Insights into the role of connexins in mammary gland morphogenesis and function

Michael K G Stewart¹, Jamie Simek¹ and Dale W Laird^{1,2}

Departments of ¹Physiology and Pharmacology and ²Anatomy and Cell Biology, University of Western Ontario, London, Ontario, Canada N6A 5C1

Correspondence should be addressed to D W Laird; Email: dale.laird@schulich.uwo.ca

Abstract

Gap junctions formed of connexin subunits link adjacent cells by direct intercellular communication that is essential for normal tissue homeostasis in the mammary gland. The mammary gland undergoes immense remodeling and requires exquisite regulation to control the proliferative, differentiating, and cell death mechanisms regulating gland development and function. The generation of novel genetically modified mice with reduced or ablated connexin function within the mammary gland has advanced our understanding of the role of gap junctions during the complex and dynamic process of mammary gland development. These studies have revealed an important stage-specific role for Cx26 (GJA1) and Cx43 (GJB2), while Cx30 (GJB6) and Cx32 (Gjb1) can be eliminated without compromising the gland. Yet, there remain gaps in our understanding of the role of mammary gland gap junctions.

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Introduction

Gap junctions are formed when six connexins co-oligomerize to form a connexon, or hemichannel, which docks with a connexon from an adjacent cell to form a gap junction channel (Laird 2006). Gap junction channels directly link the cytoplasm of adjacent cells allowing for the exchange of molecules <1 kDa in size that includes ions and second messengers (Ca²⁺, IP₃, cAMP) in a process known as gap junctional intercellular communication (GJIC) (Laird 2006). We now know that 21 connexins exist in humans, while 20 connexins are found in mouse, and generally multiple connexins are expressed within the same cell (Laird 2006). However, channels formed by one connexin cannot always compensate for the loss of channel function from another because various connexins possess distinct gating properties and conductance when forming homomeric connexons formed exclusively of one connexin isoform (Laird 2006, Harris 2007). In addition, some connexins, such as Cx26 (*Gjb2*), Cx30 (*Gjb6*), and Cx32 (*Gjb1*), are able to intermix and form heteromeric connexons composed of multiple connexin isoforms, which display altered permeability characteristics and gating sensitivities that likely reflect the biological need of the cell type *in vivo* (Harris 2007, Locke *et al.* 2007). In addition, apart from GJIC-dependent functions, connexins may also control cellular function independent of GJIC through the formation of gap junction hemichannels linking the intracellular and extracellular

environment or through poorly understood regulatory protein–protein interactions (Goodenough & Paul 2003, Laird 2010). Importantly, gap junctions are expressed in almost every cell type in the human body, including those of the breast, and are dynamically regulated throughout organ and tissue morphogenesis, suggesting a role in the regulation of developmental processes (Wei *et al.* 2004, Laird 2006). As the majority of its development occurs after birth, the breast remains a fascinating organ to study the role of connexins in development.

The human breast, similar to that of the mouse mammary gland, functions to provide both nutrition and passive immunological protection against pathogens during nursing. Both are composed of a bilayered epithelial network consisting of a single luminal layer surrounded by a layer of myoepithelial cells separated from the mammary stroma by a basement membrane (Richert *et al.* 2000, Geddes 2007). However, while the non-pregnant rodent mammary gland consists entirely of a single ductal tree consisting of blunt ended ducts that develop alveolar buds in response to the estrous cycle within a mainly adipose-rich mammary fat pad, the human mammary gland contains 15–20 independent branching epithelial networks that begin as terminal duct lobular units composed of alveoli and ducts surrounded by fibrous connective tissue (Sternlicht *et al.* 2006, Geddes 2007). Importantly, both human and mouse mammary glands undergo extensive proliferation and secretory differentiation of epithelium as the breast develops into a secretory gland during lactation, which

require tight regulation of hormonal and growth factor cues as well as epithelial–epithelial and epithelial–stromal interactions, and direct cell–cell communication through gap junctions (Hennighausen & Robinson 2005, Geddes 2007, McLachlan *et al.* 2007, Macias & Hinck 2012). Therefore, it is unsurprising that the human and mouse epithelial stem cell hierarchies share many parallels as reviewed by Visvader (2009). Ultimately, due to these similarities between the mouse and human mammary gland, the mouse mammary gland has become a useful model to further our understanding of the mechanisms associated with gland development and disease, particularly through our ability to genetically manipulate molecular functions by mutation or ablation (Howlin *et al.* 2006, Briskin 2013). The evaluation of genetically modified mice has extended our understanding of the role of connexins in the mammary gland, which is discussed in this review.

Mouse mammary gland development

The development of the murine mammary gland begins with the development of the milk lines at embryonic day 10 (Cowin & Wysolmerski 2010). By embryonic day 11.5, the milk lines develop into five pairs of mammary gland placodes which progress into mammary gland buds and ultimately a rudimentary ductal structure (Macias & Hinck 2012). Following birth, this rudimentary structure develops isometrically with body weight until the onset of puberty (Howlin *et al.* 2006). The hormonal secretion of estrogen and growth hormone (GH) then drives the first main phase of development by orchestrating ductal elongation from proliferative structures known as terminal end buds that invade the surrounding stroma and bifurcate creating new primary branches. These branches together with secondary lateral side branching loosely fill the mammary gland fat pad (Macias & Hinck 2012). The adult mammary gland continues to undergo additional tertiary branching in response to cyclical ovarian estrus cycles further developing a highly branched epithelial network with the development of alveolar-like structures that cyclically regress until the onset of pregnancy (Sternlicht *et al.* 2006).

