Physiological roles of connexins in labour and lactation

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Abstract

The connexin family of proteins are best known as oligomerizing to form intercellular membrane channels (gap junctions) that metabolically and ionically couple cells to allow for coordinated cellular function. Nowhere in the body is this role better illustrated than in the uterine smooth muscle during parturition, where gap junctions conduct the contraction wave throughout the tissue to deliver the baby. Parturition is followed by the onset of lactation with connexins contributing to both the dramatic reorganization of mammary gland tissue leading up to lactation and the smooth muscle contraction of the myoepithelial cells which extrudes the milk. This review summarizes what is known about the expression and roles of individual connexin family members in the uterus during labour and in the mammary glands during development and lactation. Connexin loss or malfunction in mammary glands and the uterus can have serious implications for the health of both the mother and the newborn baby.

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Introduction

Gap junction connexins comprise a family of 21 genes and cognate proteins in humans. Their best known function is to guarantee direct communication properties between adjacent cells. Connexins are integral membrane proteins with N- and C-terminal cytoplasmic domains linked by four transmembrane domains, an intracellular loop, and two extracellular loops. The different connexins vary greatly in size mostly due to the length of the C-terminal segment and the proteins are named with respect to their relative molecular masses: connexin43 (CX43), for example, is a ~43 kDa protein. Individual connexins oligomerize to form hexameric structures called connexons or hemichannels in which a central core forms a hydrophilic channel. When inserted into the plasma membrane of a cell, connexons can stand alone or can dock end-to-end with connexons in opposing cells to form intercellular channels that gather into tightly packed arrays known as gap junctions (see Laird (2010) for a comprehensive review of connexin biology). Gap junction channels allow direct sharing of inorganic ions such as Ca^{2+} as well as small organic metabolites and signalling molecules, including cAMP, cGMP and IP_3 , between cells without the need for passage through the extracellular space, a phenomenon known as gap junctional intercellular communication (GJIC; Söhl & Willecke 2003, Harris 2007, Yeager & Harris 2007). Many cells express multiple connexins, a situation that enables them to form heteromeric

connexons (by co-oligomerization) or heterotypic intercellular channels (by docking of connexons of different connexin composition) (Meşe *et al.* 2007, Koval *et al.* 2014). These variations in connexin composition result in changes in channel properties including ionic conductance and sensitivity to regulation by pH, transjunctional voltage and phosphorylation (Meşe *et al.* 2007, Harris & Contreras 2014). Moreover, a plethora of studies has demonstrated that connexin expression can be modulated at different post-transcriptional steps, although regulation of connexin gene transcription is accepted as an important mechanism in controlling the temporal pattern and amount of connexin protein needed for GJIC. Agents such as steroid hormones and cytokines are well known to upregulate cell-type nonspecific or specific transcription factors required for connexin gene transcription in different organs (for review, see Oyamada *et al.* (2013)). Regulation of the different connexins plays a significant role in embryogenesis, cell proliferation, apoptosis, migration and differentiation (Haass *et al.* 2004, Levin 2007, Aasen 2014, Carette *et al.* 2014). Not surprisingly, altered connexin expression and/or function have been shown to contribute to several diseases and carcinogenesis (Dobrowolski & Willecke 2009, Laird 2010, Naus & Laird 2010, Defamie *et al.* 2014). In particular, connexins are required for several female reproductive functions associated with early stages of pregnancy, which has been reviewed recently (Winterhager & Kidder 2015).

Using transgenic mice models, germline deletion of the gene encoding CX43 resulted in oocyte deficiency and impaired follicle growth; cell-specific deletion from growing oocytes reduced embryo implantation competence; and deletion of the gene encoding CX37, which forms gap junctions connecting oocytes with granulosa cells, caused arrest of oocyte development. In the uterine stroma, CX43 regulates decidualization and vascularization in mice as well as in humans. Moreover, the appropriate development and function of a mouse or human placenta is governed by several connexins (Winterhager & Kidder 2015).

