Rescue of oogenesis in Cx37-null mutant mice by oocyte-specific replacement with Cx43

Tony Y. Li[1,3], Deanne Colley[1,3], Kevin J. Barr[1,3], Siu-Pok Yee[2,3] and Gerald M. Kidder[1,3,*]

Departments of [1]Physiology and Pharmacology, Obstetrics and Gynaecology, Paediatrics, [2]Oncology and Biochemistry, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario N6A 5C1, Canada
[3]Children's Health Research Institute, 800 Commissioners Road East, London, Ontario N6C 2V5, Canada
*Author for correspondence (e-mail: gerald.kidder@schulich.uwo.ca)

Accepted 10 September 2007
Journal of Cell Science 120, 4117-4125 Published by The Company of Biologists 2007
doi:10.1242/jcs.03488

Summary

Mammalian oocytes and surrounding granulosa cells are metabolically coupled via gap junctions. In growing follicles of the mouse, gap junctions between oocytes and granulosa cells are assembled from connexin 37 (Cx37, encoded by *Gja4*), whereas those between granulosa cells are assembled from connexin 43 (Cx43, encoded by *Gja1*). This spatial separation, and the different permeability properties of gap junctions composed of Cx37 and Cx43, suggests that Cx37 channels serve a unique function in oogenesis. Female mice lacking Cx37 are sterile because oocytes do not complete their development. To test the hypothesis that the unique properties of Cx37 make it irreplaceable in oocytes, Cx43 was ectopically expressed in growing oocytes lacking Cx37. Transgenic mice were produced in which *Gja1* is expressed in oocytes under control of the *Zp3* (zona pellucida protein 3) gene promoter. When the transgene was crossed into the Cx37-null mutant line, oocyte–granulosa-cell coupling, oocyte growth and maturation, and fertility were all restored. Thus, despite their different properties, Cx43 is physiologically equivalent to Cx37 in coupling oocytes with granulosa cells.

Key words: Gap junction, Granulosa cell, Intercellular communication, Oocyte, Oogenesis

Introduction

Gap junction channels, which metabolically couple cells to allow the direct passage of small molecules between them, are assembled from a large family of homologous proteins, the connexins (reviewed by Harris, 2001). Six connexins oligomerize to form a hemichannel or connexon, and two connexons from opposing cells dock end-to-end to form the intercellular channel. To date, 20 connexin genes have been identified in the human genome and 19 in the mouse (Söhl and Willecke, 2003). Gap junction channels composed of different connexins exhibit different biophysical and permeability properties when expressed alone in cultured cells, suggesting that different types of gap junctions could perform different developmental or physiological functions in vivo. Support for this idea is provided by the fact that individual connexins are restricted in their tissue domains of expression (Söhl and Willecke, 2003). Although there is considerable co-expression within cells, this can involve segregation into different cell surface domains or cell-cell interfaces (Guerrier et al., 1995; Veitch et al., 2004). Thus, the concept that gap junction channels composed of different connexins perform distinct functions is well-established among investigators.

A limited number of studies have provided direct tests of the functional uniqueness of gap junctions formed of different connexins. The common approach has been to use gene targeting in embryonic stem cells to produce mice in which one connexin coding region replaces another, bringing expression of the replacement connexin under the control of the promoter of the targeted gene. For example, this germline 'knock-in' approach revealed that connexin 46 (Cx46, encoded by *Gja3*) can only partially replace Cx50 (encoded by *Gja8*) in the lens of the eye, in which both connexins are expressed in the fiber cells: whereas the reduction of lens clarity (cataractogenesis) caused by the loss of Cx50 was corrected, the lens growth restriction was not (White, 2002). Plum et al. described more-complex knock-in situations in which Cx43 (encoded by *Gja1*), the most widely expressed connexin, was replaced by either Cx32 (encoded by *Gjb1*) or Cx40 (encoded by *Gja5*) (Plum et al., 2000). The absence of Cx43 from mouse embryos disrupts the development of many organs, the most important of which being the heart: Cx43-deficient mouse pups die soon after birth because of dysmorphogenesis of the cardiac outflow tract (Reaume et al., 1995). This severe developmental defect did not arise when Cx43 was replaced by either Cx32 or Cx40 (Plum et al., 2000). The knock-in offspring survived, indicating that Cx32 and Cx40 can serve the same function as Cx43 during cardiac morphogenesis. By contrast, the testes, eye lenses and mammary glands of one or both knock-in strains showed defects, indicating the failure of Cx32 or Cx40 to functionally replace Cx43. Thus, some developmental processes must depend on non-redundant connexin functions.

