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Gap junction connexins in female reproductive organs: implications for women's reproductive health

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BACKGROUND: Connexins comprise a family of ~20 proteins that form intercellular membrane channels (gap junction channels) providing a direct route for metabolites and signalling molecules to pass between cells. This review provides a critical analysis of the evidence for essential roles of individual connexins in female reproductive function, highlighting implications for women's reproductive health.

METHODS: No systematic review has been carried out. Published literature from the past 35 years was surveyed for research related to connexin involvement in development and function of the female reproductive system. Because of the demonstrated utility of genetic manipulation for elucidating connexin functions in various organs, much of the cited information comes from research with genetically modified mice. In some cases, a distinction is drawn between connexin functions clearly related to the formation of gap junction channels and those possibly linked to non-channel roles.

RESULTS AND CONCLUSIONS: Based on work with mice, several connexins are known to be required for female reproductive functions. Loss of connexin43 (CX43) causes an oocyte deficiency, and follicles lacking or expressing less CX43 in granulosa cells exhibit reduced growth, impairing fertility. CX43 is also expressed in human cumulus cells and, in the context of IVF, has been correlated with pregnancy outcome, suggesting that this connexin may be a determinant of oocyte and embryo quality in women. Loss of CX37, which exclusively connects oocytes with granulosa cells in the mouse, caused oocytes to cease growing without acquiring meiotic competence. Blocking of CX26 channels in the uterine epithelium disrupted implantation whereas loss or reduction of CX43 expression in the uterine stroma impaired decidualization and vascularization in mouse and human. Several connexins are important in placentation and, in the human, CX43 is a key regulator of the fusogenic pathway

from the cytotrophoblast to the syncytiotrophoblast, ensuring placental growth. CX40, which characterizes the extravillous trophoblast (EVT), supports proliferation of the proximal EVTs while preventing them from differentiating into the invasive pathway. Furthermore, women with recurrent early pregnancy loss as well as those with endometriosis exhibit reduced levels of CX43 in their decidua. The antimalaria drug mefloquine, which blocks gap junction function, is responsible for increased risk of early pregnancy loss and stillbirth, probably due to inhibition of intercellular communication in the decidua or between trophoblast layers followed by an impairment of placental growth. Gap junctions also play a critical role in regulating uterine blood flow, contributing to the adaptive response to pregnancy. Given that reproductive impairment can result from connexin mutations in mice, it is advised that women suffering from somatic disease symptoms associated with connexin gene mutations be additionally tested for impacts on reproductive function. Better knowledge of these essential connexin functions in human female reproductive organs is important for safeguarding women's reproductive health.

Key words: connexin / gap junction / oogenesis / implantation / placental function

Introduction: the connexin family of proteins

The connexins are a family of homologous proteins (20 in mice, 21 in humans), each of which is the product of a distinct gene (Söhl and Willecke, 2003). Connexins differ greatly in size, providing a convenient method of distinguishing them: connexin23 (CX23), for example, is the smallest of the mammalian connexins at ~23 kD and connexin62 (CX62) is the largest at ~62 kD. The gene family for mice and humans is divided into five subgroups based on sequence similarity; see http://www.genenames.org/ for the human genes and http://www.informatics.jax.org/ for the mouse genes (Abascal and Zardoya, 2013). Their best known function is to form the intercellular membrane channels of gap junctions, structures which allow for direct sharing of inorganic ions and small molecules between cells in a process known as gap junctional intercellular communication, or GJIC. Six connexins oligomerize to form a connexon or gap junction hemichannel (Fig. 1). When connexons in the plasma membrane of one cell dock end-to-end with connexons in the plasma membrane of an adjacent cell, solute-permeable intercellular channels are formed. A dense array of such intercellular channels joining two cells is termed a gap junction plaque. All connexins have four membrane-spanning domains forming the channel, two extracellular loops that serve as docking domains, a cytoplasmic loop and cytoplasmic N- and C-terminal tail segments which contain sites that are involved in the regulation of channel function (Harris, 2001; Söhl and Willecke, 2003; Yeager and Harris, 2007). Sequence similarity among connexins is concentrated in their transmembrane domains and extracellular loops, whereas most of the sequence and length variation resides in their cytoplasmic loops and C-terminal tails.

Although each connexin has a characteristic distribution among different cell types, their distributions overlap because many cells express more than one member of the family. Because of this, the potential diversity of channel types can be further magnified by the existence of heteromeric connexons, formed by co-oligomerization of different connexins, and heterotypic intercellular channels, formed by end-to-end docking of connexons with different connexin composition (Meşe *et al.*, 2007; Koval *et al.*, 2014; Fig. 1). Gap junction channels comprising different connexins can exhibit widely different properties with regards to ionic conductance, solute permeability, or sensitivity to regulatory factors such as pH, transjunctional voltage, and phosphorylation, diversity that

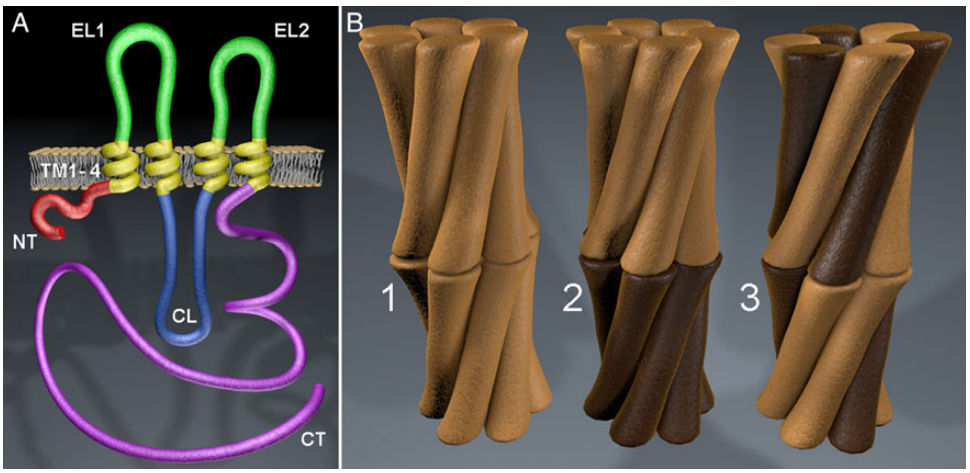

Figure 1 Schematic representation of connexins and gap junction channels. Connexins oligomerize to form hexameric connexons in the plasma membrane, which dock end-to-end with connexons from adjacent cells to form intercellular gap junction channels. (**A**) Each individual connexin has an N-terminal cytoplasmic tail (NT), four transmembrane domains (TM1-4), two extracellular loops (docking domains, EL1 and 2), a cytoplasmic loop (CL) and a C-terminal cytoplasmic tail (CT). (**B**) The permeability properties of gap junction channels depend on their connexin composition: channels can be (1) homomeric-homotypic, (2) homomeric-heterotypic or (3) heteromeric-heterotypic.