In several contractile tissues such as the heart, the smooth muscle layers of the vasculature and the myometrium, gap junctions are essential for the propagation of electrical signals via direct transfer of ions between cells. In the heart, for example, connexins are a crucial determinant of conductivity in the myocardium and, consequently, impairment of GJIC is associated with cardiac pathologies (for a review, see Lambiase & Tinker (2014)). In the vasculature, the contraction wave must be propagated efficiently from cell to cell to control vascular tone (reviewed by Johnstone *et al.* (2009)). In the pregnant uterus, GJIC allows for coordinated contraction of the myometrium during labour (reviewed by Mendelson (2009)). This review focuses on the roles played by individual connexins in the myometrium and the mammary glands, pointing out possible implications of connexin dysfunction for the health of pregnant women and newborns.

Connexin involvement in labour

Together with the oxytocin receptor, the prostaglandin receptor and cyclooxygenase2 (COX2), CX43 belongs to the so called myometrial contraction-associated proteins (CAP) or uterine activation proteins (UAP) (Cook *et al.* 2003), which are able to induce contractility and promote labour (reviewed by Mendelson (2009)). CX43 has been shown to be the dominant connexin in gap junctions connecting rodent and human myometrial cells as well as in cultured myometrial cells (Hendrix *et al.* 1992; Tabb *et al.* 1992; Miyoshi *et al.* 1998). Experiments with isolated human myometrial cells have provided direct evidence for functional ionic coupling via CX43 gap junctions by measuring junctional conductance, dye spreading and intercellular calcium waves (Sakai *et al.* 1992, Miyoshi *et al.* 1998, Young *et al.* 2002). In rodent pregnancy, high levels of progesterone acting via its receptors (PRA and PRB) in the myometrium suppress CX43 transcript and protein expression and thereby limit myometrial contractility in order to maintain pregnancy and avoid preterm labour (Ou *et al.* 1997, Shynlova *et al.* 2009). Interaction with the transcriptional co-repressor p54NRB is reported to stabilize the progesterone receptor-mediated CX43 suppression (Dong *et al.* 2009), keeping the myometrium

quiescent. Previous investigations had shown that not only progesterone but, in addition, defined levels of hCG directly suppress CX43 synthesis and thus represent another hormonal factor that downregulates gap junctional communication to maintain myometrial cell quiescence (Ambrus & Rao 1994).

As pregnancy proceeds, molecular changes in the myometrium driven by both ovarian hormones and mechanical stretch prepare the uterus for the onset of labour by inducing CAP/UAP genes (reviewed by Shynlova *et al.* (2009)). Among these changes is the dramatic upregulation of CX43 and, as a result, a large number of gap junctions is induced to establish ionic coupling among the myometrial smooth muscle cells, providing for synchronization of their contractions (Garfield *et al.* 1988). The upregulation of CX43 expression is caused by progesterone withdrawal and an increase in estrogen (Lye *et al.* 1993). It has been shown that the rodent *Gja1* gene encoding CX43 is under the control of estrogen, and its activation is probably mediated by promoter sequences resembling half-palindromic estrogen response elements (Petrocelli & Lye 1993). It has been further reported that the expression levels of the AP1 family transcription factors c-jun and c-fos are strongly increased in the myometrium before labour and are regulated by both mechanical and hormonal stimuli. The dimers drive promoter activity of the *Gja1* gene by binding to its distal AP1 binding site (Piersanti & Lye 1995, Mitchell & Lye 2005). Thus, AP1 regulation seems to play a crucial role in CX43 induction in the pregnant myometrium at term. However, other findings suggest that, in the human myometrium, the AP1 transcription factor, which is upregulated by estrogen and protein kinase C (PKC), interacts directly through an AP1 site of the *GJA1* promoter sequence and is independent from the estrogen receptor (Geimonen *et al.* 1996, 1998). Although in many species, the drop in progesterone combined with a dominance of estrogen action appears to be the crucial factor in the initiation of parturition, this may not be true for the human because plasma progesterone levels do not decline before or during labour and PRA and PRB remain elevated in the myometrium throughout pregnancy (Challis *et al.* 2000). Several studies attempt to unravel the molecular mechanism by which progesterone and its receptors contribute to the upregulation of the uterine activation proteins in humans. Condon *et al.* (2003) proposed a functional progesterone inactivation at labour by impairment of the transcriptional activity of the progesterone–progesterone receptor (PR) complex resulting in a decrease in PR-responsive genes. Xie *et al.* (2012) described that PR interacts with the *GJA1* promoter through AP1 transcription factors in the presence of progesterone, and suppression of CX43 transcription during pregnancy is mediated via the recruitment of the PR co-regulator, polypyrimidine tract binding protein-associated splicing factor (PSF), and of the yeast switch