The second phase of mammary gland development occurs following the onset of pregnancy and is characterized by massive amounts of cell proliferation and tissue remodeling (Hennighausen & Robinson 2005). The gland undergoes extensive alveolar development to acquire a secretory lobuloalveolar phenotype (Oakes *et al.* 2006). During early pregnancy, prolactin and progesterone are the main drivers of extensive branching of ducts and alveolar bud formation that continues during late pregnancy and into lactation (Macias & Hinck 2012). As a result, the volume ratio of epithelial cells to adipocytes increases as the epithelial compartment expands and adipocytes undergo delipidation (Anderson *et al.* 2007). In parallel to the

morphogenic changes in the pregnant mammary gland, luminal epithelial cells undergo differentiation as many genes involved in milk synthesis begin to be expressed during midpregnancy (Anderson *et al.* 2007). With the withdrawal of progesterone at parturition, the gland undergoes secretory activation as the mammary gland is set up as an exocrine gland to perform its main function to produce, secrete, and deliver milk (Pang & Hartmann 2007). The gland functions to produce milk until weaning of the pups where the buildup of milk within alveoli acts as a trigger for the mammary gland to undergo involution. This two-step process is characterized by extensive cell death and remodeling that returns the mammary gland back to the adult gland state (Watson & Kreuzaler 2011). The first phase of involution is characterized by extensive cell death, triggered by the activation of the LIF/Stat3 pathway, while the second phase of involution is characterized by remodeling of the extracellular matrix, adipocyte differentiation, and alveolar collapse (Macias & Hinck 2012).

Expression of connexins in the rodent mammary gland

Up until about 2004, the majority of studies evaluating connexins in the rodent mammary gland focused on characterizing connexin expression, localization, and regulation (Fig. 1). Gap junctions were first detected joining ductal epithelial cells in 3 to 9-week old mammary glands of mice (Pitelka *et al.* 1973). These likely represented gap junctions made from Cx43 (*Gja1*) as the vast majority of studies agree that Cx43 is expressed in the basal epithelium and stroma, and further represents the major connexin expressed in the non-pregnant rodent mammary gland (Pozzi *et al.* 1995, Locke *et al.* 2004, Talhouk *et al.* 2005, Lambe *et al.* 2006, Plante & Laird 2008). Cx43 is also reported to be transcriptionally upregulated in 5 to 6-week old virgin mice, suggesting a role in the development of pubertal mammary gland (Lambe *et al.* 2006). Other connexins expressed between luminal cells of the non-pregnant mammary gland are Cx26, Cx32, and Cx30 (Talhouk *et al.* 2005, Plante & Laird 2008). However, these connexins remain poorly characterized based on low connexin expression, poor sensitivity of anti-connexin antibodies, variations in tissue processing, and differences in mouse strains being investigated (Monaghan *et al.* 1994, Locke *et al.* 2000, 2004, Yamanaka *et al.* 2001).

Following the onset of pregnancy, the connexin expression landscape changes as Cx26 and Cx30 are upregulated and begin to become the dominant connexins expressed within the mammary gland during pregnancy, suggesting a role in the regulation of mammary gland alveologenesis and differentiation (Monaghan *et al.* 1994, Talhouk *et al.* 2005). In the case of Cx26, the majority of studies support a modest upregulation by day 5 of pregnancy (Monaghan *et al.* 1994, Locke *et al.* 2004),

a dramatic upregulation at day 9–10 of pregnancy (Locke *et al.* 2000, 2004, Talhouk *et al.* 2005), before peaking near parturition (Monaghan *et al.* 1994, Pozzi *et al.* 1995, Locke *et al.* 2000, 2004, Yamanaka *et al.* 2001, Stewart *et al.* 2014). Alternatively, Cx30 appears to increase between days 10.5 and 12.5 of pregnancy before peaking in late pregnancy before parturition (Talhouk *et al.* 2005, Locke *et al.* 2007). When Cx26 and Cx30 are coexpressed in mammary epithelium, they colocalize into the same gap junction plaque and form heteromeric channels *in vitro* (Locke *et al.* 2007). In contrast, Cx32 expression remains low throughout pregnancy before extensive upregulation following the onset of lactation, suggesting a role in regulation of the secretory phenotype of the gland as opposed to regulation of alveologenesis (Locke *et al.* 2000, Yamanaka *et al.* 2001, Talhouk *et al.* 2005). In myoepithelial cells, Cx43 protein expression undergoes a slight increase during pregnancy, associated with a shift to more highly phosphorylated forms of Cx43 which are commonly associated with greater GJIC (Yamanaka *et al.* 1997, El-Sabban *et al.* 2003, Talhouk *et al.* 2005). Changes in Cx43 correlate with the expression of MAP kinases ERK1/2 and JNK in primary rat mammary epithelial cells and may be indicative of more functionally active gap junctions, although this has not been demonstrated directly *in vivo* (Seo *et al.* 2006).