provides for exquisite control of the selectivity and regulation of intercellular metabolic coupling (Harris 2001, 2007; Meşe *et al.*, 2007; Harris and Contreras, 2014). Furthermore, connexin diversity implies that channels formed of different connexins play distinctive developmental or physiological roles. On the other hand, the significance of connexin diversity is still not fully understood since mouse connexin knockins (where the gene encoding one connexin has been inserted into the genome in replacement of the gene encoding a different connexin) and double knockouts have revealed unique functions in some instances and shared functions in others (Houghton *et al.*, 1999; Kirchhoff *et al.*, 2000; Plum *et al.*, 2000; White, 2003; Simon *et al.*, 2004; Li *et al.*, 2007).

To further complicate matters, it has become increasingly clear that at least some connexins play important roles in cells independent of their ability to form intercellular gap junction channels. For example, several connexins (most notably CX43) have been shown to reside in the plasma membrane as undocked connexons which can provide a conduit for the regulated exchange of certain metabolites and small signalling molecules between the cytoplasm and extracellular environment (reviewed by Evans *et al.*, 2006). The inner mitochondrial membrane is another reported location for undocked CX43 connexons, in this case their proposed function being to facilitate K^+ flux across that membrane (Miro-Casas *et al.*, 2009). Connexins also participate in multiprotein complexes involving other proteins, including cytoskeletal components (Laird, 2010; Hervé *et al.*, 2012; Palatinus *et al.*, 2012). For example, the C-terminal cytoplasmic tail of several connexins contains a PDZ motif-binding domain that links the connexin to ZO1, ZO2, and/or ZO3, components of the membrane-cytoskeletal complex associated with adherens and tight junctions (Penes *et al.*, 2005; Laird, 2010). To explore the importance of this PDZ-binding domain, Maass *et al.* (2004) generated germline replacement mice in which CX43 lacked the domain. The mutant connexin retained the ability to form functional gap junction channels but despite this, most of the homozygous mutant offspring died shortly after birth due to disruption of their epidermal permeability barrier. This result implies an essential role in epithelial integrity for at least this particular connexin based on its PDZ-binding domain. CX43 is also a well-established regulator of cell proliferation, and this too can be independent of its ability to form membrane channels (Hatakeyama *et al.*, 2013). Notably, the C-terminus of CX43 can be as effective as the full-length protein in suppressing cell growth (Moorby and Patel, 2001). In communication-deficient glioma and trophoblast tumour cells, restoration of gap junctions by transfection of CX43 significantly reduced proliferation in association with up-regulation of the growth regulator, CCN3 (Fu *et al.*, 2004; Gellhaus *et al.*, 2004). CCN is an acronym derived from the first three discovered members of its family: cysteine-rich 61 (CYR61, CCN1), connective tissue growth factor (CTGF, CCN2), and nephroblastoma-overexpressed (NOV, CCN3) (Winterhager and Gellhaus, 2014). CCN3 binds to the C-terminus of CX43, an interaction correlated with a drastic reduction in proliferation (Sin *et al.*, 2009; Gellhaus *et al.*, 2010). CX43 constructs lacking the PDZ domain interact more intensely with CCN3 causing them to be more effective in reducing proliferation (Gellhaus *et al.*, 2010). Other recent experiments have revealed a role for CX43 in cell motility due to its ability to stabilize the polarized alignment of the cytoskeleton, a function requiring the tubulin-binding domain of CX43 but independent of its ability to form gap junctions (Francis *et al.*, 2011).

Mutations in various human connexin genes have been implicated in several distinct disease syndromes, each with a unique pathophysiology (Molica *et al.*, 2014). Corresponding mouse models have been generated and have proved valuable for elucidating pathways of pathogenesis (Dobrowolski and Willecke, 2009). However, with the knowledge that connexins can play multiple roles in cellular function, it is important to exercise caution in interpreting the results of experiments with genetically altered mice where the mutant phenotype is often ascribed to loss of GJIC but may in fact be due to disruption of a different connexin function (Dobrowolski and Willecke, 2009). This caveat should be kept in mind when considering the involvement of connexins in the development and function of the female reproductive system as summarized in the next sections.

Methods

Up until the present time, there has not been a systematic review of the literature pertaining to the role of gap junction connexins in female reproductive organs. In this review, literature from the past 35 years was surveyed for information related to connexin involvement in the development and function of the female reproductive system including the ovary, the endometrium during implantation and decidualization, and in the developing placenta. Where possible, a distinction is drawn between connexin functions clearly related to the formation of gap junction channels and those possibly linked to non-channel roles. Most of the results come from research with genetically modified mice where the genetic manipulation elucidated specific connexin functions in female reproductive organs. These results are correlated with findings from research with human cell lines and patient tissue samples and reference is made to patient populations with known connexin-based diseases (connexinopathies) where reproductive impairment is a possible component of the disease syndrome that is worthy of closer scrutiny.

Connexin involvement in development of the germ line

The mammalian germ line is established very early, separate from establishment of the gonadal rudiments and the events of sex determination, necessitating a phase of migration during which primordial germ cells (PGCs) make their way into the gonadal ridges (Mamsen *et al.*, 2012). Connexin involvement in this early stage of germ line development was established with the production of the first connexin knockout mouse. Mice lacking CX43 die shortly after birth, their demise being caused by a severe developmental heart defect that obstructs blood flow into the pulmonary arteries (Reaume *et al.*, 1995). A survey to look for additional morphological abnormalities in CX43 knockout fetuses revealed a severe reduction in the number of germ cells in both sexes that was evident as early as 11.5 days of gestation, before sex determination, although the small number of oocytes remaining in the fetal ovaries was sufficient to initiate follicle formation (Juneja *et al.*, 1999). PGCs are coupled with neighbouring cells via CX43 gap junctions during their migration (Francis and Lo, 2006) but it is not yet clear whether it is the loss of this coupling and/or loss of a non-channel function of CX43 that accounts for the germ cell deficiency. Subsequent research demonstrated that the early deficiency of PGCs is associated with a decrease in their integrin-mediated cell adhesion, a decrease in their rate of motility, and an increase in their rate of apoptotic cell death (Francis and Lo, 2006) all of which could result in an impairment of PGC migration and survival. The possibility of a GJIC-independent role for CX43 in PGC migration/survival is supported by the finding that

there is no germ cell deficiency in *Gja1*^{Jrt}/+ mice expressing a dominant loss-of-function mutant of CX43 (CX43^{G60S}) which is structurally intact but provides only very weak intercellular coupling (Flenniken *et al.*, 2005; Gregory *et al.*, 2011).