A confounding issue with knock-in experiments such as these is that, because the connexin replacement occurs in the germ line, all organs in which the replaced connexin is expressed could potentially be altered to some degree, leading to widespread perturbations that could indirectly affect the organ of interest. To avoid this problem, we have tested the ability of one connexin to functionally replace another using a more precise strategy. Our cell type of interest is the developing oocyte. Mammalian oocytes develop within follicles in which each oocyte is linked with the surrounding layers of somatic (granulosa) cells via gap junctions (reviewed by Kidder, 2005). These heterocellular gap junctions serve both to transfer nutrients to the oocyte from the granulosa cells, sustaining its

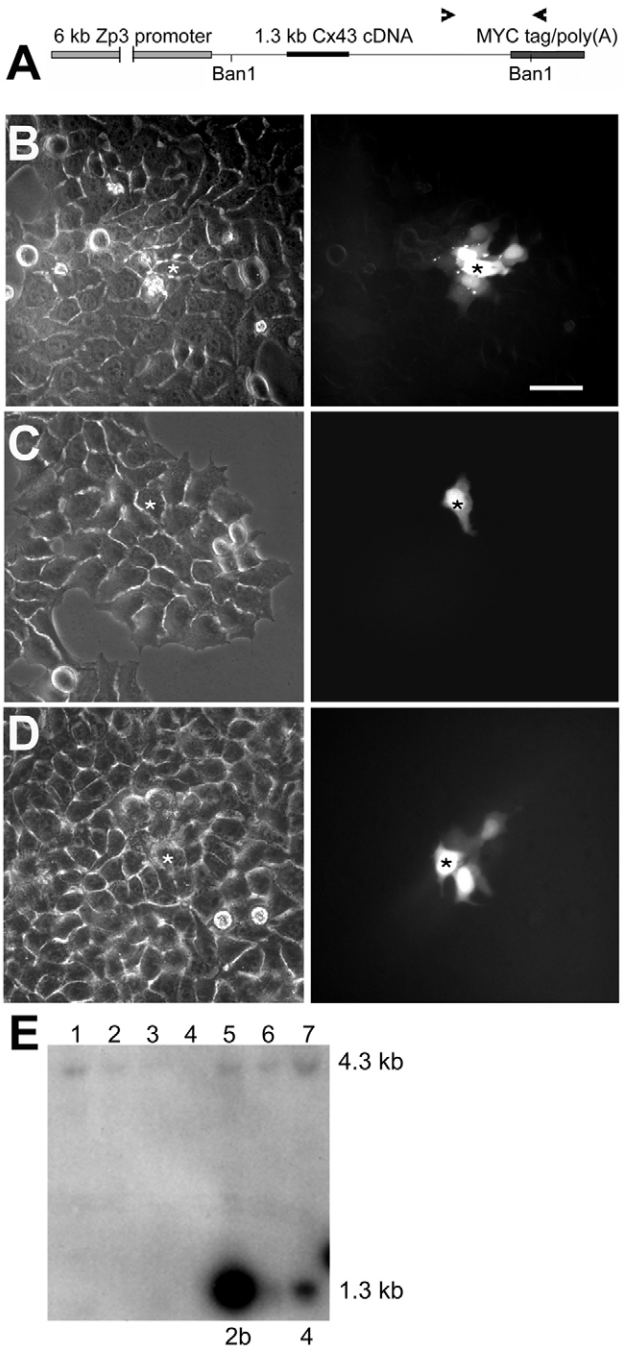

Fig. 1. Production of *Zp3-Gja1* transgenic mice. (A) Structure of the *Zp3-Gja1* transgene (not to scale). The transgene consisted of a 6 kb fragment of the *Zp3* promoter, a 1.3 kb *Gja1* exon 2 cDNA containing the entire rat Cx43 coding sequence (thin line), and a cassette consisting of a MYC-tag-encoding sequence and polyadenylation signal. The solid black section of the cDNA indicates the probe for Southern blotting and arrowheads represent the primers for PCR genotyping and RT-PCR. (B-D) Functional testing of Cx43-MYC. (B) Lucifer yellow dye was injected into a single cell (asterisk) of a population of gap-junctional-communication-deficient HeLa cells expressing the transgene. The dye readily passed to neighboring cells, as revealed by fluorescence microscopy (image at right). (C) The typical result of no dye transfer after injecting Lucifer yellow into un-infected HeLa cells not expressing the transgene. (D) Dye was injected into HeLa cells expressing native (not MYC-tagged) Cx43. (E) A typical Southern blot showing detection of the transgene in a sample of offspring. Digestion of tail-snip DNA with *Ban*I generated a 1.3 kb fragment specific to the transgene in addition to the endogenous genomic fragment at 4.3 kb. Lanes 1-7 indicate results from seven of the potential founder offspring tested; lanes 5 and 7 represent transgenic lines 2b and 4, respectively, which were analyzed in the present study. Bar, 50 μm.

