival and proliferation, and interact with various hormonal and growth factor –induced signals [12].

Cells are able to feel the mechanical properties, such as stiffness, elasticity, topography (shape), and patterning of the environment by exerting forces to the surrounding ECM through integrin-mediated adhesions. This process of mechanosensing involves the coupling of activated integrins to the actin cytoskeleton *via* FA protein complexes and acto-myosin contractility [13] (Fig. 1D). In fact, a mechanical link is formed from ECM all the way to the nuclear lamina *via* integrins, FA proteins, actin filaments, and nuclear linker proteins, which provides a means for cells directly to transduce mechanical ECM signals into biochemical signals, including changes in transcriptional regulation [13]. The dynamic transformation of mechanical signals into biochemical responses, also known as cell mechanotransduction, contributes to the regulation of cell fate in multiple ways, also in the mammary gland [14].

In this review, we provide a comprehensive view of the current knowledge regarding integrin-mediated adhesion, FA proximal signalling, actin cytoskeletal dynamics, mechanotransduction and their crosstalk with hormonal signalling in the regulation of mammary gland development after birth. Not only are these processes important for deciphering the developmental biology of the mammary gland at cell and tissue level, but they are also highly relevant in breast cancer. Thus, parallels with breast tumorigenesis and malignant progression are highlighted along the course of this review. We have placed emphasis on studies conducted *in vivo* (Table 1) or using organoid models, and highlight the open questions around the topics of this review.

## 2. Integrin-mediated adhesion

Interaction between mammary epithelial cells (MECs) and ECM is vital for the structural and functional integrity of the gland [15–17] (Fig. 1D-E). This interaction is governed predominantly through integrin-mediated adhesions which also function as important checkpoints for hormone and growth factor signalling [18]. Overall, integrin β1-, β3- and β4-subunits are expressed in both basal and luminal epithelial cell lineages [19–21]. Integrin heterodimers that have been detected in the basal epithelium include laminin-binding (α6β1, α6β4, and α3β1), collagen/laminin-binding (α1β1, α2β1), and FN/vitronectin (RGD; Arg-Gly-Asp -motif) -binding (α5β1, αvβ3) integrins [22,23]. In the luminal epithelium, laminin binding (α3β1, α6β4) and collagen/laminin-binding (α2β1) integrins are predominantly expressed [24,25] (Fig. 1B-D), and the expression of integrin αvβ3 is more restricted to progenitor cells in the luminal epithelium [21]. The laminin-binding integrin heterodimers have distinct binding affinities to different laminin isoforms, which plays a part in dictating gland development and function. Particularly α3β1, but also α6β4, binds preferentially to laminins-511/521 and laminin-332, while α6β1 can in addition interact with laminin-111 [26]. While these different laminin isoforms are expressed and deposited to the BM by distinct MEC populations [27,28], further studies are needed for detailed understanding of their patterned localization, and distinct and overlapping functions in regulating the mammary epithelial tissue dynamics.

At the cellular level, the basal myoepithelial layer physically limits the luminal cell contact with the BM composed of numerous ECM glycoproteins and proteins such as laminins (−111, −332, −511, −521), and collagen IV [29,30]. This arrangement is more pronounced in the ducts, however, in the alveoli the myoepithelial cells stretch allowing for more direct luminal-BM interaction [24]. In fact, this physical access of luminal cells to the BM is necessary for alveolar differentiation and lactogenesis [15,16]. The lack of integrin β1 specifically in luminal cells results in impaired lobulo-alveolar development and lactogenesis in mice [31,32], but also causes obstructed apico-basal polarization and lumen formation [33]. Basal MEC functions have been shown to depend on (α6β1-mediated) adhesion to laminin-111 [34]. The regenerative capacity of the mammary gland also relies on integrins α3, α6, and β1 [35,36] (Fig. 1E). Indeed, high cell surface expression of integrin α6 is commonly used as a marker for enrichment of cells with high regenerative capacity ie. mammary gland repopulating cells [37], whereas integrins α2 and β3 have been used as markers for the enrichment of luminal progenitor cells [38]. In addition, mice with integrin β4 -deficient alveolar progenitor cells (WAP-Cre) demonstrate defective