Connexin involvement in oogenesis

The ovarian follicles of mammals typically consist of a single oocyte surrounded by layers of somatic granulosa (cumulus and mural) and theca cells. Follicles pass from a non-growing (primordial) stage through primary (unilaminar) and secondary (multilaminar) growing stages, finally becoming tertiary follicles characterized by a fluid-filled cavity, the antrum. The oocyte grows along with the follicle prior to antrum formation, eventually acquiring competence to undergo first meiosis at ovulation and be fertilized. Throughout these stages of folliculogenesis, gap junctions couple the granulosa cells with each other and with the oocyte, establishing a metabolic syncytium that is essential for oogenesis (reviewed by Kidder and Vanderhyden, 2010; see Fig. 2). Research with mice has demonstrated that nutrients (including amino acids and glucose) to support the growing oocyte's metabolism, ions to regulate the oocyte's pH, and cGMP required to maintain the oocyte in a state of meiotic arrest all pass from the granulosa cells to the oocyte through the gap junctions of that syncytium. At the time of ovulation, GJIC between the oocyte and granulosa cells is attenuated, reducing the cGMP level in the oocyte thus allowing it to complete meiosis (Norris *et al.*, 2008, 2009; Santiquet *et al.*, 2012, 2013; Wigglesworth *et al.*, 2013).

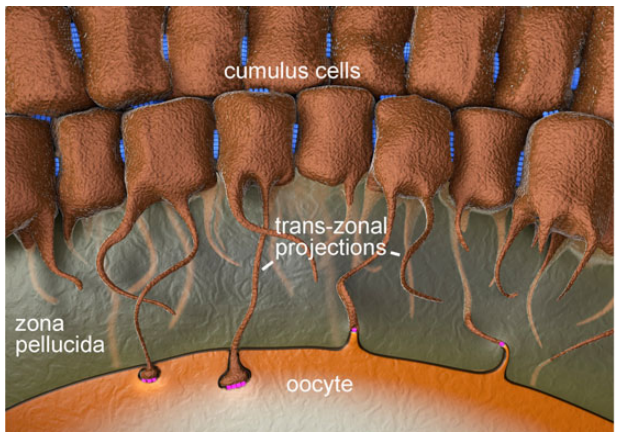

Figure 2 At least two different connexins contribute to gap junctions in ovarian follicles. In the mammalian ovary, extensions of the cumulus granulosa cells (trans-zonal projections) extend across the zona pellucida to contact the oocyte surface, where gap junctions form to metabolically couple the two cell types. Other gap junctions couple the cumulus cells with each other. Based on extensive experiments with mice, the gap junctions at the oocyte surface in growing follicles are homomeric-homotypic junctions composed of CX37 (pink) whereas those coupling the cumulus cells with each other are homomeric-homotypic junctions composed of CX43 (blue). Connexins 37 and 43 are also expressed in human follicles with CX43 being the dominant connexin forming gap junctions in the cumulus cells but the exact composition of the cumulus-oocyte gap junctions in human follicles remains to be determined. In both species, additional connexins have been detected in the granulosa cells of mature follicles.

The distribution of different connexins in the ovary has been examined in a variety of mammals (reviewed by Kidder and Mhawi, 2002 and Gershon *et al.*, 2008a; see also Teilmann 2005, Willingham-Rocky *et al.*, 2007, Wang *et al.*, 2009 and Santiquet *et al.*, 2013). CX37 and CX43 have been consistently identified in the gap junctions of both growing and mature follicles but in different locations (Fig. 2). Additional members of the family have been detected in ovarian follicles of mice and various other species but gene targeting experiments in the mouse have failed to establish clear roles for any of them in oogenesis or folliculogenesis, either because the knockout females remained fertile (CX32, Nelles *et al.*, 1996) or they died *in utero* (CX26, Gabriel *et al.*, 1998; CX45, Krüger *et al.*, 2000 and Kumai *et al.*, 2000). On the other hand, mouse gene knockouts have made it clear that both CX37 and CX43 are essential from early in follicle growth (Kidder and Vanderhyden, 2010). In mouse ovaries, CX37 localizes to gap junctions at the oocyte surface and is thereby responsible for oocyte-granulosa cell metabolic coupling whereas CX43 connects the granulosa cells with each other (Simon *et al.*, 1997; Gittens and Kidder, 2005; Fig. 2). Both the oocyte and the granulosa cells express CX37 but the granulosa cells target it to contacts with the oocyte at the tips of the trans-zonal projections, leaving CX43 to form gap junctions that couple the granulosa cells with each other (Veitch *et al.*, 2004). When the *Gja4* gene encoding CX37 was knocked out, GJIC between the oocytes and granulosa cells was eliminated with severe consequences for the oocytes: they ceased growing without acquiring meiotic competence and eventually degenerated, presumably as the result of their having been metabolically uncoupled from the granulosa cells (Simon *et al.*, 1997). Concomitant with loss of the oocytes, the granulosa cells of CX37 null ovaries underwent premature luteinization. It is therefore possible that CX37 gap junctions at the oocyte surface convey not only metabolites from the granulosa cells needed by the oocyte but also one or more signals from the oocyte to maintain the differentiated state of the granulosa cells, preventing them from luteinizing before ovulation.

Because of the neonatal lethality caused by germline knockout of CX43 (Reaume *et al.*, 1995), analysis of oogenesis in this mutant required grafting late fetal ovaries into the kidneys of adult females to follow the effect of the mutation on post-natal follicle development, a procedure which also allowed the mutant ovaries to develop post-natally in a wildtype endocrine background. It was found that loss of CX43 causes follicle development to arrest before antrum formation (Ackert *et al.*, 2001; Gittens and Kidder, 2005; Tong *et al.*, 2006). As a consequence, oocytes from CX43 null females are developmentally incompetent. Subsequent experiments revealed that the impaired growth of follicles lacking CX43 is due at least in part to reduced responsiveness of the granulosa cells to the oocyte-derived paracrine factor, growth differentiation factor 9 (GDF9), which promotes granulosa cell proliferation (Gittens *et al.*, 2005). Such interplay between gap junctional coupling and receptor-mediated signalling within growing follicles is likely a general phenomenon (Wang *et al.*, 2013).

