The contribution of adhesion signaling to lactogenesis

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Abstract The mammary gland undergoes hormonally controlled cycles of pubertal maturation, pregnancy, lactation, and involution, and these processes rely on complex signaling mechanisms, many of which are controlled by cell–cell and cell–matrix adhesion. The adhesion of epithelial cells to the extracellular matrix initiates signaling mechanisms that have an impact on cell proliferation, survival, and differentiation throughout lactation. The control of integrin expression on the mammary epithelial cells, the composition of the extracellular matrix and the presence of secreted matricellular proteins all contribute to essential adhesion signaling during lactogenesis. In vitro and in vivo studies, including the results from genetically engineered mice, have shed light on the regulation of these processes at the cell and tissue level and have led to increased understanding of the essential signaling components that are regulated in temporal and cell specific manner during lactogenesis. Recent studies suggest that a secreted matricellular protein, CTGF/CCN2, may play a role in lactogenic differentiation through binding to $\beta 1$ integrin complexes, enhancing the production of extracellular matrix components and contributions to cell adhesion signaling.

Keywords Lactogenesis · Adhesion · Integrin · Signal transduction · Mammary gland · CTGF · Connective tissue growth factor · CCN2

Mammary gland development

The mammary gland undergoes hormonally controlled cycles of pubertal maturation, pregnancy, lactation, and involution, and these processes rely on complex signaling mechanisms, many of which are controlled by cell–cell and cell–matrix adhesion. In vitro and in vivo studies, including the results from genetically engineered mice, have shed light on the regulation of these processes at the cell and tissue level.

The rudimentary mammary gland, which develops from the mammary placode, is present at birth, but the majority of glandular development is post-natal including hormone-dependent maturation of the gland during puberty, pregnancy and lactation. The mammary gland consists of ducts extending from the nipple through the mammary fat pad to the functional terminal end points referred to as the terminal end buds (TEB) in mice, or alveoli in humans. The TEB is a single bulbous structure that develops into a more complex collection of alveolar structures known as the lobulo-alveolus. In the human, multiple bulbous ends are present on multiple ducts, and these grow and expand during pregnancy (Williams and Daniel 1983). The TEBs consist of two main cellular layers: an inner layer of luminal secretory epithelial cells and an outer layer of contractile myoepithelial cells that surround a central hollow lumen. Surrounding the TEB is a basement membrane layer of highly cross-linked extracellular matrix (ECM) proteins, laminin being the predominant protein. The basement membrane proteins are secreted primarily by

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the outer layer of myoepithelial cells. Outside of the basement membrane lies the interstitial ECM, which is largely composed of collagen types I and III, as well as other common ECM proteins such as fibronectin, laminin, and tenascin. The invasion of the basement membrane and periductal stroma by the ductal epithelium is a process referred to as branching morphogenesis and is known to involve the breakdown of the ECM proteins by matrix metalloproteinases (MMP) (Wiseman and Werb 2002). MMP-3, specifically, is thought to regulate side branching (Wiseman et al. 2003). This is important because during pregnancy, lateral buds extend through the main ducts. These buds undergo massive proliferation and subsequent differentiation in order to fill the gland with lobuloalveolar structures that contain the secretory epithelial cells. Later in pregnancy, the alveolar structures increase in number and complexity, and the cells lining the alveoli and small ducts mature, acquiring the ability to secrete milk. The milk secretion, however, is kept in check by high concentrations of circulating progesterone until the initiation of lactation (Turner et al. 1992).