independent three homolog A/histone deacetylase co-repressor complex. Because of the decrease in PSF at term, the de-repressed PR transcriptional activity might abrogate the suppression of CAP genes for labour, mimicking a withdrawal of progesterone action.

The role of CX43 in labour is influenced by other signalling pathways, in addition to steroid hormones. Tan *et al.* (2012) suggested that the dominant progesterone receptor B (PRB) maintains myometrial quiescence, but at parturition, the rise in PRA expression promotes labour by stimulating proinflammatory gene expression in response to progesterone. It has been shown that upregulation of prostaglandin signalling induces the expression of labour-associated proteins, including CX43. In mice, PGF2 α could induce preterm labour by increasing the uterine activation proteins CX43, oxytocin receptor, prostaglandin receptor and PTGS-2 (Cook *et al.* 2000, 2003). In human myometrial cells, an increase of PGF2 α acts directly on CX43 expression levels as evidenced by its inhibition by indomethacin treatment. The stimulatory effect of PGF2 α on the uterine activation proteins is enhanced in combination with IL1 β (Xu *et al.* 2013). Although PGF2 α dose-dependently increased the uterine activation proteins, including CX43 (Xu *et al.* 2013), the most highly expressed prostaglandin in human myometrium is PGI2 (Omini *et al.* 1979), which is normally known as a smooth muscle relaxant (Taggart *et al.* 2008). In the pregnant human myometrium at term, however, PGI2 mediates upregulation of CX43 and contractile proteins such as SM-MHC and α -SMA via activating PKA in human myometrial cells *in vitro*, resulting in an enhanced contractile response to oxytocin (Fetalvero *et al.* 2008).

Little is known about the posttranscriptional regulation of connexin expression in myometrial cells that may occur via modulation of CX43 mRNA stability, regulation of its translation and/or modulation of CX43 turnover (reviewed by Klotz (2012)). To date, microRNAs of the miRNA-200 family, the zinc finger E-box binding homeobox proteins ZEB1 and ZEB2, have been identified as being upregulated by progesterone and its receptor in the pregnant uterus. Both miRNAs act as transcriptional repressors for the oxytocin and CX43 genes, blocking the transition into the contractile phenotype (Renthal *et al.* 2010, Zakar & Mesiano 2011). Moreover, in rat liver epithelial cells HuRn, an RNA binding protein, stabilizes CX43 expression such that silencing of HuRn lowered the level of CX43 mRNA and protein and decreased CX43 mRNA half-life (Ale-Agha *et al.* 2009). In addition, HuR silencing reduced β -catenin, which is known to be required to maintain CX43 at the plasma membrane (Ale-Agha *et al.* 2009). Despite this evidence, it is not known if HuR contributes to the regulation of CX43 in the pregnant myometrium. Altogether, a number of mechanisms undoubtedly contribute to the onset of connexin induction in the human myometrium at term, and a precise mechanism whereby transcription of

the gene encoding CX43 is regulated in this context remains elusive.

Other connexins including CX26 (in rat: Ou *et al.* (1997)), CX40 (in mice and humans: Kilarski *et al.* (2001) and Döring *et al.* (2006) respectively) and CX45 (in humans: Kilarski *et al.* (1998, 2001)) have been identified in the myometrium, but it is not known if they form heteromeric or heterotypic gap junction channels with CX43 in this context. Their involvement in human labour has not yet been elucidated. In the mouse model, the genes encoding connexins 26, 40 and 45 have been ablated, but the results have not been informative as to their potential importance for labour: CX26 and CX45 knockout mice die *in utero* (Gabriel *et al.* 1998, Krüger *et al.* 2000, Kumai *et al.* 2000) whereas CX40 knockout females were reported to be fertile without any reproductive problems being noted (Kirchhoff *et al.* 1998). See Table 1 for a summary of mouse connexin mutants affecting labour and/or lactation.