Although the available evidence does not support an essential contribution of CX43 to the gap junctions connecting oocytes with granulosa cells in the mouse, a surprising result was obtained when the *Gja1* gene was specifically knocked out in oocytes: embryos derived from the

CX43-depleted oocytes developed to the blastocyst stage in apparently normal fashion as would have been predicted, but some failed to implant (Gershon *et al.*, 2008b). One possible explanation for this is that a non-junctional pool of CX43 in the oocyte contributes to post-fertilization implantation competence in such a way that the deficiency in the CX43-depleted oocytes could not be overcome by expression of the *Gja1* gene from the zygote genome (Davies *et al.*, 1996). It remains to be determined what non-junctional role oocyte-derived CX43 plays in post-fertilization development.

In addition to providing for gap junction-based metabolic coupling among the granulosa cells, CX43 could support oogenesis by forming undocked connexons (gap junction hemichannels) in the granulosa cell plasma membranes. Such channels could allow the regulated release of small signalling molecules such as ATP, which is known to be released from cells upon opening of CX43 hemichannels (Leybaert *et al.*, 2003), to interact with receptors on nearby cells, thus establishing a paracrine signalling pathway. In developing follicles, extracellular ATP acts through P2 purinergic receptors to induce Ca^{2+} release from the granulosa cells, resulting in a corresponding Ca^{2+} increase in the oocyte (Tai *et al.*, 2000; Webb *et al.*, 2002). Given this information, it was considered possible that the essential function of CX43 in folliculogenesis is to enable paracrine signalling via ATP release rather than (or in addition to) providing for GJIC. This possibility was examined by Tong *et al.* (2007), who constructed recombinant follicles by combining wildtype mouse oocytes with granulosa cells expressing a mutant form of CX43 which was able to form functional hemichannels in the plasma membrane but not capable of docking to form intercellular gap junction channels. The result was that follicles constructed from granulosa cells exclusively assembling CX43 hemichannels failed to support folliculogenesis, implying that there is an absolute need for CX43 to form intercellular gap junction channels in mouse granulosa cells. The role of CX43 hemichannels, if any, in folliculogenesis remains to be determined.

The PDZ-binding domain is another part of the CX43 molecule that plays an important role in oogenesis. In the study by Maass *et al.* (2004) cited above, very few mice homozygous for the PDZ-binding domain deletion survived as newborns. Those few females that did survive were infertile: follicles began to show morphological irregularities in early antral stages and none reached the size of pre-ovulatory follicles. Interestingly, as with CX37 knockout follicles (Simon *et al.*, 1997), premature luteinization was noted in association with the presence of degenerating oocytes. At present, there are no published data to explain why the PDZ-binding domain of the CX43 molecule is critical for oocyte survival but the answer could shed light on the failure of blastocysts derived from CX43-depleted oocytes to implant (Gershon *et al.*, 2008b).

CX37 and CX43 are also expressed in human follicles (Furger *et al.*, 1996; Cepni *et al.*, 2008; Ratchford *et al.*, 2008; Wang *et al.*, 2009). Studying cultured cumulus cells, Wang *et al.* (2009) found that CX43 forms prominent gap junctions but CX37 was not detected, suggesting that it is restricted to the cumulus-oocyte interface in intact human follicles, as it is in the mouse. It is not clear whether the several other connexins detected in that study (CX26, CX30, CX30.3, CX32, and CX40) assemble into follicular gap junctions *in vivo*. The importance of CX43 as a major contributor to gap junctions in human cumulus cells was confirmed by demonstrating that the level of CX43 expression is positively correlated with the strength of intercellular coupling measured electrophysiologically.

Connexin involvement in implantation and decidualization

Uterine receptivity

During cycling and in early pregnancy, the hormonally regulated transformation of the mammalian endometrium is needed to achieve endometrial receptivity for embryo implantation and placentation, and connexins are intimately involved in this process. The expression and hormonal regulation of endometrial connexins has been studied in a variety of species including ruminants, rodents, rabbits, baboons and humans (see below). In the cycling endometrium of rodents, the uterine epithelium is characterized predominantly by expression of CX26 whereas in the endometrium of humans and baboons during the menstrual cycle, CX32 is strongly co-expressed with CX26 (Winterhager *et al.*, 1993; Jahn *et al.*, 1995). In all species investigated, CX43 is only expressed in the stromal compartment (Jahn *et al.*, 1995; Winterhager *et al.*, 2009). The expression levels of CX26 and CX43, but not of CX32, are ruled by the ovarian hormone profile during cycling and upon experimentally induced estrogen or progesterone dominance in ovariectomized rodents: both CX26 and CX43 expression increase upon estrogen administration and are suppressed by progesterone during the phase of receptivity to the implanting blastocyst (Winterhager *et al.*, 1993; Grümmer *et al.*, 1994). Surprisingly, the rodent and rabbit uterine epithelium lacks detectable GJIC during this critical phase (Winterhager *et al.*, 1988, 1993). In human endometrium, the ratio between progesterone and estrogen levels in serum determines the level of expression of both connexins resulting in high expression in the proliferative phase and strong down-regulation in the secretory phase (Jahn *et al.*, 1995).

Implantation

Successful implantation requires not only an endometrium appropriately transformed by ovarian steroid hormones but also a preimplantation embryo (blastocyst) competent to engage in precise crosstalk with the endometrium via various signals. This crosstalk produces a local developmental programme change in the endometrium to establish the implantation chamber (Wang and Dey, 2006). In rodents and rabbits, the suppression of connexin expression and GJIC during the receptive phase is reversed by local up-regulation in response to the implanting embryo: where an embryo is present, there is strong expression of CX26 (in rodents) and CX32 (in rabbits) in the uterine epithelium immediately surrounding it (Winterhager *et al.*, 1988, 1993). Given that estrogen induces CX26 expression, experiments were conducted to test the hypothesis that it is estradiol or a closely related estrogen, presumably released from the blastocyst, which is responsible for the local connexin up-regulation (Grümmer *et al.*, 2004). On the contrary, anti-estrogen treatment of pregnant rats during implantation did not interfere with CX26 up-regulation in the epithelium. This result was confirmed in the same study using mice lacking either the α or the β estrogen receptor (ER): the up-regulation of CX26 was clearly missing in ERα knockout mice but still present in ERβ knockout mice demonstrating that an estrogenic response mediated by ERα regulates the connexin expression pattern during cycling but is not required for implantation itself. However, the local up-regulation of CX26 was not impaired in pseudopregnant (i.e. hormonally primed) animals after a traumatic stimulus mimicking the implanting blastocyst, indicating that it is not simply a

mechanical stimulus by the blastocyst that causes CX26 up-regulation. Further experiments with pseudo-pregnant uteri in organ culture revealed that the implanting blastocyst mediates up-regulation of CX26 in the uterine epithelium by means of an inflammatory cascade (Grümmer *et al.*, 2004).