Following parturition, gap junctions between luminal epithelial cells lining alveoli become larger and less numerous than those during pregnancy (Pitelka *et al.* 1973) because elevated and intermixed luminal Cx26, Cx30, and Cx32 channels continue to be colocalized (Locke *et al.* 2000, 2007, Yamanaka *et al.* 2001). The different temporal onset of Cx26, Cx30, and Cx32 as the gland transitions from pregnancy through lactation promotes discrete heteromeric and homomeric gap junctions with distinct permeabilities of regulatory molecules IP₃, cAMP, and cGMP, as well as differences in channel insensitivity to closure by the osmolyte taurine present during milk protein synthesis (Locke *et al.* 2004, 2007). The physiological importance of this remains chiefly unknown and understudied due largely to a lag in the development of assays to measure channel activity *in vivo*. Heteromeric channels consisting of Cx30 appear to become obsolete following day 7 of lactation because Cx30 levels decrease to a pre-pregnant state (Talhouk *et al.* 2005, Locke *et al.* 2007). Typical of exocrine glands, most studies agree that both Cx26 and Cx32 are reported to maintain elevated expression throughout lactation compared with the virgin gland, until the onset of involution (Locke *et al.* 2000, 2004, Yamanaka *et al.* 2001, Serre-Beinier *et al.* 2002, Talhouk *et al.* 2005). Myoepithelial cells on the other hand continue to readily express Cx43 in surrounding alveoli throughout lactation (Pitelka *et al.* 1973, Yamanaka *et al.* 1997). The majority of studies suggest that there is a lack of gap junctions between luminal and myoepithelial cells of alveoli as historically assessed by electron

microscopy (Pitelka *et al.* 1973, Yamanaka *et al.* 1997). However, more recent evidence has suggested that Cx43 may be localized near myoepithelial–luminal cell contacts based on immunofluorescence, suggesting possible heterocellular interactions (Talhouk *et al.* 2005). However, as Cx43 has not readily been described to be expressed in luminal cells of mice, it remains difficult to interpret which connexin expressed in luminal cells would be able to dock with Cx43 in myoepithelial cells. Future electron microscopy studies may best answer whether gap junctions are present between the luminal and myoepithelial cells of alveoli.

Unlike lactation, our knowledge of the expression of connexins during involution is limited. During the first 48 h of involution, large gap junctions between luminal cells of alveoli, similar to those found in lactating mice, are present (Pitelka *et al.* 1973). These gap junctions likely represent Cx26 as Cx32 levels drop dramatically following the onset of involution (Locke *et al.* 2004). Cx43 appears also to be expressed and has been described to increase between 6 and 48 h in the involuting mammary gland following weaning of the pups (Lambe *et al.* 2006). However, both Cx43 and Cx26 return to the pre-pregnant state of expression following 1 week of gland involution (Monaghan *et al.* 1994, Pozzi *et al.* 1995, Talhouk *et al.* 2005).

Expression of connexins in the human breast

Unlike that of the rodent gland, knowledge of connexin expression in the human breast is currently restricted to the non-pregnant adult (Fig. 2; Wilgenbus *et al.* 1992, Pozzi *et al.* 1995, Monaghan *et al.* 1996, Jamieson *et al.* 1998, Laird *et al.* 1999, Teleki *et al.* 2014). Spatial and temporal connexin expression in human breast development remains understudied due to the difficulty in obtaining pubertal, pregnant, and lactating human samples. Similar to that of the rodent gland, Cx43 is readily detected in the nonpregnant breast, localizing mainly with the myoepithelial marker keratin14 forming gap junctions between basal cells of both the major ducts and lobules (Wilgenbus *et al.* 1992, Pozzi *et al.* 1995, Monaghan *et al.* 1996, Jamieson *et al.* 1998, Laird *et al.* 1999, Teleki *et al.* 2014). In addition, sparse Cx43 gap junction plaques between luminal cells have been reported, raising the potential for Cx43 gap junctions between myoepithelial and luminal cells, although this is likely a rare event based on a lack of evidence for luminal–myoepithelial gap junctions at the ultrastructural level (Monaghan *et al.* 1996, Laird *et al.* 1999). In addition, Cx26 has been observed localized between luminal epithelial cells (Monaghan *et al.* 1996, Teleki *et al.* 2014). Cx26 expression is likely low and its expression may reflect dynamic hormonal variation due to monthly cycles or inter-patient variability as many studies have failed to detect Cx26 in ducts or lobules based on immunolabeling and electron

microscopy (Wilgenbus *et al.* 1992, Pozzi *et al.* 1995, Monaghan *et al.* 1996, Jamieson *et al.* 1998).

Until recently, no other connexin was reported in the human mammary gland. However, Teleki *et al.* (2014) have recently described both Cx32 and Cx30 in human breast luminal epithelial cells, similar to that found in the mammary glands of rodents, and Cx30 appeared to colocalize with Cx26. Cx30 was also described to be expressed within the microvasculature and myoepithelial cells, which in the latter case raises the possibility of heterocellular gap junctions between Cx26 and Cx30 expressed in luminal cells (Teleki *et al.* 2014). Intriguingly, these authors also identify Cx46 between luminal, myoepithelial, and inflammatory cells, despite a lack of expression in the rodent mammary gland, suggesting possible species-specific differences between human and rodent mammary glands (Locke *et al.* 2004, Teleki *et al.* 2014). Limitations of the Teleki study include low sample size and a lack of double immunolabeling with cell type-specific markers. However, while others have found only Cx43 and Cx26 in the human breast, the Teleki *et al.* study argues that a more rigorous re-evaluation of connexin expression at all stages of human breast development is needed. Importantly, these findings also suggest that connexin expression in the human breast may parallel more closely to that found in the mammary glands of rodents than previously thought.