**Table 1**
Mammary gland phenotypes in genetically-engineered mouse models related to integrin-mediated mechanosensing.

| Gene | Transgene Promoter/ Null | Cell type | Mammary gland phenotype | Reference |
|---|---|---|---|---|
| *Integrins* | | | | |
| *Itgb1* | Blg-Cre | Luminal | Impaired lobulo-alveolar development and lactogenesis | [31] |
| *Itgb1* | WAP-Cre | Luminal | Alterations in alveolar integrity; Decreased density of lobulo-alveolar structures; Decreased luminal cell–ECM adhesion | [32] |
| *Itgb1* | Blg-Cre | Luminal | Surplus luminal cells within the ducts; Obstructed apico-basal polarization | [33] |
| *Itgb1* | Krt5-Cre (transplant) | Basal | Largely disorganized general branching pattern and few side branches; Abolished regenerative potential of the mammary epithelium | [35] |
| *Itgb3* | Null | | Normal development of the virgin gland, but defective alveologenesis during pregnancy and lactation | [45] |
| *Itgb4* | WAP-Cre | Luminal | Defective alveologenesis and impaired milk production during pregnancy | [39] |
| *Itga3; Itga6* | Krt5-Cre | Basal | Decreased capacity of basal cells to regenerate mammary epithelium following their transplantation into cleared mammary fat pads; Increased Rho activity<br>Increased Rho activity | [36] |
| *Itga3* | Krt5-Cre | Basal | Normal differentiation of the mammary epithelium but impaired ductal contractility and failed lactation<br>Impaired contractility, failed lactation | [40] |
| *Extracellular matrix* | | | | |
| *Lama5* | Krt8-CreERT2 | Luminal | Delayed development of the mammary epithelium; Diminished TEB structures during branching morphogenesis | [28] |
| *Fn1* | MMTV-Cre | Luminal/Basal | Delayed mammary branching morphogenesis and defective alveologenesis | [44] |
| *Focal adhesion* | | | | |
| *Fermt2* (Kindlin-2) | MMTV-Kindlin2 | Luminal/Basal (overexpression) | Enhanced mammary gland branching morphogenesis and alveologenesis; Enlarged ductal lumen; Spontaneous mammary tumor formation | [98] |
| *Ptk2* (FAK) | MMTV-Cre | Luminal/Basal | Defective alveologenesis during pregnancy and lactation | [108] |
| *Ptk2* (FAK) | Cre-ERT2 (transplant) | Luminal/Basal | Dilated ducts with thin epithelium; Spatial orientation of epithelial cell populations disturbed within the epithelium. | [109] |
| *Ptk2* (FAK) | Blg-Cre | Luminal | Normal lactational differentiation | [110] |
| *Ilk* | Blg-Cre | Luminal | Compromised lactational differentiation of luminal epithelial cells | [110] |
| *Pxn* | MMTV-Cre | Luminal/Basal | Ductal dilation; Altered epithelial organization (apico-basal polarity); Constricted branching morphogenesis | [101] |
| *Src* | Null | | Reduced oestrogen responsiveness; Delayed mammary gland branching morphogenesis; Defective milk secretion during lactation | [103]<br>[104] |
| *Bcar1* (p130Cas) | MMTV-p130Cas | Luminal/Basal (overexpression) | Reduced oestrogen responsiveness; Impaired mammary branching morphogenesis | [118] |
| *Actin cytoskeleton and contractility* | | | | |
| *Rac1* | WAP-Cre | Luminal | Loss of phagocytotic function in luminal alveolar cells during involution | [139] |
| *Cdc42* | WAP-Cre | Luminal | Under-developed alveoli with disorganized epithelium; Lacking polarity and lumen | [142] |
| *Mrtfa* | Null | | Disturbed myoepithelial differentiation upon lactation and milk ejection | [125] |
| *Arhgap35* (p190ARhoGAP) | Null | | Hindered ductal outgrowth and condensation of stroma around mammary ducts | [136] |
| *Arhgap5* (p190BRhoGAP) | MMTV-rtTA | Luminal/Basal | Altered TEB organization, with structural changes in the ductal tree and surrounding stroma | [129] |
| *Nuclear mechanosensing* | | | | |
| *Taz* | MMTV-Taz | Luminal/Basal (overexpression) | Accelerated branching morphogenesis | [147] |
| *Taz* | Null | | Branching defects; Loss of the basal MEC population | [148] |
| *Yap1* | MMTV-Cre | Luminal/Basal | Reduced alveolar structures in pregnancy | [149] |
| *Yap1* | MMTV-rtTA; TRE-Yap | Luminal/Basal (overexpression) | Defective alveologenesis in pregnancy | [149] |

Blg, beta-lactoglobulin; WAP, whey acidic protein; Krt5, keratin-5; Krt8, keratin-8; MMTV, mouse mammary tumor virus.