To explore the importance of CX26-containing membrane channels for implantation, Diao *et al.* (2013) injected the non-specific channel blocker carbenoxolone (CBX) into mice before embryo attachment. CBX disrupted the implantation process, presumably by inhibiting the local increase in CX26 channel function induced by the blastocyst, but this experimental treatment also disturbed the decidualization process. It therefore remains uncertain whether CX26 or gap junction-based intercellular communication in the uterine epithelium is essential for implantation in the mouse. Recent experiments with mice in which the *Gjb2* gene encoding CX26 was specifically deleted in the uterine epithelium failed to reveal any obvious effect on implantation (E. Winterhager, unpublished results).

Decidualization

Experiments with animal models have indicated that perturbations of connexin function within the implantation chamber disrupt early pregnancy. Impaired uterine receptivity concomitant with failure to decidualize the endometrial stroma is one of the major reasons for implantation failure (Cha *et al.*, 2012). The decidual cells are extensively connected by gap junctions which, in rodents and humans, are mainly composed of CX43 (Abrahamsohn and Zorn, 1993; Winterhager *et al.*, 1993; Jahn *et al.*, 1995; Pauken and Lo, 1995). GJIC among the decidual cells is thought to play an important role in decidual angiogenesis. To confirm this, the *Gja1* gene encoding CX43 was specifically deleted in the mouse uterine stroma with the result that differentiation of the stromal cells into decidual cells was impaired (Laws *et al.*, 2008). Expression of pro-angiogenic factors such as VEGF (vascular endothelial growth factor) and angiopoietin was reduced, resulting in reduced angiogenesis and a loss of embryos at implantation. Similar results have been obtained using cultured human endometrial stromal cells where knockdown of CX43 or disruption by a gap junctional coupling inhibitor impaired decidualization as well as secretion of prolactin and VEGF (Laws *et al.*, 2008, Yu *et al.*, 2011). In contrast, decidual angiogenesis was not impaired but instead enhanced in mice carrying the dominant loss-of-function $Gja1^{Jrt}$ mutant allele encoding $CX43^{G60S}$ in which the level of CX43 is severely reduced (Flenniken *et al.*, 2005; Winterhager *et al.*, 2013). Enhanced angiogenesis in the implantation chambers of $CX43^{G60S}$-expressing females was correlated with increased expression of the genes encoding several angiogenic factors including VEGFA, VEGF receptor 1 (VEGFR1), and VEGFR2. The mutation was also associated with fetal growth restriction which was independent of the genotypes of individual fetuses developing in the same mother, implying a strictly maternal effect. Because of an irregular distribution of the maternal sinusoidal network in the mutant females, invasion of the trophoblast was disoriented and placentas developed mostly laterally (as opposed to centrally) in the implantation chamber, an effect that could potentially explain the fetal growth restriction (Winterhager *et al.*, 2013).

It would be reasonable to conclude from the above studies that coupling of decidual cells via gap junction channels composed of CX43 influences the expression of paracrine factors that control neovascularization, but the stark differences between the effects of the stromal cell-specific *Gja1* knockout and the global $Gja1^{Jrt}$ mutation remain unexplained. One possibility is that the signals passing through $CX43^{G60S}$ mutant channels differ from those passing through wildtype channels, a phenomenon that has been already observed for other connexin isoforms, e.g. CX31 in the placenta (Schnichels *et al.*, 2007). However, the G60S mutation is not known to alter channel permeability properties but rather is predicted to reduce the total number of intercellular channels in apposed membranes due to impairment of connexon-connexon docking (Flenniken *et al.*, 2005). In effect, therefore, the *Gja1* knockout and the $Gja1^{Jrt}$ mutation both reduce the number of gap junction channels in decidual membranes, although to different extents. The explanation for the differing mutant phenotypes may therefore be that, rather than assembling into gap junctions, another function of the CX43 molecule—perhaps its participation in the membrane cytoskeletal complex via its C-terminal tail, which remains intact in the G60S mutant—may be the critical factor for decidual angiogenesis. In any case, the results from these mouse studies suggest that some problems in early pregnancy may have their origin in reduced or altered connexin function in the maternal side of the maternal–fetal interface, resulting in aberrant placental development despite the placental organ itself being intact.

Connexin involvement in placental development and function

Placental development starts in the preimplantation embryo during blastocyst formation when the embryonic cells separate into the inner cell mass, progenitor of the embryo proper, and the trophectoderm, generating the placenta, an event which represents the first lineage decision in embryonic development. Gap junction assembly occurs prior to this event: in the mouse it begins after completion of the third cleavage of the zygote with the result that the cells of the preimplantation embryo remain metabolically coupled thereafter (Lo and Gilula, 1979a). Interestingly, rodent and human preimplantation embryos express multiple connexin genes (reviewed by Kidder and Winterhager, 2001; Houghton, 2005; Brison *et al.*, 2014). Of those connexins that have been localized by immunofluorescence to date, all are expressed in both the inner cell mass and the trophectoderm of the blastocyst; hence they are not at this early stage restricted to the trophoblast progenitors. Preimplantation embryos could potentially make use of several different types of gap junction channels, each permeable to a subset of molecules, but the knockout of individual mouse connexin genes has not yet revealed an essential role for any particular connexin in preimplantation development or implantation (Kidder and Winterhager, 2001). This could be ascribed to functional redundancy among connexin isoforms, but neither the double knockout of CX31 and CX43 (Kibschull *et al.*, 2005) nor use of the non-specific gap junction channel blocker 18α-glycyrrhetinic acid to block GJIC altogether (Houghton *et al.*, 2002) had any effect on the development of preimplantation embryos, implying that GJIC is not essential during this very early developmental period. It remains a mystery why preimplantation embryos express multiple connexins.

The ubiquitous expression pattern of connexins in the rodent blastocyst changes directly after implantation: co-expression of CX31 and CX31.1 comes to characterize the trophoblast lineage whereas CX43 and CX45 become restricted to the embryo proper (Dahl *et al.*, 1996; Reuss *et al.*, 1997). The separation of communication compartments

(embryo versus placenta, with an apparent barrier to GJIC between them) shown previously by Lo and Gilula (1979b) and Kalimi and Lo (1989) may be explained by this distribution of connexins and could have a role in establishing positional information for cell differentiation. Human preimplantation embryos exhibit the same variety of connexin isoforms as mice with CX43 and CX31 dominating (Bloor *et al.*, 2004). Thus, although placental connexins differ between rodents and human (see below), these species share the same isoforms during the preimplantation period, pointing to an evolutionarily conserved developmental mechanism. As in rodents, the spatial pattern of connexin expression in the human placenta does not replicate the lineage separation seen in the blastocyst stage (Bloor *et al.*, 2004).