Role of connexins in mouse mammary gland development

With the development of genetically modified mice expressing either loss-of-function connexin mutants or lacking a specific connexin (Table 1), the depth and understanding of connexins in the mammary gland has dramatically increased.

Embryonic mammary development

Mammary gland embryogenesis is mediated mostly by local mechanisms through epithelial–stromal interactions, suggesting a possible role for gap junctions

in this process (Parmar & Cunha 2004). To date, no study has evaluated connexin expression in prenatal mammary gland development, which is not surprising given that the majority of gland development occurs after birth (Levin 2002). However, a limited number of indirect studies suggest that connexins are not critical mediators of this process. The strongest argument for this stems from the fact that all Cx43, Cx26, Cx30, and Cx32 genetically modified mice where the connexin is ablated or mutated can proceed to develop at least some kind of a rudimentary mammary gland, while others appear to exhibit no gland defects at all (Table 1). An obvious explanation for this may be that the impairment or loss of one connexin is compensated by another during this stage of development. Alternatively, another connexin family member may be expressed, which is not typically expressed at a similar stage of development, although no evidence exists for either of these two possibilities. In fact, adult stem/progenitor cells isolated from human mammary gland (Kao *et al.* 1995, Trosko & Chang 2003) or from the bovine mammary gland (Holland *et al.* 2003) were found to lack GJIC, inferring that stem/progenitor cells in the mammary gland during embryogenesis may also lack GJIC. An in-depth screening of connexins during embryogenesis is necessary to determine if, and when, connexins are embryonically expressed in the developing gland.

Virgin mammary gland

Cx43 is the most highly expressed connexin in the virgin mammary gland, and is reported to be upregulated during puberty (Lambe *et al.* 2006). Hormonal control of ductal elongation at puberty is driven by estrogen and GH signaling, as estrogen receptor alpha null (ER α KO) and GH receptor null (GHRKO) mice both exhibit absent or impaired ductal elongation, respectively, without affecting rudimentary gland growth before puberty (Gallego *et al.* 2001, Mueller *et al.* 2002). Although estrogen, GH, as well as hormone insulin-like growth factor 1 has been implicated in the regulating the expression of Cx43 in other *in vitro* models, it still

Figure 1 Connexin expression throughout mouse mammary gland development. (A) Schematic rendering of Cx43 (green), Cx26 (red), Cx32 (blue), and Cx30 (yellow) expression throughout mammary gland development based on the body of evidence discussed in this review. (B) The first main phase of mammary gland development occurs through puberty as a rudimentary duct structure extends and branches throughout the fat pad. The virgin mammary gland epithelium contains luminal cells (brown) expressing low basal levels of Cx26, Cx30, and Cx32 (not shown) throughout virgin gland development. Luminal cells surrounded by myoepithelial cells (peach) connected by gap junctions composed of Cx43 (green) that are upregulated during puberty. The epithelium is embedded in the stroma that contains mainly adipocytes (clear), as well as fibroblasts (yellow) that express Cx43, separated by a basement membrane (dark blue). (C) The second main phase of development occurs following the onset of pregnancy as extensive lobuloalveolar development prepares the mammary gland to produce and deliver milk during lactation before the gland undergoes involution where extensive cell death and remodeling returns the gland to a pre-pregnant state. Cx26 and Cx30 are dramatically upregulated in luminal alveolar cells during pregnancy, while Cx32 is upregulated following parturition during early lactation. By mid lactation, Cx30 expression reverts back to the levels expressed in the virgin mammary gland, while Cx32 expression occurs at the onset of involution. Cx26 remains elevated throughout lactation and in the early stages of involution in luminal alveolar cells. In some instances, these connexins may selectively intermix to form heteromeric and heterotypic gap junction channels. Myoepithelial cells continue to express Cx43 gap junctions throughout pregnancy, lactation, and involution. Each developmental stage is paired with a representative epithelial architecture of the mice mammary gland as revealed by whole-mount analysis using carmine alum staining.

Figure 2 Connexin expression in the non-pregnant human breast. (A) Alveoli (blue) are connected to openings at the nipple by multiple converging duct systems (yellow). (B) The mammary epithelium consists of luminal cells (brown) connected by gap junctions composed of Cx26 (red), surrounded by myoepithelial cells (peach) connected by Cx43 (green) gap junctions. The epithelium is separated by a basement membrane (dark blue) from the connective tissue containing fibroblasts (yellow) linked by Cx43 gap junctions.

remains unclear whether these hormones regulate Cx43 in the pubertal mammary gland (Aberg *et al.* 2000, 2003, Ren *et al.* 2013). Thus, it remains important to assess whether the levels of Cx43 change in ER α KO or GHRKO mice during puberty to identify upstream regulators of Cx43. Importantly, genetically modified mice where Cx43 is expressed but not fully functional have revealed impaired ductal phenotypes similar to ER α KO or GHRKO mice (Table 1).