At the onset of pregnancy, the anterior pituitary gland is stimulated to produce prolactin, a single peptide hormone that has two main functions in reproduction. Through the preservation of the corpus luteum, prolactin ensures the secretion of estrogen and progesterone (Gallego et al. 2001), an important event for regulating the morphological changes in the mammary gland during pregnancy. Progesterone receptor studies and knockout in mice revealed that progesterone is required for ductal side branching (Humphreys et al. 1997) (Plaut et al. 1999; Atwood et al. 2000), while prolactin knockout studies revealed that it is required for alveologenesis during pregnancy (Briskin et al. 1999). While the main sources of prolactin are the lactotroph cells of the pituitary, the production of prolactin by mammary epithelial cells has been reported, where it functions as a paracrine mediator of mammary epithelial cell development (Bonnette and Hadsell 2001; Oakes et al. 2006). At parturition, a fall in progesterone levels, accompanied by the maintained elevation of prolactin, leads to secretory cell activation and lactation (Neville et al. 2002).

The establishment of functional alveoli during pregnancy depends on the polarization of the luminal cells and the formation of junctions between them (Barcellos-Hoff et al. 1989). Proteins required for the polarization of the cells and for the formation of tight junctions are regulated, in part, by adhesion-mediated signals. During pregnancy, the myoepithelial cell barrier of the TEB is stretched, thereby allowing many luminal cells to make direct contact with the basement membrane, directly altering cell adhesion and subsequent signaling (Oakes et al. 2006). After parturition, the act of suckling stimulates the release of oxytocin from the posterior pituitary gland, which causes the myoepithe-

lial cell layer to constrict, forcing the milk out of the secretory cells and into the lumen of the alveolus and out through the ductal structures to the nipple. Successful lactation depends upon a pulsatile release of prolactin from the pituitary gland (Wynick et al. 1998). While it is known that prolactin instructs the proliferation and the differentiation of the mammary epithelium through mechanisms specific to the prolactin receptor (Briskin et al. 2002), evidence also suggests that prolactin-mediated signaling, similar to signaling resulting from adhesion to the basement membrane, activates transcriptional programs that are shared between several receptors, such as integrins (Briskin et al. 1999).

Multiple secretory processes are utilized in the epithelial cells of the lactating mammary gland: exocytosis, lipid synthesis and secretion, transmembrane secretion of ions and water, and transcytosis of extra-alveolar proteins such as immunoglobulins and albumins as well as hormones from the interstitial space. The luminal epithelial cells convert precursor proteins into milk components which are transported into the lumen of the alveolar structures. To ensure the properly polarized secretion of milk components, the formation of tight junctions between secretory cells is required. Main components of tight junctions, specifically occludin and zona occludens 1 (ZO-1), are induced by both prolactin and glucocorticoids (Stelwagen et al. 1999). Aside from their contractile function, the physical and paracrine interactions between luminal and myoepithelial cells are critical for maintaining luminal cell polarity, as well as regulating proliferation and apoptosis (Kouros-Mehr et al. 2006). The interaction between the epithelial cells and the stromal cells is also essential for mediating proliferation and survival.

Regulation of milk protein gene expression

During pregnancy, the luminal secretory epithelial cells are induced to produce caseins, the predominant milk proteins in all species, and whey acidic protein (WAP), the primary whey protein. The genes encoding these proteins display both developmental and tissue-specific patterns of expression (Hobbs et al. 1982) and are used as molecular markers of functional differentiation in the mammary gland (Topper and Freeman 1980). The casein genes, including α s1, β , γ , δ , and κ , are encoded by a 250 kb cluster on chromosome 5 of the mouse genome (Rijnkels et al. 1997) with β -casein being expressed at the highest levels in the mouse (Schmidhauser et al. 1992).

The precise contribution of specific transcription factors and their binding sites in the regulation of milk protein gene expression have established that hormonal and developmental regulation of the β -casein gene requires a complex

DNA element referred to as a composite response element (CoRE) (Liu et al. 1997; Robinson et al. 1998; Seagroves et al. 1998; Blakely et al. 2006). These CoRE units are defined as a cluster of transcription factor binding sites containing both positive and negative regulatory elements which integrate signal transduction pathways through protein-DNA and protein-protein interactions (Jiang and Levine 1993). The primary factors associated with activation of the β -casein CoRE include signal transducer and activator of transcription 5 (Stat5), glucocorticoid receptor (GR), and CCAAT enhancer binding protein β (C/EBP β), while Yin Yang-1 (YY-1) associates with the CoRE as a negative regulator of gene expression (Schmitt-Ney et al. 1991; Meier and Groner 1994; Raught et al. 1994; Wakao et al. 1994; Doppler et al. 1995; Raught et al. 1995; Lechner et al. 1997; Seagroves et al. 1998). Interestingly, none of the transcription factors associated with the β -casein CoRE is mammary gland-specific or even restricted to the lactation phase of development.