During the formation of the murine chorio-allantoic placenta, the labyrinth starts to differentiate to take over fetomaternal exchange. This process involves elongation of trophoblast cells from the chorion and fusion to form a two-layered syncytiotrophoblast in conjunction with chorionic mesoderm invasion and formation of embryonic blood vessels. The two syncytial trophoblast layers that form the placental barrier are connected with each other by dense arrays of CX26-containing gap junctions to establish a metabolically coupled unit (Shin *et al.*, 1996), the importance of which was demonstrated by the dramatic effect of deleting the *Gjb2* gene encoding that connexin (Gabriel *et al.*, 1998). Loss of CX26 resulted in an early embryonic death around day 10.5 which was caused by impaired transport of metabolites across the two-layered syncytial trophoblast as indicated by severely decreased glucose uptake by the embryo.

The functions of CX31 and CX31.1 in murine placental development have also been illuminated by targeted gene deletions (reviewed by Kibschull *et al.*, 2008). Both knockouts had a direct influence on trophoblast lineage differentiation, the result of which was a partial loss of embryos between embryonic days 11.5 and 14.5: 60% in CX31-deficient dams and 30% in CX31.1-deficient dams (Plum *et al.*, 2001; Zheng-Fischhofer *et al.*, 2007). Phenotypic analysis of the CX31-deficient placentas showed a transient, strong reduction of the labyrinthine and spongiotrophoblast layers and a shift to the parietal trophoblast giant cell population. The phenotype of CX31.1-deficient placentae includes a shift in placental subpopulations, a reduced area of fetal blood spaces, and a reduced number of secondary trophoblast giant cells. In contrast to CX31-deficient trophoblast stem (TS) cells, TS cells from CX31.1 knockout mice revealed enhanced proliferation and delayed trophoblast differentiation (Kibschull *et al.*, 2014). These findings suggest that CX31.1 and CX31 exert opposite effects to balance trophoblast lineage differentiation during placental development. In addition, Dupressoir *et al.* (2011) reported that, although syncytin-B knockout mice displayed an impaired formation of syncytiotrophoblast layer II, the embryos survived *in utero* and, because the researchers found an up-regulation of the gene encoding CX30 (but not of that encoding CX26), they postulated that CX30 serves as a compensatory transport channel in the mouse placenta.

As mentioned above, the human placenta differs morphologically from that of rodents and other species in respect to the connexins involved in its development and function (Kibschull *et al.*, 2004, 2008). The functional analogue of the rodent labyrinthine trophoblast in the human placenta is the villous trophoblast, which consists of one syncytial layer (the syncytiotrophoblast) and an underlying cytotrophoblast (Fig. 3). The cytotrophoblast maintains the syncytiotrophoblast during pregnancy by continuous fusion with it (Benirschke *et al.*, 2006). Because there is only one syncytial

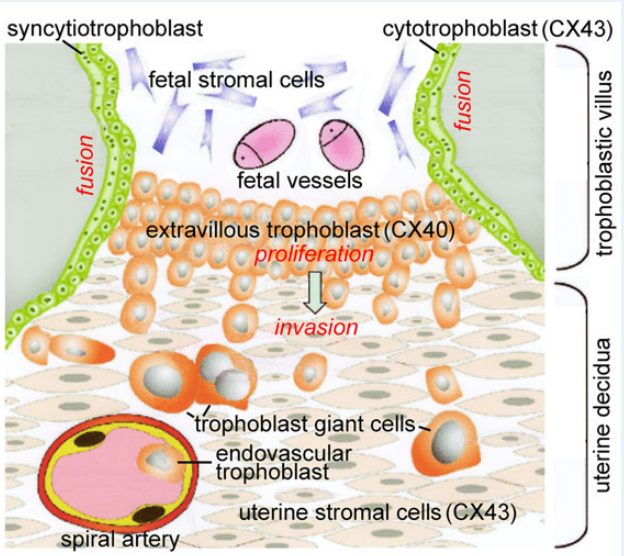

Figure 3 Different connexins characterize cell populations in the human placenta. The drawing represents a placental anchoring villus attached to the maternal decidua with the villous cyto- and syncytiotrophoblast and the extravillous trophoblast of the cell column along with invading giant cells. CX43 is expressed in the cytotrophoblast and regulates the process of cytotrophoblast fusion to form the syncytial layer. CX40 is expressed in the proliferating cytotrophoblast cells of the cell column. The extravillous trophoblast cells stop proliferating and escape from the cell column to invade into the maternal decidua and the spiral arteries. CX40 has been shown to be involved in this trophoblast lineage differentiation leading to reduced proliferation and at the same time to increased migration properties. Adapted from Winterhager and Gellhaus (2014).

trophoblast layer in human, GJIC via CX26 channels is not necessary for transplacental transport as in rodents; instead, the underlying cytotrophoblast cells express CX43 which, as has been convincingly shown using *in vitro* systems, is mandatory for the fusion process (Cronier *et al.*, 1994, 2002, 2003). Investigations by Dunk *et al.* (2012) revealed that CX43 exerts this function via the transcription factor GCM1 and its downstream target HERV-W/syncytin-1 together with its receptor ASCT2. The CX43 C-terminal cytoplasmic tail is crucial for this regulatory action as evidenced by the observation that its removal prevented the connexin's interaction with either syncytin-1 or ASCT2, abrogating fusion. CX43 is therefore a key regulator of the fusogenic pathway, ensuring placental growth and controlling placental aging. It is still not clear if the CX43 channel contributes to transport across the placental barrier as indicated by *in vitro* experiments using multilayered trophoblast cell lines or placental explants (Sood *et al.*, 2011).