One mutant mouse line that has proven to be insightful in our understanding of the mammary gland is Cx43^{G60S/+} mutant mice. These mice mimic the human developmental disease known as oculodentodigital dysplasia

(ODDD). ODDD mice harbor a systemic autosomal-dominant mutation in the gene that encodes Cx43, which yields a glycine to serine substitution at position 60, rendering total Cx43 function to only <30% of normal levels (Flenniken *et al.* 2005). Evaluation of 4 to 10-week virgin mutant mice revealed a severe delay in ductal elongation, as well as stromal impairment compared, with control mice in prepubertal, pubertal, and adult mice, highlighting a role for Cx43 in the regulation of epithelial morphogenesis and stromal development in the virgin gland (Plante & Laird 2008). In addition, a second mutant mice model of ODDD that harbors a Cx43^{I130T} mutant, yielding an isoleucine-to-threonine substitution at position

Table 1 Genetically modified mice used to evaluate the role of connexins in the mammary gland.

Mouse line	Background (DNA; ES cell; Blastocyst; Cross)	Modification	Mammary gland phenotype	Reference
Cx43 ^{+/-}	129 SV; 129 R1; C57BL/6	Loss of one allele of Cx43	No defect reported	Reaume <i>et al.</i> (1995)
Cx43 ^{Cx40/+}	129 SV; HM1; C57BL/6; C57BL/6	Cx43 allele replaced with Cx40	Normal development and function	Plum <i>et al.</i> (2000)
Cx43 ^{Cx26/+}	129 SV; HM1; C57BL/6; C57BL/6 and Flp	Cx43 allele replaced with Cx26	Reduced branching of ductuli	Winterhager <i>et al.</i> (2007)
Cx43 ^{Cx32/+}	129 SV; HM1; C57BL/6; C57BL/6 mammary transplant into cleared fat pads of nude mice	Cx43 allele replaced with Cx32	Normal gland development	Bry <i>et al.</i> (2004)
Cx43 ^{Cx32/+}	129 SV; HM1; C57BL/6; C57BL/6	Cx43 allele replaced with Cx32	Milk ejection defect	Plum <i>et al.</i> (2000)
Cx43 ^{I130T/+}	129 R1; 129 R1; C57BL/6; CD1 and C57BL/6	Point mutation in the <i>Gja1</i> gene causes I130T substitution in one allele	Virgin – delayed ductal development in pre-pubertal mice Lactation – normal development and function	Stewart <i>et al.</i> (2013)
Cx43 ^{G60S/+}	ENU mutagenized C3H/HeJ; FVB; C3; C57BL/6	Point mutation in the <i>Gja1</i> gene causes G60S substitution in one allele	Virgin – delayed ductal and stromal development Lactation – milk stasis and defect in milk ejection	Plante & Laird (2008) and Plante <i>et al.</i> (2010)
Cx32 ^{-/-}	BALB/c; 129 SV J1; C57BL/6; C57BL/6	Loss of Cx32	Normal Gland Development	Bry <i>et al.</i> (2004)
Cx30 ^{-/-}	129 SV; HM1; C57BL/6; C57BL/6	Loss of Cx30	Fertile, lactate; no rigorous assessment reported	Teubner <i>et al.</i> (2003)
Cx26 ^{fl/fl} x MMTV-Cre	Cx26 ^{fl/fl} mice – BALB/c; HM1; C57BL/6; C57BL/6 MMTV-Cre mice – not described	Conditional deletion of Cx26 prior to birth	Virgin – normal development Pregnancy – increased apoptosis and decreased alveologenesis	Bry <i>et al.</i> (2004)
Cx26 ^{fl/fl} x WAP-Cre	Cx26 ^{fl/fl} mice – BALB/c; HM1; C57BL/6; C57BL/6 WAP-Cre mice – not described	Conditional deletion of Cx26 ~D17 of pregnancy	Normal development and function	Bry <i>et al.</i> (2004)
Cx26 ^{fl/fl} x BLG-Cre	Cx26 ^{fl/fl} – BALB/c; HM1; C57BL/6; C57BL/6 BLG-Cre mice – CBA; C57BL/6	Conditional deletion of Cx26 ~D12.5 of pregnancy	Normal development and function	Stewart <i>et al.</i> (2014)

130, further elucidated our understanding of the role of Cx43 in puberty. $Cx43^{T130T/+}$ mice retain 50–60% normal Cx43 function and revealed similar ductal development following puberty as compared with control mice despite showing delayed ductal elongation in prepubertal mice. In addition, these mice maintained normal stromal development (Stewart *et al.* 2013). The combination of these Cx43 mutant mice studies highlight that a specific threshold of Cx43 is needed to maintain normal ductal morphogenesis that is correlated with GJIC. This is supported by the finding that virgin glands develop normally despite maintaining only one allele of Cx43 as observed in heterozygous $Cx43^{Cx32/+}$ mice, in which one allele of Cx43 was replaced with that of Cx32, as well as $Cx43^{+/-}$ mice, in which one allele for Cx43 has been removed (Reaume *et al.* 1995, Bry *et al.* 2004). However, as defects are observed in both, whether Cx43 is important in the epithelial and/or stromal compartments remains to be determined. Future studies where mutant $Cx43^{G60S/+}$ mouse mammary epithelium is transplanted into WT mouse stroma and vice versa are needed to clarify the role that Cx43 plays in ductal morphogenesis.