Adhesion signaling in mammary epithelial cell differentiation and survival

The interaction between mammary epithelial cells (MECs) and the basement membrane (BM) is critical for successful lactogenic differentiation (Fig. 1). While MECs adhere to the BM via various types of ECM receptors, the primary class of receptors is composed of heterodimeric α - and β -chain integrins (Taddei et al. 2003). Other receptors whose specific roles remain undefined include dystroglycan,

syndecan, and galactosyl transferase (Streuli 2003). In MECs stimulated to undergo lactogenic differentiation, signals from the matrix are primarily mediated through integrins, since a function-blocking anti-integrin antibody severely diminishes the ability of cells to synthesize β -casein (Streuli et al. 1991). Laminin is the primary component of the BM responsible for the activation of milk protein production, and Muschler et al. (Muschler et al. 1999) determined that signals from laminin for β -casein transcription are inhibited in the presence of function-blocking antibodies against both the $\alpha 6$ and $\beta 1$ integrin subunits.

The extracellular matrix (ECM) induces a complex interaction between bound transcription factors, the basal transcriptional machinery, and a chromosomally integrated template responsive to the acetylation state of the histones at the β -casein enhancer and promoter regions (Myers et al. 1998). There is evidence suggesting that the ECM maintains a high level of histone H4 acetylation upstream of the $\alpha s 1$ -casein gene, especially at the level of a distal prolactin and ECM-sensitive enhancer region (Jolivet et al. 2005). Recently, Xu et al. determined that extracellular matrix molecules cooperate with prolactin to induce histone acetylation and the binding of transcription factors as well as the ATP-dependent SWI chromatin remodeling complex to the β -casein promoter (Xu et al. 2007).

Integrin binding to ECM proteins promotes the formation of multi-protein adhesion complexes at the membrane of mammary epithelial cells (Geiger et al. 2001); the complexes include a variety of structural (i.e., vinculin), adaptor (i.e., paxillin, p130cas, parvin, ILK), and kinases (i.e., focal adhesion kinase [FAK], Src) (Cabodi et al.

Fig. 1 Cell adhesion regulates essential pathways in lactogenesis. The binding of lactogens in conjunction with signals from integrin engagement initiate the critical changes that take place during lactogenic differentiation. The diagram includes the central pathways known to contribute to the initiation and control of lactogenesis. Dotted lines (.....) indicate pathways with multiple or incompletely known enzymatic steps

2006). Complex formation results in integrin clustering and activation of downstream signals primarily involving p130cas, paxillin, ILK, and Src (Cabodi et al. 2006). The focal adhesion complex is formed downstream of the adhesion-induced phosphorylation of FAK-Y397. Recruitment of Src causes tyrosine phosphorylation of additional sites on FAK and the subsequent FAK-mediated phosphorylation of p130cas and paxillin (Schlaepfer et al. 1997). Src contributes to both MEC differentiation and proliferation through its effects on ER α , ErbB, prolactin receptor and p130cas (Kim et al. 2005). FAK activation may also contribute to cell survival by direct activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Bouchard et al. 2007).