Two independent studies in the mouse have supported that GJIC among the myometrial smooth muscle cells is required for coordination of the uterine contractions that expel the fetus during parturition. Döring *et al.* (2006) used the inducible Cre-LoxP system to generate female mice in which the gene encoding CX43 was ablated in the myometrial smooth muscle cells 1 week before the females were mated. Not surprisingly, the knockout markedly decreased GJIC among the smooth muscle cells, but the expression of several selected myometrial CAP genes was not affected, indicating that, despite being uncoupled, the cells' ability to contract remained intact. The result of this targeted knockout was that the females exhibited a delay in parturition in comparison with their unaffected counterparts, with less than one-fifth of the knockout dams being able to deliver their pups within the normal temporal window. The researchers failed to find any hormonal differences that could explain the delayed parturition, hence it was attributed to reduced ionic coupling that impaired myometrial contraction. A similar effect on parturition was seen in the second study (Tong *et al.* 2009) in which myometrial function was analysed during parturition in female mice expressing a dominant mutant form of CX43 (CX43^{G60S}), which reduced the amount of the connexin in cell membranes and the strength of GJIC by $\sim 85\%$ measured electrophysiologically. Myometrial contraction strength in response to oxytocin was correspondingly impaired, resulting in delayed parturition along with, in some cases, failure of some pups or placentas to be expelled. Collectively, these two studies make it clear that CX43 is essential for parturition in the mouse. Given that the two mutations severely reduced GJIC between the myometrial cells by different means (gene knockout vs mutation that impairs the ability of CX43 to assemble into gap junctions), it is most likely that the role of CX43 in parturition is to provide for ionic coupling of the myometrial cells to coordinate contraction.

Table 1 Connexin importance for labour and lactation revealed in mutant mice.

Connexin	Genetic alteration	Effect on labour	Effect on lactation	References
CX26	Mammary ductal epithelium-specific knockout	None reported	Impaired gland development, impaired lactation	Bry <i>et al.</i> (2004)
	Mammary gland-specific knockdown of lactational expression surge	None reported	Comparatively normal gland development and function	Stewart <i>et al.</i> (2014)
CX30	Germline knockout	No reproductive impairment	No reproductive impairment	Teubner <i>et al.</i> (2003)
CX32	Germline knockout	None reported	Gland development not affected	Bry <i>et al.</i> (2004)
CX40	Germline knockout	None reported	None reported	Kirchhoff <i>et al.</i> (1998)
CX43	Myometrial smooth muscle-specific knockout	Delayed parturition	None reported	Döring <i>et al.</i> (2006)
	Dominant loss-of-function germline mutation (G60S)	Delayed and incomplete parturition	Delayed mammary duct development; impaired milk ejection	Tong <i>et al.</i> (2009), Plante & Laird (2008) and Plante <i>et al.</i> (2010)
	Dominant loss-of-function germline mutation (I130T)	None reported	Delay in ductal elongation; glands smaller but milk ejection unaffected	Stewart <i>et al.</i> (2013)
	Heterozygous germline knockin (CX26 replaced CX43)	None reported	Impairment of lactation due to gland under-development	Winterhager <i>et al.</i> (2007)
	Germline knockin (CX32 replaced CX43)	None reported	Impaired milk ejection	Plum <i>et al.</i> (2000)
	Germline knockin (CX40 replaced CX43)	None reported	None reported	Plum <i>et al.</i> (2000)