In the case of Cx26 and Cx32, which are normally expressed in mammary luminal cells, it is not surprising that mammary glands with conditionally ablated Cx26 and global Cx32-null mice exhibit normal virgin gland architecture as their expression is low in the non-pregnant mammary gland (Bry *et al.* 2004). Similarly, Cx30 appears dispensable in the virgin gland and also in the developing gland, as these mice are fertile and able to lactate, suggesting that a ductal network has been formed during adolescence, although a detailed evaluation of the virgin mammary gland in Cx30-null mice has not been performed (Teubner *et al.* 2003). Taken together, Cx43 appears to regulate ductal elongation and stromal development in the virgin mammary gland.

Mammary gland during pregnancy

Following the onset of pregnancy, Cx26 upregulation is likely driven by prolactin signaling because transcript profiling of mammary glands from prolactin receptor knockout mice (PrIRKO) revealed a downregulation of Cx26 compared with control mice during early pregnancy (Ormandy *et al.* 2003). In support of this, a binding site for STAT5, a downstream mediator of prolactin signaling, has been reported in the promoter of the Cx26 gene (Kiang *et al.* 1997). As Cx30 is also upregulated during pregnancy and colocalizes with Cx26, it is likely that it is also regulated by prolactin. Indeed, Talhouk *et al.* (2005) observed that exogenous administration of prolactin and the corticosteroid dexamethasone were able to upregulate Cx30 expression in mice mammary epithelial cells *in vitro*. Thus, both Cx26 and Cx30 appear to be regulated by the hormone prolactin.

Apart from prolactin signaling, Cx26 has also been shown to be upregulated by the administration of exogenous human chorionic gonadotropin (hCG) in rat mammary glands and *ex vivo* organ culture, through downstream activation of Sp transcription factors (Tu *et al.* 1998, You *et al.* 1998). However, as hCG treatment coincided with an increase in both 17 β -estradiol and progesterone, it remains unclear how each hormone individually affects the levels of Cx26 or how the presence of multiple hormones alters the expression of Cx26 (You *et al.* 1998). Importantly, both estrogen and progesterone have been reported to regulate Cx26 expression in the endometrium of the uterus, suggesting that ovarian steroidal hormones may also regulate Cx26 in the mammary gland (Grummer *et al.* 1994). It remains unknown whether Cx30 can also be hormonally regulated by hCG and ovarian steroids. Further studies are needed to understand hormonal regulation of Cx26 and Cx30, particularly in the more physiological context of multiple hormones.

Cx26 was first identified to be important in alveolar development during pregnancy following conditional deletion of Cx26 before birth by crossing $Cx26^{fl/fl}$ mice with mice expressing *Cre* under the mouse mammary tumor virus promoter (MMTV; $Cx26^{fl/fl}$) (Bry *et al.* 2004). These mice exhibited impaired alveologenesis and reduced the ability of the dams to feed their pups as a result of increased apoptosis (Bry *et al.* 2004). As such, the working model suggested that in response to prolactin and progesterone-driven alveologenesis, Cx26 is upregulated to promote epithelial cell survival within the pregnant mammary gland (Bry *et al.* 2004). However, two other conditionally ablated Cx26 mice models were created crossing $Cx26^{fl/fl}$ mice with mice expressing *Cre* under the β -lactoglobulin (BLG; $Cx26^{fl/fl}$) or whey acidic acid (WAP) promoter (WAP; $Cx26^{fl/fl}$), yielding Cx26 silencing during mid pregnancy or Cx26 ablation during late pregnancy respectively. Unexpectedly, these mice revealed normal gland development and function, suggesting one of three possibilities (Bry *et al.* 2004, Stewart *et al.* 2014). Firstly, as low levels of Cx26 were observed in BLG; $Cx26^{fl/fl}$ mice, a basal level of Cx26, and not the physiological surge, is sufficient to maintain normal mammary gland development. Secondly, Cx26 may be more important earlier in gland development than at day 12.5 when Cx26 is ablated in BLG; $Cx26^{fl/fl}$ mice. Based on the MMTV; $Cx26^{fl/fl}$ and BLG; $Cx26^{fl/fl}$ mutant mice, they predict the critical window for Cx26 in mammary gland development between ~ 9.5 and ~ 12.5 of pregnancy (Bry *et al.* 2004, Stewart *et al.* 2014). Finally, it remains to be seen whether the loss of Cx26 can be compensated by other connexins, such as Cx30. However, mutant mice studies thus far would suggest that this is not likely the case as conditional silencing of Cx26 in mid pregnancy also delayed the upregulation of Cx30 during pregnancy, suggesting that there is crosstalk between

Cx26 and Cx30 (Stewart *et al.* 2014). A detailed evaluation of the mammary gland in Cx30^{-/-} mice is lacking, but it would be interesting to evaluate changes in Cx26 to determine whether there is a reciprocal crosstalk between Cx30 and Cx26 (Teubner *et al.* 2003). In addition, if Cx26 and Cx30 are coregulated, breeding of Cx30^{-/-} mice with mammary gland-ablated Cx26 mice may yield a more severe mammary gland phenotype that would also address the issue of compensation.