growth, and to regulate oocyte meiosis. Recent evidence indicates that the gap junctions connecting mouse oocytes and granulosa cells during oocyte growth consist of homomeric, homotypic channels composed of Cx37 (encoded by *Gja4*) (Veitch et al., 2004). The cumulus and mural granulosa cells are themselves connected by gap junctions, which are, in this case, composed of Cx43 (Gittens et al., 2003; Tong et al., 2006). Mouse granulosa cells therefore express both Cx37 and Cx43 but target them to different cell-surface-membrane domains: Cx37 to contacts with the oocyte and Cx43 to contacts with each other (Veitch et al., 2004). The developmental importance of the gap junctions that couple growing oocytes with granulosa cells was clearly demonstrated when the gene encoding Cx37 was knocked out (Simon et al., 1997). Although viable and without overt pathogenesis, female mice lacking Cx37 are sterile because folliculogenesis in the ovaries is disrupted. In the absence of oocyte–granulosa-cell coupling provided by Cx37, null mutant oocytes suffer growth retardation and do not survive to become meiotically competent (Carabatsos et al., 2000). Follicle growth is also interrupted: the mutant granulosa cells form structures resembling corpora lutea, which would normally develop only after the mature oocyte has been expelled from the follicle during ovulation. Thus, Cx37 gap junctions are essential to maintain oocyte growth and survival, which in turn is necessary to maintain proper granulosa-cell function (Sugiura and Eppig, 2005).

Given the different locations of Cx37 and Cx43 within developing mouse follicles, we hypothesized that they play non-redundant roles in oogenesis, possibly transmitting different metabolites or second messengers between the different compartments of the follicle. This hypothesis is supported by the fact that these two connexins differ markedly in their biophysical properties and permeabilities (Elfgang et al., 1995; Veenstra et al., 1995; Weber et al., 2004; Ek-Vitorín and Burt, 2005). To test our hypothesis, we generated mice in which Cx37 is replaced by Cx43 specifically in growing oocytes (hereafter called connexin-replacement mice). An oocyte-specific promoter was fused to the *Gja1* coding region to produce a transgene that could then be crossed into the Cx37-knockout mouse line so that Cx43 would replace the missing Cx37 from the beginning of oocyte growth. Because the granulosa cells would also lack Cx37, any gap junctions assembled at the oocyte surface would be composed entirely of Cx43. Our prediction was that, although coupling between the oocytes and granulosa cells would be restored, the females would remain sterile, unable to produce viable oocytes.

Results

Generation of transgenic mice

The 7.75 kb transgene (Fig. 1A) was designed to target expression of MYC-tagged rat Cx43 in growing mouse oocytes (rat and mouse Cx43 sequences are 95% identical at the nucleotide level and 99% identical at the amino acid level). We

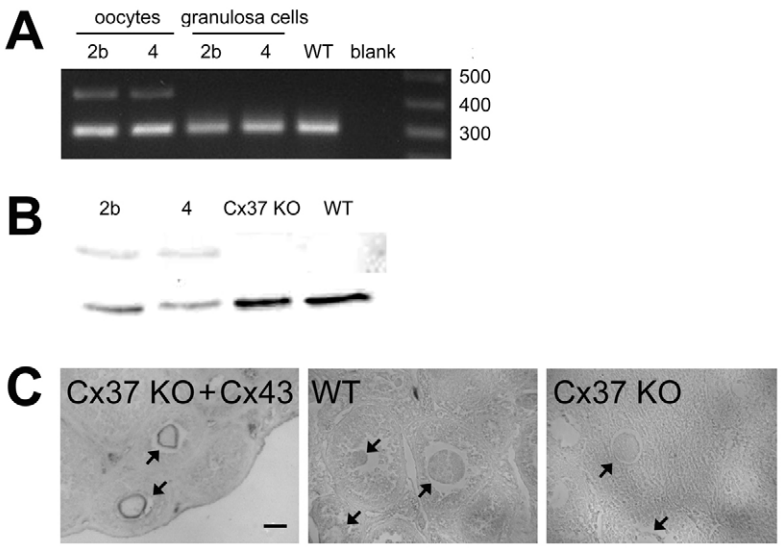

Fig. 2. Verification of transgene expression by probing for the MYC tag. (A) RT-PCR amplified a segment of the MYC-encoding sequence (upper band, 420 bp) from oocyte RNA, but not granulosa cell RNA, of both transgenic lines. A segment of mRNA encoding glyceraldehyde phosphate dehydrogenase (GAPDH; lower band, 299 bp) was amplified as a positive control. WT, wild-type ovary; blank, negative control (no template). (B) The MYC epitope (upper band) was detected by western blotting in ovaries of both transgenic lines, but not in ovaries of Cx37-knockout (Cx37 KO) or wild-type (WT) mice. The lower band is GAPDH (loading control). (C) Immunostaining for the MYC epitope in ovary sections revealed its localization at the oocyte surface in connexin-replacement mice and its absence from wild-type or Cx37-deficient mice. Oocytes are indicated by arrows. Bar, 30 μm.