Detachment from the ECM leads to a type of apoptosis of epithelial cells called anoikis (Frisch and Francis 1994) (Gilmore 2005), thus one of the roles most commonly ascribed to integrin-mediated adhesion is cell survival. Mammary-specific dominant-negative β 1 integrin has been shown to result in a decrease in MEC proliferation and increased apoptosis during pregnancy and lactation (Faraldo et al. 2001). Upon the genetic deletion of β 1 integrin, MECs undergo cell cycle arrest and glands display defective development *in vivo*, suggesting that mammary epithelial cell proliferation requires integrin-mediated ECM adhesion (Li et al. 2005), and cells that lack a functional β 1 integrin display enhanced apoptosis *in vitro* culture (Li et al. 2005). The loss of β 1 integrin function contributes to defective milk protein production in mammary epithelial cells, though the exact mechanism involved remains unclear. Mice genetically lacking functional β 1 integrin display disrupted alveolar morphology and the absence of Stat5 nuclear translocation in response to prolactin stimulation (Li et al. 2005; Naylor et al. 2005). A study of the mechanism of β 1 integrin *in vivo* by Faraldo et al. (2002) determined that the disruption of β 1 integrin function induced precocious dedifferentiation of the secretory epithelium in the mammary gland. This was shown by a premature decrease in β -casein and WAP mRNA levels, accompanied by the inactivation of Stat5 and an upregulation of NF- κ B (Faraldo et al. 2002). The link between β 1 integrin signaling and prolactin-induced signaling likely involves the integrator of adhesion signaling, Rac1, because integrin-containing adhesion complexes cooperate with Rac1 to allow prolactin-mediated Stat5 nuclear translocation and the resulting transcription of milk proteins (Akhtar and Streuli 2006).

In MECs, overexpression of FAK can prevent anoikis (Gilmore et al. 2000). FAK mammary conditional knockout mice display severe lobulo-alveolar hypoplasia and secretory immaturity during pregnancy and lactation, and the transplantation of FAK knockout MECs into a cleared fat pad of immune-deficient mice also resulted in glands with a

disordered myoepithelial and luminal epithelial cell multilayer and abnormal ductal morphogenesis during pregnancy (Nagy et al. 2007; Luo et al. 2009; van Miltenburg et al. 2009). Mammary specific knockout of ILK also resulted in a post-pregnancy defect in alveologenesis; decreased milk fat production and secretion was observed and attributed to reduced lumina, all of which contributed to a lactation defect (Akhtar et al. 2009). These studies revealed a significant decrease in active Rac1 due to ILK deletion and demonstrated that ILK deletion had a more profound effect on milk protein expression than FAK deletion (Akhtar et al. 2009).

A conditional knockout of fibronectin in the mammary gland resulted in decreased β 1 integrin and FAK phosphorylation and abrogated alveologenesis and ductal outgrowth in pregnant mice (Liu et al. 2010). The cells of these glands also showed a decrease in phosphorylated Erk1/2, cyclin D1, phosphorylated Stat5, and diminished proliferative capacity (Nagy et al. 2007). Moreover, knockouts of Src blocked ductal development and lactational ability of the mammary gland (Kim et al. 2005) (Watkin et al. 2008). Because of its role in blocking anoikis and its function in adhesion complexes, the role of Akt in lactogenesis has been examined. Expression of constitutively activated Akt1 in the mammary gland resulted in elevated lipid synthesis and secretion due to an AKT-dependent activation of glucose uptake and formation of cytosolic lipid droplets, whereas Akt1 deletion resulted in failure to regulate glucose uptake and lipid synthesis (Schwertfeger et al. 2003; McManaman et al. 2004; Boxer et al. 2006). Mammary gland specific knockout of Akt 2 and Akt 3 revealed that these proteins are critical for the regulation of involution (Maroulakou et al. 2008).