Implicating connexins in birth complications

There is sufficient evidence that CX43 in the human myometrium serves the same function that has been demonstrated in the mouse. For example, reduced expression of this connexin correlates with prolonged labour (Cluff *et al.* 2006). Moreover, early onset of CX43 induction or impaired suppression of CX43 expression may be correlated with preterm birth, a leading cause for neonatal morbidity and mortality and thus a major health problem (Blencowe *et al.* 2012). A decrease in progesterone level and/or a functional progesterone withdrawal are known reasons for the onset of expression of CAP genes, including CX43, at birth (Mendelson 2009). Research with women or using animal models has shown that prolonged administration of progesterone is able to prevent preterm birth (Norman *et al.* 2011). Progesterone probably acts as an anti-inflammatory hormone and prevents prostacyclin activation (Fetalvero *et al.* 2008). Interfering with prostaglandin synthesis as a therapeutic treatment to arrest myometrial contraction was considered to be a promising approach, but there is little evidence that COX2 inhibitors could prevent preterm labour as shown by a recent Cochrane study (Khanprakob *et al.* 2012). Recently, Gonzalez *et al.* (2014) uncovered another mechanism of CX43 induction involving complement C5a activation in human and mouse myometrium; inhibition of C5a activation by statins can prevent myometrial contractions. The posttranscriptional regulation by ZEB miRNA-200 could serve as another target for therapeutic treatment by creating inhibitors for the signalling cascades controlling this specific miRNA as mentioned by Zakar & Mesiano (2011) and Klotz (2012). It must be kept in mind, however, that clinical therapies

to prevent preterm birth are always accompanied by a risk for fetal development. Nevertheless, a better understanding of the molecular events that regulate the transition from the quiescent pregnant myometrium to the contractile state would improve therapies for preterm labour. In this context, direct functional studies on the regulation of myometrial gap junction channels may suggest new strategies for preventing preterm birth.

Connexin involvement in lactation

Several connexins have been identified as forming gap junctions in various compartments of the developing and mature mammary glands of rodents and humans (reviewed by Stewart *et al.* (2015)). The mammary gland is one of several secretory organs that develop from an interaction between epithelial and mesenchymal rudiments; in the mammary gland, this culminates in a series of ducts emanating from milk-producing alveoli. The ducts are surrounded by myoepithelial cells whose contraction in response to the pituitary hormone, oxytocin, drives milk ejection. During pregnancy, duct expansion occurs as the alveolar cells ramp up for postpartum milk production. In both mouse and human, CX43 is expressed in the glandular stroma and luminal cells in addition to forming gap junctions connecting the myoepithelial cells (Fig. 1). CX26 is also prominent in human luminal cells, whereas in mice, CX26, CX30 and CX32 all contribute to gap junctions connecting the luminal epithelial cells (Stewart *et al.* 2015). Surprisingly, a recent study also identified CX46 in both luminal and myoepithelial cells of the human mammary gland despite its not having been reported in the mouse (Teleki *et al.* 2014).

Figure 1 Two connexins are prominent in the human mammary gland. (A) Alveoli are the milk-producing elements of the gland, with the milk being conducted to the nipple via a converging system of ducts. (B) CX43 gap junctions (blue) couple the contractile myoepithelial cells (ME), whereas CX26 (yellow) predominates in gap junctions coupling the luminal cells (LU) of the alveoli and ducts.

The roles of connexins 26, 30 and 32 in mammary gland development and lactation have been explored through mouse gene knockouts (Table 1). Bry *et al.* (2004) used a transgene to drive Cre recombinase expression in the ductal epithelium of the mouse mammary glands to effect deletion of the gene encoding CX26 before puberty. This early loss of CX26 abrogated the growth and functional differentiation of the gland,

with the consequence that post-parturition lactation was impaired and pup survival reduced, demonstrating an essential role for CX26 in glandular development. In contrast, when CX26 expression was knocked down later during lactation, gland development was essentially normal, as indicated by the production of milk proteins (Stewart *et al.* 2014). Interestingly, Bry *et al.* (2004) also reported that mammary gland development was not affected by the germline knockout of the gene encoding CX32. Likewise, Teubner *et al.* (2003) did not find any obvious reproductive impairment in female mice lacking CX30, although the mammary glands were not specifically examined in that study. The roles of CX43 in mammary gland development and lactation have been explored using the CX43^{G60S} mutant mouse line (Plante & Laird 2008, Plante *et al.* 2010). A temporary delay in postnatal ductal development and a reduction of the gland's size were noted in CX43^{G60S} expressing females in comparison with their WT littermates. Not surprisingly, considering the presence of this connexin in their myoepithelial gap junctions, oxytocin-stimulated milk ejection from the mutant mammary glands was impaired. In contrast, in mice expressing a less severe loss of function mutant (CX43^{I130T}), the mammary glands were reduced in size but milk ejection was not affected (Stewart *et al.* 2013).