Apart from extensive lobuloalveolar development that occurs in the mammary gland during pregnancy, the gland must also undergo differentiation as it prepares for secretory activation. To date, reduced or ablated Cx26, Cx32, Cx43, or Cx30 function suggests that loss of one connexin does not impair epithelial differentiation. For example in MMTV;Cx26^{fl/fl}-mutant mice, evaluation of numerous markers of epithelial differentiation including β -catenin, keratin 5, smooth muscle actin, E-cadherin, and the NaKCl cotransporter 1 suggested that the gland differentiated normally, despite reduced lobuloalveolar development, suggesting that Cx26 is not required to maintain normal epithelial differentiation (Bry *et al.* 2004). Similarly, loss of Cx43 function in Cx43^{G60S/+} mice was not associated with changes in the expression of E-cadherin, P-cadherin, β -catenin, occludin, or claudin 1 molecules involved in tight and adherens junctions (Plante *et al.* 2010). In both these mice models, as well as in Cx32^{-/-} and Cx30^{-/-}-mutant mice, milk was produced, suggesting normal secretory differentiation of the gland and that no single connexin is essential for the production of milk, although a more rigorous assessment has not been performed (Teubner *et al.* 2003, Bry *et al.* 2004, Plante *et al.* 2010). Taken together, it is apparent that the loss of one connexin does not critically impede normal epithelial and secretory differentiation of the gland but the loss of Cx26 does impair normal alveologenesis.

Lactating mammary gland

Following parturition, Cx32 is dramatically upregulated and appears to be under the control of prolactin signaling as both *PrLrko* and *Stat5*-null mice failed to express Cx32 during lactation (Miyoshi *et al.* 2001). Importantly, as both Cx26 and Cx30 are also suggested to be upregulated by prolactin but exhibit distinct temporal regulation compared with that of Cx32, additional hormonal or local mechanisms also likely influence the expression of Cx32. The expression of Cx32 and Cx26 during lactation is similar to that of all exocrine glands and is believed to function through the diffusion of ionic and molecular gradients allowing for coordinated secretion of adjacent cells in exocrine glands (Serre-Beinier *et al.* 2002). Despite this, both Cx32-null and mammary gland ablated Cx26-mice have revealed histological evidence of milk and lipid droplets in the

lumen of alveoli, suggesting normal secretion of the gland (Bry *et al.* 2004, Stewart *et al.* 2014). However, the reduction in Cx43 function in the lactating gland of Cx43^{G60S/+} mice led to the presence of large cytoplasmic lipid droplets in luminal cells that have previously been implicated in delayed secretory activation (Anderson *et al.* 2007, Plante *et al.* 2010). As a result, only myoepithelial Cx43, and not luminal cell, that expressed Cx26 and Cx32 have been implicated in regulating the luminal secretory phenotype of the mammary gland albeit only when Cx43 function is greatly impaired.

Apart from the production and secretion of milk, the lactating mammary gland must coordinate the contraction of myoepithelial cells to propel milk from the alveoli through the converging duct system toward the nipple. Cx43 was first suggested to contribute to the coupling of myoepithelial cells during milk ejection based on a shift in the phosphorylation state of Cx43 in the myoepithelial cells during lactation (Yamanaka *et al.* 1997, Talhouk *et al.* 2005). Although the hormonal control regulating the Cx43 phosphorylation shift and its increased expression are unknown. Likely candidates include estrogen and oxytocin because these have been shown to regulate the levels of Cx43 in the uterus and mice embryonic stem cells respectively (Risek *et al.* 1995, Yun *et al.* 2012).

Evidence of a role for Cx43 in milk ejection *in vivo* was not obtained until the assessment of knockin Cx43 mouse models in which one allele of Cx43 was replaced by Cx32 (Cx43^{Cx32/+}), Cx26 (Cx43^{Cx26/+}), or Cx40 (Cx43^{Cx40/+}). For example, mammary glands of lactating Cx43^{Cx32/+} and Cx43^{Cx26/+} were unable to feed their pups despite evidence of milk production and secretion (Plum *et al.* 2000, Winterhager *et al.* 2007). Surprisingly, Cx43^{+/-} mice have no reported defect in mammary gland development or function, thus it may be the additional expression of Cx26 and Cx32 in myoepithelial cells, rather than the 50% loss of function of Cx43, which is ultimately responsible for the gland defects (Reaume *et al.* 1995). Although not directly demonstrated, myoepithelial cells engineered to express Cx26 and Cx32 could allow for gap junctions between luminal and myoepithelial cells creating a gap junction channel-mediated exchange that does not normally exist (White & Bruzzone 2000). Conversely, when Cx43 is replaced with Cx40 in Cx43^{Cx40/+} mice, there is no luminal/myoepithelial exchange possibility because Cx40 in the myoepithelial cells is unable to form heterotypic channels with the Cx26, Cx30, or Cx32 found in luminal cells, and these mice continue to lactate and feed their pups (Plum *et al.* 2000).