Laminin-1 (LM-1) plays a significant role in the integrin-mediated survival pathway and suppressed anoikis more efficiently than other ECM proteins in MECs (Pullan et al. 1996). Laminin binding to the β 1 integrin complex (Mercurio 1995) is directly associated with the induction of lactogenesis and β -casein transcription (Mercurio 1995; Roskelley et al. 1995), but the α integrin partner has not been definitively identified (Klinowska et al. 2001; Chen et al. 2002). The cell-BM interaction is commonly viewed as a positive checkpoint to suppress apoptosis, ensuring that MECs are only maintained within ducts and alveoli (Prince et al. 2002). In addition, MECs proliferate in response to hormonal stimulation only if the adhesion context is permissive (Xie and Haslam 1997). For example, while the estrogen receptor α (ER α) is associated with a highly proliferative MEC phenotype, elevated expression of the ER β receptor enhances the expression of β 1 integrin and promotes the formation of focal adhesions and inhibits MEC proliferation (Lindberg et al. 2010). Furthermore, adhesion to the BM regulates

insulin-mediated signaling (Lee and Streuli 1999) (Farrelly et al. 1999) and this is through the RhoA/Rok pathway, which is critical to lactogenic differentiation (Lee et al. 2009).

CCN2 protein contributes to lactogenesis

Connective Tissue Growth Factor (CTGF/CCN2) was identified as a gene highly upregulated in mouse mammary epithelial cells that had been stimulated to undergo lactogenic differentiation, and in primary mammary gland tissue CTGF/CCN2 levels increase during late pregnancy and early lactation (Wang et al. 2008). In HC11 mammary epithelial cells stimulated to undergo lactogenic differentiation, the elevated expression of CTGF/CCN2 was dependent on glucocorticoids, not prolactin or TGF β (Wang et al. 2008) whereas progesterone elevation, which typically occurs during late pregnancy, regulated CTGF/CCN2 in bovine mammary gland (Forde et al. 2010) (Fig. 2). CCN proteins are matricellular effectors that are secreted into the extracellular environment where they can associate with the cell surface and ECM components (Tettamanti et al. 2006). They function as both as growth factors (Bradham et al. 1991) and as ECM-associated cell adhesion molecules (Kireeva et al. 1997) and exhibit diverse functions regulating many important biological processes including cell attachment (Babic et al. 1999; Chen et al. 2001; Hoshijima et al. 2006), migration (Grzeszkiewicz et al. 2001; Lin et al. 2005), survival (Babic et al. 1999; Leu et al.

2002; Lin et al. 2003), growth (Abreu et al. 2002; Ivkovic et al. 2003), differentiation (Mori et al. 1999; Brigstock 2003) and angiogenesis (Babic et al. 1999; Mo et al. 2002; Lin et al. 2003; Mo and Lau 2006). The CCN proteins (CYR61/CCN1, CTGF/CCN2, NOV/CCN3, and the Wnt-induced secreted proteins 1-3 (Wisp) Wisp1/Elm1/CCN4, Wisp2/Rcop1/CCN5, and Wisp3/CCN6) share a modular structure of an N-terminal signal peptide followed by four homology domains (Perbal 2004). Domain 1 bears sequence homology to IGF binding proteins (IGFBP), domain 2 is homologous to a von Willebrand type C repeat (vWC), domain 3 resembles thrombospondin 1 (TSP-1), and domain 4, the carboxyl-terminal (CT) domain, contains a cysteine knot motif (Bork 1993; Lau and Lam 1999). Tissue-specific CCN isoforms, derived from post-translational processing, proteolytic cleavage between the distinct modules or alternate splicing, have been detected (Perbal 2004). The diverse functions of the CCN family members have been well reviewed elsewhere (Lau and Lam 1999; Brigstock 2003; Perbal 2004; Brigstock et al. 2005; Kubota and Takigawa 2007; Chen and Lau 2009).