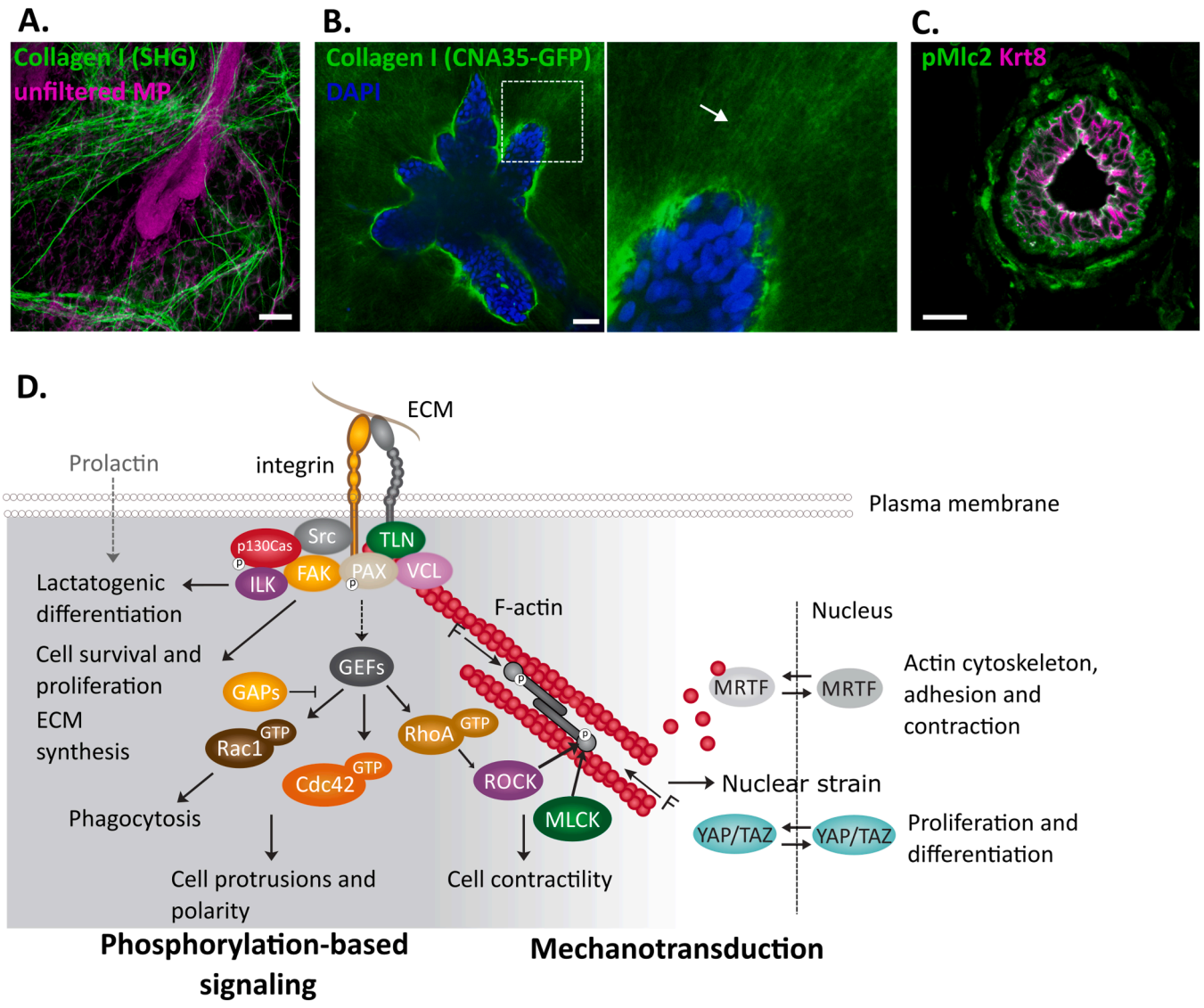


**Fig. 2.** Integrin-mediated mechanosensing and signalling in mammary gland development and function. A. Pubertal mouse TEB imaged by multiphoton (MP) microscopy. Collagen I fibers were imaged by second harmonic generation (SHG, green) and epithelial structures by unfiltered MP signal (magenta). B. Confocal imaging of a primary human mammary epithelial organoid in a floating collagen gel. Collagen I was labelled with recombinant CNA35-GFP peptide probe and nuclei were labelled with DAPI (blue). The arrow in ROI (right) indicates aligned collagen fibers that are presumably under tension. C. Phosphorylation of myosin light-chain 2 (pMlc2, green), indicative of acto-myosin contractility, can be detected in basal cells and at the apical side of luminal cells by immunofluorescence labelling of pubertal mouse mammary gland frozen sections. Luminal cells were labelled for Krt8 (magenta). D. Upon binding to ECM ligands, integrins assemble a protein complex at their cytosolic domains. These complexes mature into focal adhesions (FA) that are linked to actin cytoskeleton and contain adaptor proteins (PAX, paxillin; p130Cas), actin binding proteins (TLN, talin; VCL, vinculin), kinases (FAK, Src, ILK, ROCK, MLCK) and phosphatases (not shown) that convey signals to multiple pathways, including prolactin-mediated lactogenic differentiation, cell survival and proliferation, and ECM synthesis. Rho GTPases (RhoA, Rac1, Cdc42) can be activated downstream of FA signalling, and their activity is controlled by GAPs and GEFs. Rho GTPases modulate actin polymerization and acto-myosin contractility (actin filaments with myosin motor proteins are shown). Actin polymerization and cytoskeletal tension also affect the nuclear translocation and activity of mechanosensitive transcription factors such as MRTF and YAP/TAZ that regulate cell fate and feedback to the mechanosensitive cytoskeletal and adhesive machinery. F, force. Scale bars represent A-B. 50 μm, C. 20 μm.

alveologenesis and impaired milk production during pregnancy, which are indicative of a significant role of integrin α6β4 in alveolar function [39]. From a functional perspective, the laminin-binding α3β1 integrin was shown to be necessary for oxytocin-induced myoepithelial contraction that enables the ejection of milk from the alveolar lumen into the ducts [40].

Branching morphogenesis comprises the outgrowth and branching of the epithelial ducts in an invasive manner through the adipose tissue, taking place most actively during puberty. MECs mostly utilize α2β1, rather than α1β1, for collagen-binding during epithelial duct formation and cell proliferation in branching morphogenesis [41] (Fig. 1E). It has been demonstrated that α2β1 preferentially recognizes fibrillar collagen I, whereas α1β1 binds non-fibrillar collagen IV that is enriched in the BM [42]. FN is deposited in the cleft at sites of ductal bifurcation in the salivary gland [43], and similar mechanisms might operate also in mammary gland branching morphogenesis. Indeed, mammary ductal outgrowth is delayed in mammary epithelial FN knockout mice, and adhesion to FN appears particularly important for lobulo-alveolar differentiation of the gland [44] (Fig. 1E). Mouse mammary gland branching morphogenesis proceeds until the ductal network reaches the edges of the fat pad. However, the specific signals that terminate branching morphogenesis at that point remain elusive.

Integrin αvβ3-expression is essential for lobulo-alveolar differentiation of mouse mammary gland [45], and the expression of α5β1 is elevated in

actively proliferating basal cells during branching morphogenesis and early pregnancy [46]. In general, the FN-binding integrins α5β1 and αvβ3 have distinct features but they also co-operate. They both require the RGD motif of FN for interaction, but α5β1 also requires engagement of the nearby synergy site to reach maximal binding strength [47]. The maturation of α5β1-initiated focal adhesions has been shown to necessitate co-recruitment of αvβ3 integrin [48], and in fact, force is required for the conformational changes in αvβ3 to enable binding to FN instead of vitronectin [49]. Interestingly, the engagement of the FN synergy site by integrin α5β1 promotes tension-dependent malignant transformation in mammary gland [50], and interaction of epithelial cells with FN can stimulate epithelial-to-mesenchymal transition (EMT) [51], a process linked to breast cancer progression. The functional impact of the co-operative and force-dependent features of integrin-FN adhesion on mammary gland development and function warrants further investigation.