The EVT takes on the function of the mouse spongiotrophoblast in humans. These villi, which anchor the trophoblast cells to the decidua, form the cell columns and constantly generate giant cells (Georgiades *et al.*, 2002). EVT cells detaching from the column escape from the cell cycle, achieve polyploidy, and invade into the spiral arteries to become endovascular trophoblasts or into the decidua to become interstitial EVT cells (Zybina *et al.*, 2002). The endovascular trophoblast is essential to remodel the spiral arties in order to guarantee a continuous blood flow into the intervillous space of the placenta (Pijnenborg *et al.*, 1991). The proximal EVT cells are characterized by CX40 expression which

terminates when the cells detach from the column to invade the decidual compartment (Winterhager *et al.*, 2000; Fig. 3). The same expression pattern was found in the villous explant model *in vitro* and the malignant trophoblast cell lines BeWo and Jar, providing an experimental model for exploring the function of CX40 in trophoblast proliferation and invasion (Hellmann *et al.*, 1996; Winterhager *et al.*, 2000; Nishimura *et al.*, 2004). Outgrowth of the EVT was strongly enhanced and proliferation was reduced when placental explants were treated with gap junctional coupling inhibitors or CX40 expression was knocked down using small interfering RNA, providing evidence that CX40 supports proliferation of the proximal EVTs while preventing them from differentiating into the invasive pathway (Nishimura *et al.*, 2004). It is possible, therefore, that CX40 dysfunction could contribute to the shallow invasion of trophoblast cells found in pre-eclampsia (McMaster *et al.*, 2004). In spite of this growing fund of information about the role of connexins in placental development and function, there is still much to be discovered about how they contribute to human placental diseases and pregnancy outcomes.

Implications for women's reproductive health

It is clear from the research described in the previous sections of this review that several connexins play essential roles in the development and/or function of the mammalian female reproductive system. Given this, it is likely that connexins are also important in women's reproductive health—indeed, it would be surprising if this were not the case. In this final section we summarize evidence pointing to important roles for connexins in human female reproduction and their possible involvement in reproductive problems.

Fertility

The critical role for cumulus cell CX43 in oogenesis, established using knockout and $CX43^{G60S}$ mice, was the impetus for the study by Wang *et al.* (2009) cited above that was aimed at testing the importance of this connexin for human female fertility. Their study involved patients undergoing IVF by ICSI, a process which involves prior stripping of the cumulus cells from the oocyte to facilitate sperm injection, making the cells readily available for research to discover biomarkers of oocyte/embryo competence. The researchers looked for an association between cumulus cell CX43 expression or gap junctional coupling strength and embryo quality, the latter indicated by morphological assessment of the embryos produced *in vitro* and the pregnancy outcomes after embryos had been transferred back into the patients' wombs. It was found that cumulus cell samples from different patients differed with respect to both CX43 expression and cumulus coupling strength. Most importantly, when patients were partitioned into subgroups depending on whether or not they became pregnant, those who did so registered significantly higher mean scores with respect to both CX43 expression and coupling strength within their cohort of retrieved follicles. These results imply that CX43 itself and/or the metabolic coupling which it provides within the cumulus-oocyte complex is a determinant of oocyte and embryo quality in women just as it is in mice. It is possible therefore, that inadequate expression or mutationally altered function of CX43 could contribute to human female infertility by reducing the number and/or quality of oocytes available to be ovulated. To date, however, none of the recognized syndromes involving primary ovarian insufficiency, which involves premature cessation of ovarian function due to reduced oocyte reserve, have been linked to the *GJA1* gene (Zhao and Chen, 2013). On the other hand, there could be manifestations of subfertility associated with CX43 functional deficiency which remain unidentified. An example of this is provided by $Gja1^{Jrt}/+$ female mice which remain fertile despite reduced CX43 in the granulosa cells, but ovulate fewer oocytes and thus produce smaller litters than wildtype littermates, even when mated with wildtype males (Tong *et al.*, 2009).

Examples of reproductive impairment in $Gja1^{Jrt}/+$ female mice bear special interest for women's reproductive health because those mice model the human dysmorphogenesis syndrome, oculodentodigital dysplasia (ODDD; Flenniken *et al.*, 2005). ODDD is caused by some 73 different mutations in *GJA1*, most of them inherited as autosomal dominants (Paznekas *et al.*, 2003, 2009; reviewed by Laird, 2014). As its name implies, this pleiotropic syndrome characteristically involves the eyes (along with other facial features), teeth, and digits but also affects many other parts of the human body where CX43 is expressed, with the different ODDD mutant alleles generating a range of impairments of differing severity. In addition to the $Gja1^{Jrt}/+$ mutant mouse line expressing $CX43^{G60S}$, additional lines have been generated to model the human $CX43^{I130T}$ and $CX43^{G138R}$ ODDD mutants (Kalcheva *et al.*, 2007; Dobrowolski *et al.*, 2008). Although detailed analyses of reproductive function in those females have not been reported, they are, like $CX43^{G60S}$ mutant females, fertile. Furthermore, there is only one report to date of a woman with ODDD having compromised fertility (early menopause, a symptom of primary ovarian insufficiency) associated with a *GJA1* mutation (see Supplemental Table 5 in Paznekas *et al.*, 2009). Given the prominent expression of CX43 in the human ovarian follicle, the correlation between CX43 expression and pregnancy success resulting from IVF, and the extensive evidence frommutant mice demonstrating the importance of this connexin for oogenesis, it is possible that there are many more women in the ODDD population with unrecognized subfertility. Additional indications of reproductive impairment in ODDD women could include delayed menarche and problems with placental function leading, for example, to intrauterine growth restriction. It is important to note that different ODDD mouse mutants can differ greatly in the severity of their effects on organ development and function despite being associated with the same diagnostic dysmorphogenesis features of the human ODDD syndrome (Stewart *et al.*, 2013). Such variable expressivity among different mutant alleles would make it difficult to recognize reproductive problems as part of the ODDD syndrome in women.

CX37, by virtue of its expression in human oocytes, is another candidate for influencing women's fertility and this inference is also supported by research with mice: there is evidence that expression of this connexin is reduced, as is oocyte-cumulus GJIC, in a mouse model of type 1 diabetes (Ratchford *et al.*, 2008). It is possible, therefore, that CX37 insufficiency could contribute to the reproductive problems of diabetic women.

Endometrial function

Given its expression in the endometrium, CX26 is also likely to play roles in women's reproductive health. The gene encoding CX26 is very sensitive to estrogen administration (Grümmer *et al.*, 1994). Interestingly, endometrial CX26 expression is not only highly sensitive to estrogen

Table I Women's reproductive problems potentially associated with inherited connexin-based diseases (connexinopathies).