The critical role for Cx43 in milk ejection was established later as the reduction in Cx43 by 70% in Cx43^{G60S/+} mice led to a defect in the ability of the mammary gland to respond to oxytocin despite normal expression of the oxytocin receptor (Plante & Laird 2008). Thus, it was concluded that Cx43 functions to coordinate

synchronous contractions of myoepithelial cells for proper milk ejection (Plante & Laird 2008). This was corroborated by others where WNT5A overexpression in human MCF10A cells led to an increase in Cx43 phosphorylation at serine P368 and a decrease in GJIC, suggesting that WNT5A negatively regulates Cx43 function (Baxley *et al.* 2011). Indeed, WNT5a overexpression in the mammary glands of lactating mice resulted in a similar inability of these mice to respond to exogenous oxytocin, further supporting a critical role of Cx43 in coordinating timely and proper milk ejection (Baxley *et al.* 2011). Oxytocin signaling is mediated through the oxytocin receptor coupled to $G_{\alpha q}$ 11 and phospholipase C activation, triggering the release of Ca^{2+} from intracellular stores (Reversi *et al.* 2005). Ca^{2+} has previously been shown to pass through Cx43 gap junctions, making it interesting to speculate that Cx43 functions via GJIC-dependent mechanisms to coordinate synchronous contraction via transfer of Ca^{2+} (Harris 2007). This is supported by the fact that Cx43^{1130T/+} mice with ~50% Cx43 function retain full myoepithelial function (Stewart *et al.* 2013). In summary, the evidence to date points to a critical GJIC-dependent role for Cx43 in coordinating myoepithelial contraction and milk ejection; however, a milk ejection defect is only present when Cx43 function is found to be well below 50%.

Involuting mammary gland

Only Cx26 and Cx43 are described as being expressed during the first 48-h involution. To date, no evidence exists that would suggest connexins are critical for the mammary gland to undergo involution. Both genetically modified mice with either a Cx26-knockdown mammary gland or expressing the loss-of-function Cx43^{1130T} mutant have revealed similar epithelial remodeling and cell death during involution compared with control mice (Stewart *et al.* 2013, 2014). In addition, Cx26-ablated mammary gland did not initiate precocious involution of the gland despite an increase in the number of apoptotic cells during pregnancy because STAT3 phosphorylation, a marker and critical regulator of involution, did not increase (Bry *et al.* 2004). On the basis of limited evidence to date, connexins appear not to be drivers in the initiation or progression of epithelial cell death during mammary gland involution, but instead act like passengers that are altered following transition from the lactation phase of mammary gland development.

Concluding remarks

Our assessment of gap junctions in the mammary gland using genetically modified mice has established a role for Cx43 in ductal elongation during puberty, Cx26 in alveologenesis during pregnancy, and Cx43 in milk ejection during lactation. Future studies may use these

tools to further expand our understanding of connexins in the mammary gland. Firstly, knock-in strategies using the *LacZ* reporter gene may be useful to support previous connexin expression studies as was used to assess the expression of Cx30 in the heart and Cx26 in the brain (Filippov *et al.* 2003, Gros *et al.* 2010). Secondly, Cx43-mutant mice models or conditional knockout mouse models of Cx26 may be used to more closely evaluate specific features of development, which are not possible *in vitro*. These could include a closer examination of the possible role for Cx43 in terminal end-bud growth during the pubertal stage of development or more rigorous assessment of involution using multiple time points respectively. In addition, to provide further insights into the cell-specific function of Cx43, the role of Cx43 in myoepithelial cells may be evaluated using conditional deletion under the K14 promoter. Alternatively, to better assess how loss of connexins affects mammary gland function, a quantitative evaluation of milk components following the milking of mice with reduced connexin function may be useful to reveal more subtle changes in milk secretion (DePeters & Hovey 2009). Perhaps most importantly, the cross-breeding strategy of Cx30 and Cx32-knockout mice with *Dox*-inducible conditional knockout of Cx26 may be best to tackle issues of compensation and establish a clear role for luminal connexins in the mammary gland during pregnancy (Gunther *et al.* 2002). Although the details by which gap junctions regulate the development or function of the gland remains unclear, genetically modified mice have provided the tools to more rigorously assess previous predictions based on temporal connexin expression patterns *in vivo* and *in vitro* studies.

Importantly, it has become clear that our understanding of connexins in the mouse mammary gland may have implications extending to a wider appreciation of connexins in human health. For example, mutations in the genes encoding Cx43 and Cx26 give rise to ODDD and hearing loss respectively (Apps *et al.* 2007, Paznekas *et al.* 2009). In the case of Cx43, the rarity of ODDD in the human population and scarcity of clinical records make it difficult to assess whether these patients have breast function or development abnormalities (Paznekas *et al.* 2009). Nevertheless, our mutant mouse model studies suggest that distinct populations of ODDD patients may present with breast feeding problems that is dependent on the severity of the Cx43 mutant on overall Cx43-based GJIC (Plante & Laird 2008, Stewart *et al.* 2013). However, unlike ODDD, deafness linked to Cx26 mutations is incredibly prevalent in the human population. In fact, it has been estimated that 17.3% of worldwide hearing loss is associated with biallelic mutations in the gene that encodes Cx26 (Chan & Chang 2014). On the basis of mice studies where loss of Cx26 function impaired normal lobuloalveolar development of the gland, these patients may represent a yet unidentified population with increased risk of

developing breast feeding defects (Bry *et al.* 2004). However, as yet, there are no epidemiology studies to determine whether female patients harboring a subclass of loss-of-function or gain-of-function Cx26 mutants have any breast defects as the data set is difficult to ascertain. In addition, both Cx43 and Cx26 are implicated in other breast-related diseases such as cancer, which has previously been reviewed by El-Saghir *et al.* (2011), although it remains unclear whether patients with loss-of-function mutations in the genes that encode Cx43 or Cx26 are predisposed (Naus & Laird 2010). Ultimately, our understanding of the role of gap junction proteins in normal breast physiology will provide future insights into the pathologies of the breast and the possible development of targeted therapeutics.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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