Appropriate time and cell-type specific patterns of integrin expression are involved in proper mammary gland development, differentiation and cell-specific functions. Integrin activity also affects the spatial patterning of ECM [52–54], which can provide important developmental cues and niche-regulatory features. Furthermore, other receptors that interact with integrins also regulate integrin-mediated adhesion. For example, the tetraspanin CD151, an interactor of laminin-binding integrins, limits MEC proliferation, ECM deposition, and differentiation, as well as tertiary mammary ductal branching in mice [55]. CD151 was suggested to maintain the luminal progenitor niche in the mammary gland [55], which might be associated with the role of CD151 and integrin α3β1 in the stabilization of integrin α6β4-containing cell–matrix adhesions [56]. Also CD44, the hyaluronan receptor utilized as a marker for putative breast cancer stem cells [57], can form a complex and modulate the activity of integrin β1 leading to increased FN-adhesion of breast cancer cells in the presence of hyaluronic acid [58]. Thus, multiple overlapping and interacting mechanisms contribute to MEC–ECM adhesion, although the full complexity and function of these mechanisms remains to be deciphered.

## 3. Extracellular matrix stiffness and topography

In addition to the biochemical composition, the topography and stiffness of ECM are of high importance to mammary gland development and homeostasis. Fibrillar type I collagen is an integral component of the mammary gland stromal ECM and its expression is regulated both spatially and temporally during branching morphogenesis [59]. Collagen I strongly contributes to tissue mechanics, along with FN and other collagen associated proteins such as elastin, fibrillin 1, decorin, lumican, and biglycan [1]. In human breast tissue, fibrillar collagen content around the mammary epithelium correlates with mammographic density [60], which in turn is associated with increased risk of breast cancer [61]. Distinct patterns of collagen fibre organization (tumor-associated collagen signatures, TACS) have also been linked to disease progression and prognosis in breast cancer [62–64]. The clinical and fundamental data related to mammary gland density and its links to breast cancer are reviewed in more detail in another review article of this special issue.

Besides malignant transformation, collagen organization contributes to normal mammary gland morphogenesis. Invading cells within the mammary ducts are able to sense the mechanical properties of the surrounding ECM, and this information is transduced into decisions that guide cell proliferation, differentiation, and collective migration, as well as further ECM remodelling along the path forward. Patterning of collagen fibres into thicker bundles in the mammary gland stroma regulates the orientation of TEBs during branching morphogenesis (Fig. 2A-B) [65]. Accordingly, the loss of stromal cell capacity to contract and organize collagen fibres due to deregulated integrin activity in fibroblasts correlates with reduced mouse fat pad stiffness and mammary ductal invasion [54,66]. Stromal macrophages also contribute to collagen fibrillogenesis and thus promote mammary ductal outgrowth [67,68]. Further studies generating dynamic information of the stromal processes that mediate ECM remodelling and stromal-epithelial interaction during mammary gland morphogenesis *in vivo* could turn out to be relevant for understanding breast cancer invasion.

Mammary ductal branching is regulated by matrix metalloproteases (MMP), proteolytic enzymes that remodel the ECM [69]. Mmp14 (MT1-MMP), for instance, has been shown to regulate invasive processes in both neoplastic and normal mammary gland *in vivo* [70]. Normal MECs have not been reported to cross the BM, although it appears thinner at the leading edge of the invading TEBs possibly due to partial enzymatic degradation and/or incomplete BM synthesis [71]. The BM separating the mammary epithelium from the stromal ECM network is very thin, only 50–100 nm in thickness [72], and therefore, patterns or changes in stromal stiffness and topography are indirectly accessible also to the mammary epithelium. Recent data has also revealed the dynamic turnover and movement of distinct BM components [73,74]. How this turnover is reflected in mammary gland BM functions remains unknown. Altogether, alterations in BM or stromal ECM organization, and the associated mechanical properties, are likely to have a profound influence on normal breast epithelial functions as well as malignant transformation. Interestingly, previous evidence suggests that mammary ECM can even reprogram non-mammary cells to form mammary glands [75,76].

BM is a major component of the mammary stem cell (MaSC) niche, and therefore regulation of BM adhesion has important implications for the regenerative capacity of the mammary gland [34,35]. The hemidesmosomal integrin α6β4, which binds to laminin-332 in BM and connects to cytoplasmic intermediate filaments, has been shown to limit FA formation, cell spreading, and traction-force generation in basal keratinocytes [56]. Therefore, it is plausible that hemidesmosomes in basal MECs may also influence mechanosensing in the mammary epithelium. There is also a fair possibility that MaSC identity is more of a context-dependent state that emerges from the collective dynamics of the tissue and cues from the local niche. A compelling idea where the dynamic competition of the stem cells for niche access acts as regulator of stem cell lineage survival was recently examined by a mathematical model [77]. The authors considered niche geometry and the dynamic movement that pushes the cells away from the niche, and predicted the number of functional stem cells with fairly good accuracy in different tissues, including the mammary gland [77]. The niche could also actively promote differentiation, as loss of laminin-521 expression in luminal cells was shown to cause an overall increase of luminal cell content in mouse mammary gland while somewhat reducing the number of hormone receptor-positive luminal cells [28]. This luminal-derived laminin-521 also promoted branching morphogenesis and alveolar differentiation in the mouse mammary gland [28]. Together, these data support a view of MaSC niches as spatially confined spaces providing the required mechanical and biochemical ECM cues along with the key signalling factors such as Wnt, Hedgehog and Notch ligands [78].