Connexinopathies	Mutated connexin	Possible female reproductive problems*
Congenital non-syndromic deafness; Vohwinkel syndrome; Bart-Pumphrey syndrome; keratitis-ichthyosis-deafness (KID) syndrome; palmoplantar keratoderma/hyperkeratosis	CX26	Implantation failure and/or endometriosis susceptibility due to altered or inappropriate uterine epithelial cell development
X-linked Charcot–Marie–Tooth disease	CX32	Endometriosis due to altered or inappropriate uterine epithelial cell development
Congenital atherosclerosis; congenital heart disease	CX37	Difficulty conceiving due to impaired oocyte/follicle development
Congenital or idiopathic heart arrhythmia (atrial fibrillation)	CX40	Pre-eclampsia due to reduced number of extravillous trophoblast cells and shallow invasion
Oculodentodigital dysplasia; Hallermann–Streiff syndrome; palmoplantar keratoderma; congenital heart malformations; congenital non-syndromic deafness	CX43	Difficulty conceiving due to deficiency of oogonia and/or impaired oocyte/follicle development; endometriosis due to impaired decidualization; intrauterine growth restriction due to placental insufficiency; pre-eclampsia due to maladaptive uterine blood flow regulation

Further information about connexin involvement in the listed diseases can be found in Dobrowolski and Willecke (2009).
*Possible reproductive problems likely to be more prevalent in women with a connexinopathy are suggested based on the localization of expression of the connexin within the human female reproductive tract and the consequences of loss of function mutations in female mice.

but also to the ER modulators raloxifen and tamoxifen and the phytoestrogen, genistein (Heikaus *et al.*, 2002). The use of these drugs in breast cancer treatment or to alleviate symptoms of menopause could therefore contribute to changes in endometrial physiology. Another problem of concern is endometriosis, a benign endometrial disease which is accompanied by ectopic endometrial lesions growing outside the uterine cavity. Endometriosis is characterized by an altered genetic programme of the human eutopic endometrium at the time of uterine receptivity. This inappropriate differentiation is correlated with aberrant connexin expression in ectopic endometriotic lesions, with expression of CX43 in the epithelium, loss of CX32, and strongly reduced CX26 (Regidor *et al.*, 1997). Such an aberrant expression pattern is also observed in baboon uteri with experimentally induced endometriosis resulting in a loss of CX26 and CX32 in the epithelium but an up-regulation of CX26 in the stromal cells (Winterhager *et al.*, 2009). These altered connexin expression profiles indicate a loss of the appropriate differentiation programme of both the epithelial and stromal compartments of the primate uterus and may be a factor that contributes to endometriosis-associated infertility.

Pregnancy

The severe disruption of decidual angiogenesis in *Gja1* conditional knockout mice (Laws *et al.*, 2008) and in mice harbouring the *Gja1*^{Jrt} mutant allele (Winterhager *et al.*, 2013) suggests the possibility that decidualization is a process early in human pregnancy that could be affected by aberrant expression or function of CX43. In humans, stromal decidualization has already been induced in the secretory phase of the menstrual cycle, with CX43 expression being a characteristic feature (Jahn *et al.*, 1995). Nair *et al.* (2011) reported that women with recurrent early pregnancy loss exhibit significantly reduced levels of CX43 expression in their decidua. These findings were supported by Yu *et al.* (2014), who found a reduced level of CX43 in the endometrial stromal cells of women with endometriosis who suffer from subfertility. Primary cultures of the diseased stromal cells did not only reveal strongly reduced CX43 expression but also they responded with a less pronounced transformation upon being induced to decidualize *in vitro*. Other evidence for linking CX43-based GJIC and aberrant decidualization comes from the use of mefloquine, an antimalarial drug that is also widely used as a potent blocker of GJIC (Cruikshank *et al.*, 2004; Nevin, 2011). Mefloquine is responsible for increased risk of early pregnancy loss and stillbirth as evidenced in several epidemiological studies (Smoak *et al.*, 1997; Irvine *et al.*, 2011; summarized by Nevin, 2012). These phenomena could be explained by inhibition of GJIC in the decidua. Alternatively, stillbirth under mefloquine treatment may be caused by the blockage of GJIC between cyto- and syncytiotrophoblast followed by an impairment of the fusion process.

It is well established that connexins are important regulators of the cardiovascular system, where gap junctions conduct the myocardial contraction waves underlying heart rhythm (reviewed by Lambiase and Tinker, 2014) and the endothelium-derived hyperpolarization waves that regulate arteriolar blood flow (reviewed by de Wit and Griffith, 2010 and Tyml, 2011). Indeed, connexin gene mutations have been linked to cardiac, arterial and lymphatic diseases (reviewed by Molica *et al.*, 2014). With respect to pregnancy, recent studies have shown that CX43 gap junctions in the uterine vascular endothelium are an essential contributor to the adaptive response to pregnancy since they convey the cell-to-cell Ca^{2+} signals which underlie production of the vasodilator, nitric oxide (reviewed by Boeldt *et al.*, 2011, 2014). Indeed, there is evidence that pre-eclampsia, a disease of pregnancy characterized by hypertension, is associated with phosphorylation-induced closure of CX43-based gap junction channels (Boeldt *et al.*, 2011, 2014).

Conclusion

As summarized in this review, numerous connexins are expressed in mammalian female reproductive organs and when mutated or their expression is reduced or eliminated in mice, reproductive impairment or

even outright infertility can result. It is thus surprising that there are no reports in the literature directly linking reproductive problems in women to connexin gene mutations. Whereas one would not expect every reproductive problem caused by a connexin mutation in mice to be phenocopied in the human population, there is generally good correspondence between mouse and human disease symptoms when considering connexin mutations affecting other parts of the body. Examples of this include the cochlea, where mutations in the mouse genes encoding CX26 and CX30 cause deafness (Cohen-Salmon *et al.*, 2002; Teubner *et al.*, 2003); the heart, where CX40 and CX43 mutations affect conductance of the contraction wave, resulting in arrhythmias (Simon *et al.*, 1998; Bevilacqua *et al.*, 2000; Kalcheva *et al.*, 2007; Tuomi *et al.*, 2011); the skeleton, where CX43 mutations are associated with a variety of deformities (Flenniken *et al.*, 2005; Dobrowolski *et al.*, 2008); and the nervous system, where CX32 mutations cause peripheral neuropathy (Anzini *et al.*, 1997; Scherer *et al.*, 1998). As summarized in Table I, women identified as suffering from the various recognized 'connexinopathies'—diseases caused by connexin mutations—should be examined more closely for signs of reproductive impairment or complications of pregnancy, such as pre-eclampsia or intrauterine growth restriction, that could be associated with their genetic diagnosis. The ability to link specific connexin gene mutations with specific aspects of reproductive impairment is essential for better understanding the contributions of these proteins to women's reproductive health.

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Conflict of interest

The authors have no conflict of interest to declare.

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