In the HC11 mouse mammary epithelial cell background, CTGF/CCN2 expression enhanced the early transcription of β -casein in response to lactogenic hormone, and exogenous addition of CTGF/CCN2 contributed to the formation of mammospheres and MCF10A acini, hallmarks of terminal differentiation (Wang et al. 2008; Debnath and Brugge 2005; Morrison et al. 2010). CTGF/CCN2 enhanced the expression of laminin in mammary epithelial cells resulting in a decreased requirement for exogenous laminin for the activation of β -casein transcription (Morrison et al. 2010). CTGF/CCN2 increased expression of fibronectin and stabilized the surface expression of the $\alpha 6$ and $\beta 1$ integrins; PINCH1 and Rsu1, proteins found in an integrin-ILK-linked protein complex were also elevated (Wang et al. 2008; Morrison et al. 2010). CTGF/CCN2 expression blocked anoikis in the absence of serum and correlated with enhanced focal adhesion formation in HC11 cells. This may have resulted from direct interaction between integrins and secreted or cell-bound CTGF/CCN2, as suggested by studies using $\alpha 6$ and $\beta 1$ integrin blocking antibodies. Alternatively, the elevation of matrix protein expression may have increased RGD-binding integrin engagement in HC11 cells (Morrison et al. 2010). Syndecan 4, a heparin sulfate proteoglycan, which has been reported to stabilize integrin complexes, including $\beta 1$ integrin complexes (Morgan et al. 2007; Chen et al. 2004; Kennedy et al. 2007), was also upregulated in CTGF/CCN2-stimulated or -expressing HC11 cells (Cutler and Morrison, unpublished data). Collectively, these results suggest that the mechanism by which CTGF/CCN2 enhances lactogenic differentiation is through stabilization of the interaction between laminin and $\alpha 6\beta 1$ integrin that is required early in the lactogenic differentiation

Fig. 2 The contribution of CTGF/CCN2 to lactogenic differentiation. The diagram includes the pathways known to be affected by CTGF/CCN2 during lactogenic differentiation of mammary epithelial cells in primary cells or established cell lines

process. Lactogenic differentiation may also depend on expression and stabilization of CTGF/CCN2 -integrin complexes that promote prolactin-induced Stat5 activity (Xu et al. 2007, 2009).

CTGF/CCN2 promotes cell adhesion and migration in a variety of cell types (Kireeva et al. 1997; Babic et al. 1999; Crean et al. 2002) and performs many of its adhesion-related functions through interaction with integrin complexes, as well as through the interaction with heparin sulfate proteoglycans (HSPGs) and the LDL-related protein (LRP) (Chen et al. 2001; Segarini et al. 2001; Ball et al. 2003). CTGF/CCN2 enhanced the levels of $\beta 1$ integrin on the surface of the cells (Weston et al. 2003; Morrison et al. 2010) and contributed to the adhesion via binding to $\alpha 6\beta 1$ integrin, a common laminin receptor (Chen et al. 2001; Leu et al. 2003; Tong and Brigstock 2006). CTGF/CCN2 also enhanced binding of cells to the common fibronectin receptor, $\alpha 5\beta 1$ integrin (Frazier et al. 1996; Blom et al. 2001; Weston et al. 2003). Disruption of focal adhesions and $\beta 1$ integrin binding via either FAK or ILK inhibited CTGF/CCN2 expression, suggesting regulation by a pathway dependent on $\beta 1$ integrin (Graness et al. 2006a, b). CTGF/CCN2 enhanced survival in some cells (Hishikawa et al. 2001; Tong and Brigstock 2006; Wahab et al. 2007). This may have involved the induction of MAP kinase phosphatase-1 (Mkp-1) to dephosphorylate MAP kinases p38 and JNK, allowing for accumulation of the anti-apoptotic protein Bcl-2 (Wahab et al. 2007). Because these activities have been observed in mammary epithelial cells expressing CTGF/CCN2, it appears that CTGF/CCN2 may have an essential function in the developing and differentiating mammary gland.

Conclusions

The adhesion of epithelial cells to the extracellular matrix initiates signaling mechanisms that control cell proliferation, survival, and differentiation throughout the phases of mammary gland development. There are multiple levels of control ranging from the control of integrin expression on the mammary epithelial cells to the composition of the extracellular matrix and the presence of secreted matrix proteins. The targeted deletion of specific proteins and the use of three-dimensional in vitro systems are leading the way to increased understanding of the essential signaling components that are regulated in temporal and cell specific manner during development, differentiation and lactogenesis.

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