Stiffness, patterning, and tissue geometry are also guiding factors during mammary gland development. Atomic force microscopy of tissue sections suggests that healthy mammary gland tissue has an average stiffness (elastic modulus) of roughly 1 kPa [54,79,80]. Increased stiffness of the mammary gland ECM can be considered both a cause and a consequence of breast tumour progression [81], and the contribution of ECM stiffness in reprogramming of breast cancer precursors was recently described [82]. The fact that mammary gland is much softer than many other tissues [83], has its impact on cellular mechanosensing. For example, mouse mammary gland fibroblasts exhibit maturation of integrin FA and increased force generation at low (2 kPa) matrix stiffness [66] demonstrating adaptation of the cell mechanotransduction to the surrounding stiffness range. The underlying mechanisms of rigidity adaptation seem to involve the expression of distinct integrin heterodimers with specific integrin-ECM binding dynamics [66,84]. Further studies will shed light on how distinct patterns of integrin expression

and mechanotransduction translate into coordinated cell behaviour in the mammary gland tissue environment.

Studies utilizing three-dimensional (3D) micropatterns demonstrate that ductal branches are initiated from sites of high mechanical stress, namely the tips, based on tissue geometry [85]. Also, optimal collagen I content providing both sufficient stiffness and fibrillar patterns in 3D ECM matrix is necessary for branching morphogenesis *in vitro* (Fig. 2B). Consequently, mouse mammary gland organoids cultured in pure basement membrane extract have attenuated branch elongation [86]. Accordingly, excessive collagen I content with higher stiffness can perturb alveologenesis in human mammary epithelial 3D organoids [87]. After weaning of the offspring, milk accumulates in the mammary gland, which stretches the ductal lumen. In fact, stretching of MECs was found to induce involution-associated signalling and transcriptional regulation suggesting that mechanical strain participates in the triggering of involution [88]. These data exemplify the optimum range for stiffness and ECM structure for normal mammary gland development, and demonstrate how experimental *in vitro* models can be utilized to reveal mechanosensitive aspects of mammary gland development and function.

## 4. Integrin focal adhesions

The integrin-mediated mechanisms that regulate mammary gland development and function, as mention in the previous sections, are ultimately governed by the protein machinery that operates at the intracellular integrin tails, the FA complex. Through FA, the ECM-bound integrins are coupled to the actin cytoskeleton [13] and to the intracellular signalling pathways that regulate a range of cell function such as cell migration, differentiation, survival and proliferation. In addition to different integrin heterodimers, FA can contain a large variety of cytoplasmic molecules that can be classified into different categories including adaptor proteins (*e.g.*, paxillin and zyxin), actin-associated proteins (*e.g.*, α-actinin, filamin, talin, and vinculin), tyrosine kinases (*e.g.*, FAK; FA kinase, and Src; proto-oncogene tyrosine-protein kinase Src), and phosphatases [89–92] (Fig. 2D). The consensus adhesome, a set of 60 proteins that have been most commonly identified in isolated integrin-adhesion complexes on FN, and a partially overlapping group of over 100 proteins linked to acto-myosin contractility [93], contribute to cell adhesion and mechanotransduction. While many studies have investigated the role of specific integrin heterodimers in basal and luminal mammary epithelium, the role of the intracellular pathways, particularly related to mechanosensing, remain less characterized. Due to the large number of proteins involved, this review focuses only on selected key adhesion and contractility-related genes that have been implicated in mammary gland development.

Mature FAs are elongated cell-attachment structures located mainly at the cell periphery, and form when sufficient mechanical tension is applied on small nascent integrin adhesions (focal complexes) [94]. Talin is a key integrin activator that directly connects integrins to the actin cytoskeleton. The release of talin from its auto-inhibited state by the small GTPase Rap1 and Rap1-interacting adaptor molecule (RIAM), and its following recruitment to integrin β tails, promote the high ligand-affinity conformation of integrins [95]. There is limited information about integrin activity regulation in the mammary epithelium *in vivo*. Mice that lack talin 1 isoform or another integrin activating protein, kindlin-2, are embryonic lethal [96,97], demonstrating the fundamental role of these proteins in cell adhesion. However, mammary epithelial overexpression of Kindlin-2 (MMTV-Kindlin-2) results in enhanced mammary gland branching morphogenesis and alveologenesis, enlarged ductal lumens, as well as spontaneous mammary tumour formation [98], indicating that integrin activity needs to be controlled for normal mammary gland development. Integrin activity can also be regulated by inhibitor proteins, such as the cytoplasmic protein SHARPIN (Shank associated RH-domain interactor) that binds to inactive integrin α tails [99]. However, mammary epithelial SHARPIN expression was dispensable for ductal outgrowth [51], suggesting that in mammary gland development, epithelial integrin activation might be more important than integrin inhibition.

### 4.1. Phosphorylation-based focal adhesion signalling

The activated integrin tails recruit adaptor proteins that convey adhesion signal towards kinase-mediated pathways. The scaffold protein paxillin is one of the FA core components, and regulates the recruitment of several proteins to FA, including focal adhesion kinase (FAK) [100]. Mammary epithelial knockout of paxillin (MMTV-Cre) causes ductal dilation, alters epithelial organization (apico-basal polarity), and attenuates branching morphogenesis [101]. In addition, the level of active integrin β1 and laminin at the BM was significantly reduced in the paxillin-depleted mammary ducts [101]. However, it is currently not known if paxillin partakes in the regulation of lobulo-alveolar differentiation. Paxillin is also a phosphorylation target of Src kinase, a regulator of FA dynamics and signalling [102]. Src knockout mice are fertile, but exhibit a significant delay in mammary gland branching morphogenesis, reduced oestrogen responsiveness [103], as well as defective milk secretion [104].