Given the contrasting roles of the different mammary gland compartments – secretion vs contraction in response to different hormonal stimuli – it is tempting to speculate that the connexins expressed in mammary glands are spatially restricted based on the different properties of the intercellular membrane channels that they form, either singly or, where co-expressed in the same cells, in various connexin combinations (see Locke *et al.* 2000, 2004). The principle of functional uniqueness of CX43 in the mouse mammary gland was tested by Plum *et al.* (2000), who replaced the genomic CX43 coding sequence with that encoding either CX32 or CX40 (termed CX43^{K132} and CX43^{K140} mice respectively). Whereas CX40 was able to replace CX43 without impairing mammary gland function, CX32 was not, resulting in restricted postnatal growth and diminished survival of CX43^{K132} pups, even those born of mothers heterozygous for the gene replacement. As expected, the CX43^{K132} pups could be rescued by cross-fostering to WT mothers. Histological analysis indicated that lactation failure was at the core of the CX43^{K132} mutant phenotype, due not to impaired milk production but rather to impaired myoepithelial contraction. When the same analysis was applied to mice in which the CX43 coding sequence had been replaced by that of CX26 (CX43^{K126} mice), the result was again an impairment of lactation, but in this case it appeared to be due to underdevelopment of the alveoli and ducts (Winterhager *et al.* 2007). Collectively, these gene replacement experiments point to unique (i.e., nonredundant) roles

for CX43 in different aspects of mammary gland development and function.

Conclusion and future directions

It is clear from the research summarized above that several connexins play important roles in parturition and lactation in the mouse and, based on similarities in expression domains, likely also in the human. This implies that perturbations of connexin function could pose risks for pregnant women and their newborn babies. Prolonged labour, for example, would be a likely consequence of any reduction in the level of expression or the function of CX43 in the myometrium, conditions that would reduce the strength of contractions and possibly necessitate delivery by Caesarean section. On the other hand, promoting the expression of CX43 in the quiescent myometrium in pregnancy contributes to preterm birth. Similarly, a delay in mammary gland maturation during late pregnancy would be a possible consequence of reduced CX26 expression/function, whereas CX43 deficiency would be expected to blunt the effect of oxytocin in inducing postnatal milk extrusion. The seriousness of this latter effect could be compounded if, as in the mouse (Winterhager *et al.* 2013), a reduced level of CX43 in the decidual tissue results in intrauterine growth restriction, a condition that increases the importance of breastfeeding (Bozzetti *et al.* 2013, Tudehope *et al.* 2013). Such scenarios are not merely hypothetical, given that numerous human diseases and disabilities have been linked with mutations in connexin genes (Dobrowolski & Willecke 2009), including those encoding connexins mentioned in this review.

Going forward, more research with mutant mice will be needed to refine our understanding of connexin involvement in labour and lactation. For example, there is still much to be learned about the intercellular signalling pathways and connexin modifications involved in the endocrine-regulated acquisition of myometrial contractility that presages labour. Phosphorylation of CX43 undoubtedly plays a role in that process, given the importance of phosphorylation in regulating gap junction assembly and turnover (Solan & Lampe 2014) and the fact that CX43 phosphorylation is known to be disrupted in the myometrium of CX43^{G60S} mutant mice with impaired parturition (Tong *et al.* 2009). We also need to know why CX43 is critical for mammary duct development, in addition to its well-understood role in conducting the contraction waves that effect milk extrusion. For each of the several connexins known to be involved in parturition or lactation, its critical role – whether as undocked hemichannels in the plasma membrane, as intercellular gap junction channels, or as performing some intracellular function – must be determined. Above all, it will be important to track pregnancies in women carrying disease-causing

connexin gene mutations (Gerido & White 2004, Richard 2005, Laird 2008, Paznekas *et al.* 2009) in order to identify possible adverse effects on labour and/or lactation.

Declaration of Interest

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

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