FAK activity regulates cell survival, actin cytoskeleton, and contractility [105], increases the turnover of integrin adhesions [102], and is also implicated in breast cancer [106,107]. Conditional deletion of FAK in the mammary epithelium (MMTV-Cre) [108] or cleared fat pad transplantation of FAK null MECs [109], led to defective alveologenesis during pregnancy and lactation. Another study demonstrated that loss of FAK specifically in luminal MECs (Blg-Cre) does not compromise the lactational differentiation of luminal epithelial cells, whereas loss of luminal integrin-linked kinase (ILK) does [110]. Similar to paxillin-depleted mammary epithelium [101], ductal dilation and loss of normal bilayer organization were observed in the FAK null mammary gland transplants [109]. Furthermore, defective branching morphogenesis of FAK null organoids was linked to increased Rho-associated protein kinase (ROCK) -mediated contractility in the absence of FAK *in vitro* [109]. Together, these studies suggest that the expression of FAK is more important in basal epithelial cells in alveologenesis, while the expression of ILK in luminal epithelium mediates milk production in mouse mammary gland. The role of ILK in lactogenic differentiation appears to function through its interaction partners, Parvins and αPix, that were shown to strongly affect milk protein expression [111].

Despite the extensive crosstalk, some patterns emerge in the regulation of mammary epithelium by FA signalling: Src seems to integrate cell adhesion and oestrogen responses in the mammary epithelium [103], Src and ILK convey signals that are critical for milk production [104,110], and paxillin-FAK signalling appears to be involved in the regulation of the mammary ductal polarity, bilayer structure and diameter [101,109]. FAK also regulates RhoA-ROCK-mediated contractility and thus affects mammary epithelial motility required for branching morphogenesis [109]. How the signalling inputs are integrated from distinct ECM-integrin adhesions in the mammary epithelium, and whether some of the adhesions preferable activate certain pathways is not yet known.

### 4.2. Mechanotransduction at focal adhesions

The acto-myosin contractility and the mechanosensitive nature of FA assembly are essential features in the ability of cells to read mechanical cues in their environment ie. mechanosensing. Mechanosensors like talin and vinculin form a structural link from integrins to the actin cytoskeleton. The maturation of nascent adhesions to mature FA involves the binding of vinculin to talin when the mechanosensitive talin domains gradually unfold under strain between integrins and the actin cytoskeleton [112,113]. Vinculin recruitment reinforces the link to actin cytoskeleton and provides both mechanical strength and enhanced signal transduction to FA [113]. Also, the stretching of vinculin results in

the exposure of additional binding sites for proteins, such as talin and α-actinin [114].

Although stiffness sensing is highly relevant to mammary gland biology [61,81,82,86,87], there is limited information on how mechanotransduction at FA regulates mammary gland development and functions. The interaction of vinculin with talin appears essential for mammary epithelial differentiation and milk protein expression in organoid cultures, but it is not required for epithelial polarization [115]. To date, *in vivo* mouse studies addressing the role of vinculin in mammary gland development are yet to be conducted. Activation of the FAK-Rho-ERK (ERK; extracellular signal-regulated kinase) signalling pathway by increased collagen density and stiffness are sufficient to induce an invasive cell phenotype and increased growth of non-transformed MECs [116]. FAK, as well as Src, can phosphorylate p130Cas, a mechanosensitive adaptor protein implicated in multiple signalling pathways including activation of Rho GTPase Rac1 [117], thereby influencing for instance actin filament dynamics. Similar to Src knockout mice [103], over-expression of p130Cas affects oestrogen responsiveness and impairs mammary branching morphogenesis [118,119]. Future studies might elucidate further this interesting crosstalk between mechanosensitive FA signalling and hormonal regulation in the mammary epithelium.

## 5. Actin cytoskeleton in integrin-mediated mechanosensing

As described above, coupling of integrins and FA to the actin cytoskeleton forms the basis for mechanosensing. The dynamic flow of actin filaments (retrograde flow) and acto-myosin -based contractility are the main sources of the forces that allow cells to pull and examine their substratum [120]. Actin filaments form a dynamic network structure that enables cell movement and contractility, helps retain cell shape, and participates in cellular signalling. Actin is regulated at many levels, including polymerization, filament cross-linking, and myosin-mediated contraction. Kinases, such as ROCK and myosin-light chain kinase (MLCK), phosphorylate non-muscle myosin II and induce acto-myosin contractility [121,122], which in turn promotes force transduction *via* integrin mediated adhesions (Fig. 2D). Importantly, the activity of these kinases in the basal alveolar epithelium contributes to the synchronized, $Ca^{2+}$-dependent contractions that lead to milk ejection from the lactating mammary gland [123].

### 5.1. Actin regulation by MRTFs

Actin polymerization is increased in response to mechanical strain, which is sensed in cells through the ratio of monomeric to filamentous (G/F-) actin content through the serum response factor (SRF) pathway transcriptional co-regulators, the myocardin-related transcription factors (MRTFs) [124]. When MRTFs accumulate in the nucleus in response to actin polymerization, they co-activate the transcription of genes related to actin dynamics, cell adhesion, and acto-myosin contraction [124] (Fig. 2D). MRTF-A has been shown to be required for myoepithelial differentiation upon lactation and milk ejection in mice [125,126]. In a MEC cell line, MRTFs were required for normal morphogenesis and epithelial differentiation in 3D organoid cultures, and overexpression of MRTFs restricted the anoikis required for lumen formation [127]. MECs overexpressing MRTFs also exhibit reduced integrin α6 expression and increased integrin α5 expression, and EMT [127]. Thus, experimental evidence implies that the adaptive transcriptional responses to actin polymerization are particularly important for MEC anoikis-resistance, and for basal MEC identity and functions *in vivo*.

### 5.2. Actin regulation by Rho GTPases

Rho GTPases are the key mediators of actin cytoskeletal dynamics, and their activity is closely coupled to FA signalling (Fig. 2D). Guanyl exchange factors (GEFs) promote and GTPase activating proteins (GAPs) inhibit the activity of Rho GTPases, and these regulatory proteins can be recruited to FA [128]. The multiple forms of actin filament structures, such as stress fibres, lamellipodia, or filopodia are produced in response to the activity of distinct Rho GTPAse family members RhoA, Rac1, or cell division control protein 42 homolog (Cdc42), respectively [129,130]. The activity of Rho GTPses, in turn, feeds back to FA dynamics [131].

Active RhoA promotes FA assembly and contractile stress fiber formation through ROCK-mediated tyrosine phosphorylation of FA proteins and non-muscle myosin II, respectively [132,133]. In stiff ECM environment, RhoA/ROCK pathway is upregulated in MECs which helps retain the intracellular tension, but also results in a loss of prolactin responsiveness, β-casein expression and lactogenic differentiation [134], suggesting that excessive tissue stiffness may inhibit pregnancy associated morphogenetic changes. In turn, inhibition of ROCK results in disorganized epithelial branching and prevents uniform bilayer formation in organoids [135]. Overexpression of p190B RhoGAP, and hence decreased Rho activity, was shown to cause structural changes in TEBs and thickened, disorganized stroma surrounding them [129]. Lack of another isoform, P190A RhoGAP, inhibited the condensation of stroma around mammary ducts, and ductal outgrowth in transplantation studies [136]. These findings suggest that control of mammary epithelial Rho activity is particularly important for the stromal crosstalk.

ROCK inhibitors also promote the viability of mammary repopulating cells during *in vitro* culture [137], suggesting that attenuated contractility is linked to the maintenance of regenerative capacity in the mammary gland. Interestingly, integrin α3 and α6 double knockout MECs that are laminin-binding deficient exhibit increased RhoA activity and reduced regenerative potential, and inhibition of ROCK activity restored the phenotype [36]. In all, these data suggest that adhesion to BM limits Rho/ROCK activity to promote stem cell capacity [36,137].

Rac1 can be activated at the FA in response to integrin–ECM interaction [138]. In the mammary gland, lack of Rac1 causes the loss of phagocytotic function in alveolar luminal cells during involution, resulting in impaired clearance of milk and apoptotic cells from the gland lumen [139]. This implies that Rac1-induced actin lamellipodia are involved in mammary gland involution. Rac1 was also shown to be involved in the maintenance of mammary repopulating cells through integrin β1 -mediated regulation of Wnt signalling [140]. During mammary epithelial morphogenesis, Rac1 promotes the initiation of branching [135], whereas Cdc42, a Rho GTPAse regulating the formation of thin actin protrusions, appears to be involved in MEC migration and contractility [141]. Loss of Cdc42 in mammary epithelium (WAP-Cre) results in under-developed alveoli with disorganized epithelium, lacking polarity and lumen, thus impairing lactation *in vivo* [142]. Altogether, these data demonstrate the important role of dynamic actin polymerization, acto-myosin contractility, and formation of actin-rich protrusions in the regulation of mammary gland morphogenesis. Integrin-mediated adhesion and mechanotransduction are structurally coupled to actin dynamics, and hence, their functions are interconnected. Investigation of cellular force transduction in the regulation of morphogenetic processes in the mammary gland requires dynamic information that for example intravital imaging of force-sensitive fluorescent reporters may provide in future investigations.

## 6. Nuclear mechanotransduction *via* YAP/TAZ signalling

Mechanical ECM cues can directly influence the transcriptional regulation that governs cell fate decisions [143]. YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) can function as nuclear mechanosensors for ECM rigidity and cell shape [144]. These functions of YAP/TAZ are independent of their role in Hippo/LATS signalling pathway that controls organ size by modulating cell growth, proliferation and apoptosis [145], and is frequently deregulated in cancers [145]. Upon activation, YAP/TAZ translocate into the

nucleus where they co-activate the expression of cell-proliferative and anti-apoptotic genes [145]. Cell adhesion -mediated cytoskeletal forces can also trigger YAP nuclear entry by regulating its transport across nuclear pores [146]. Thus, integrin-mediated mechanotransduction regulates the transcriptional activity of YAP/TAZ, which in turn modulates cell proliferation and differentiation (Fig. 2D).

Transgenic mouse model with mammary gland -specific expression of constitutively active TAZ (MMTV-TAZ) was associated with an accelerated branching morphogenesis [147], whereas loss of TAZ in mice led to branching defects and loss of the basal MEC population [148]. YAP, in turn, is dispensable for pubertal mammary gland development but specifically required for the morphogenetic changes during pregnancy [149]. Hyper-activation of YAP in the mammary gland does not induce hyperplasia but also results in a failure of MECs to undergo alveolar differentiation in pregnancy [149], highlighting the importance of correct level of YAP activity in alveologenesis.

Interestingly, mechanosensitive transcription factors can even overrule the physical environment in dictating cell behaviour [144]. Overexpression of active YAP or TAZ alone can convert luminal MECs to basal lineage [148,150], and increases the regenerative capacity *in vitro* and *in vivo* [150]. YAP-dependent mechanotransduction was even required for reprogramming of oncogene-transformed MECs to tumour precursors [82]. However, the role of YAP in regulation of breast epithelium may be more complex in tissue environment since breast cancer progression appeared independent of YAP signalling in 3D culture conditions or in clinical breast cancer samples [151].

Ageing seems to also play a role in mammary epithelial mechanotransduction. When older multipotent mammary epithelial progenitors were exposed to extra-physiologically stiff (3 GPa) substrata, they exhibited predominantly luminal differentiation, whereas progenitors from younger donors differentiated towards the basal lineage when cultured on a stiffer matrix [152]. In both old and young cells, the mechanoresponses depended on YAP/TAZ. Overall, the data suggest that mechanotransduction regulates mammary epithelial differentiation, and YAP/TAZ signalling may function as regulator of mechanical memory in lineage commitment. However, this hypothesis remains to be investigated further in mammary gland development.

## 7. Interplay between ECM, integrins, and systemic hormones

In the mammary gland, particularly oestrogen, progesterone and prolactin have profound influence on development and function. Integrin-mediated adhesion is also tightly coupled to the signalling in response to endocrine hormones and growth factors [18]. On one hand, BM proteins collagen-IV and laminin-111 were shown to regulate the expression and function of oestrogen receptor α (ERα) [153], and Src and p130Cas downstream of integrins also modulates ER signalling [103,118]. On the other hand, the expression of several ECM proteins is under endocrine control, and their levels are upregulated during puberty and pregnancy [46,154]. Furthermore, integrin β1 null MECs are not capable of differentiation in response to prolactin due to defective STAT5 (signal transducer and activator of transcription 5) activation [31], demonstrating the importance of appropriate cell adhesion in hormonal responses of the mammary epithelium (Fig. 2D).

Laminin-521 produced by mature and hormone responsive luminal cells in mouse mammary gland promotes luminal identity and also regulates basal cells *via* Wnt4 paracrine signalling [28]. Interestingly, a recent study showed that oestrogen and progesterone stimulation of mature luminal cells induces the expression of a secreted protease Adamts18 (adam metallopeptidase with thrombospondin type 1 motif 18) in basal myoepithelial cells, also through paracrine signalling [155]. *In vivo*, Adamts18 deletion caused increased collagen deposition in pubertal mammary gland, resulting in impaired YAP/TAZ signalling and reduced expression of fibroblast growth factor receptor 2 (Fgfr2), both of which are important regulators of mammary repopulating cells [155]. Collectively, these studies demonstrate how ECM remodelling and integrin-mediated adhesion in the mammary epithelium are under complex endocrine and paracrine regulation, and directly linked to cell identity.

Hormone responsiveness of the human mammary gland epithelium decreases in response to pregnancies as the basal-to-mature luminal cell ratio increases [156]. During menopause, the levels of oestrogen and progesterone also decrease, while the proportion of glandular epithelium decreases [157], and the tissue stiffness increases [158]. Interestingly, the mammary gland has a circadian rhythm that is affected by the stiffness of the surrounding ECM; the amplitude of circadian oscillations is smaller in mammary tissue explants from old mice as compared to younger mice [159]. Overall, the mechanical properties of the ECM are evolving alongside the age- and parity-related changes in the mammary gland, which in turn, are linked to the risk of breast cancer. Increased understanding of these interrelations may reveal important information that could benefit breast cancer risk assessment and diagnostics.

## 8. Concluding remarks

Cell adhesion signalling and mechanotransduction integrate information about the biochemical and physical status of the environment in the mammary gland. Thereby, changes in the ECM environment can be conveyed into altered cell fate, whether it means active proliferation, differentiation to a particular cell type, apoptosis, collective migration to form an organ, or quiescence. However, how exactly mechanosensing modulates mammary gland development and function *in vivo* is still not well understood.

Luminal and basal MECs, and their respective progenitor cells, are exposed to the ECM of the BM but also sense the mechanical cues from the stromal ECM. The patterns of ECM and integrin expression, and their co-operative and redundant functions in different developmental stages of the mammary epithelium, indicate the importance of particular integrin-ECM signals in the distinct morphological processes. Moreover, the cytoplasmic signalling pathways that originate from integrin-mediated adhesions and branch into cascades modulating cell proliferation and viability, cytoskeletal tension and contractility, and ECM remodelling have an impact on all aspects of mammary gland biology. Consequently, altered mechanical properties in the environment also have a strong influence. Indeed, the gradual stiffening of the mammary gland microenvironment in breast cancer appears to be an integral part of malignant progression. Future advances in mechanobiological techniques, including reporter systems for detection of dynamic changes in force transduction and mechanosensing *in vivo*, will help to close the gaps in our knowledge. Once the different aspects of mechanosensing in the regulation of normal mammary gland morphogenesis as well as breast cancer initiation and metastasis are well-understood, new strategies for therapeutic targeting of breast cancer may emerge.

## Funding

This work was supported by Academy of Finland [grant numbers 323096, 328892].

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

We thank J. Englund and A. Isomursu, and the Peuhu Lab members for insightful comments and suggestions to the manuscript. We also thank P. Hartiala and N. Brück for the clinical collaboration; M. Saari for assistance in multiphoton imaging; Cell Imaging Core facility of Turku Bioscience Centre and Biocenter Finland for providing the imaging infrastructure; and University of Turku Histocore for sectioning and staining of tissues.

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