chose the *Zp3* promoter because, during oogenesis in the mouse, the *Zp3* gene is active from the beginning of oocyte growth to direct the synthesis of ZP3, one of the three glycoproteins that constitute the oocyte-derived zona pellucida (Epifano et al., 1995). The MYC tag was added to ensure that Cx43 expressed from the transgene could be detected against a background of robust expression of endogenous Cx43 in developing follicles. To confirm that the transgene would encode functional Cx43, the construct was expressed in communication-deficient HeLa cells for dye-transfer tests. Thirty-five of the 40 Cx43-MYC-infected HeLa cell clusters tested showed strong evidence of dye coupling, whereas only six of the 30 un-infected clusters tested did so, demonstrating that the Cx43-MYC fusion protein could form functional gap junctions (Fig. 1B,C). The extent of dye coupling among Cx43-MYC-expressing HeLa cells was comparable to that among cells expressing native Cx43 (Fig. 1D).

In total, 48 offspring were obtained after pronuclear microinjection and transfer of zygotes to 11 pseudopregnant foster mothers. The transgene was detected in nine of those offspring by Southern blotting (a sample blot is shown in Fig. 1E). Total RNA from ovaries of each of the nine founder lines was subjected to semi-quantitative reverse transcriptase (RT)-PCR directed at the MYC-encoding sequence to compare transgene expression levels. Lines 2b and 4 were chosen for further study as having high and low expression levels corresponding to estimated transgene copy numbers of 20 and three, respectively (our unpublished data). These two lines were maintained for five generations by backcrossing transgenic males with C57BL/6 females; to minimize genetic background differences, we analyzed pools of littermates issuing from transgenic males of the same backcross generation. When transgenic females heterozygous for the Cx37-null mutation were crossed with Cx37-knockout males to produce connexin-replacement offspring, both transgenic lines generated litters of normal size (Table 1) with pups that were free of obvious developmental abnormalities. In addition, the transgene was inherited in Mendelian fashion. Thus, ectopic expression of Cx43 in developing oocytes containing Cx37 did not cause reproductive impairment.

Confirmation of transgene expression in oocytes

RT-PCR applied to oocyte RNA from 3-week-old juveniles of the two selected transgenic lines, from the Cx37-knockout line and from wild-type mice revealed the MYC-encoding sequence in the transgenic females, restricted to oocytes (Fig. 2A). Likewise, when western blots of whole-ovary lysates were probed with an anti-MYC antibody, the MYC epitope was only detected in ovaries of the transgenic mice (Fig. 2B). Probing for the MYC epitope in ovary sections by immunocytochemistry revealed staining at the oocyte surface in connexin-replacement ovaries but not in ovaries of wild type or in Cx37-knockout ovaries (Fig. 2C). To confirm Cx43 protein expression in the oocytes of transgenic females, 600 oocytes were isolated from each of the two selected transgenic lines and from wild-type females, and 700 oocytes were isolated from Cx37-null females. The entire oocyte lysate from each strain/line was loaded into one well of a polyacrylamide gel. The resulting western blot was probed for total Cx43 (i.e. endogenous or from the transgene) using a Cx43-specific antibody. Cx43 was detected in oocytes from both of the transgenic lines but not from the wild-type and Cx37-null lines (Fig. 3A). Interestingly, we did not detect the slower-migrating phosphorylated Cx43 bands that are typically seen in somatic

Table 1. Number of offspring

	Cx37 KO + Cx43		Cx37 KO		Cx37 het + Cx43		Wild type	
Line	No. females	Pups/female	No. females	Pups/female	No. females	Pups/female	No. females	Pups/female
2b	8	6.9±0.9	4	0	9	7.1±0.8	10	6.9±2.3
4	12	7.3±0.6	5	0	8	7.0±0.8		

Numbers of offspring (± s.d.) from connexin-replacement females (Cx37 KO + Cx43) in comparison with Cx37-knockout females (Cx37 KO), females expressing both Cx37 and Cx43 in oocytes (Cx37 het + Cx43), and wild-type females.

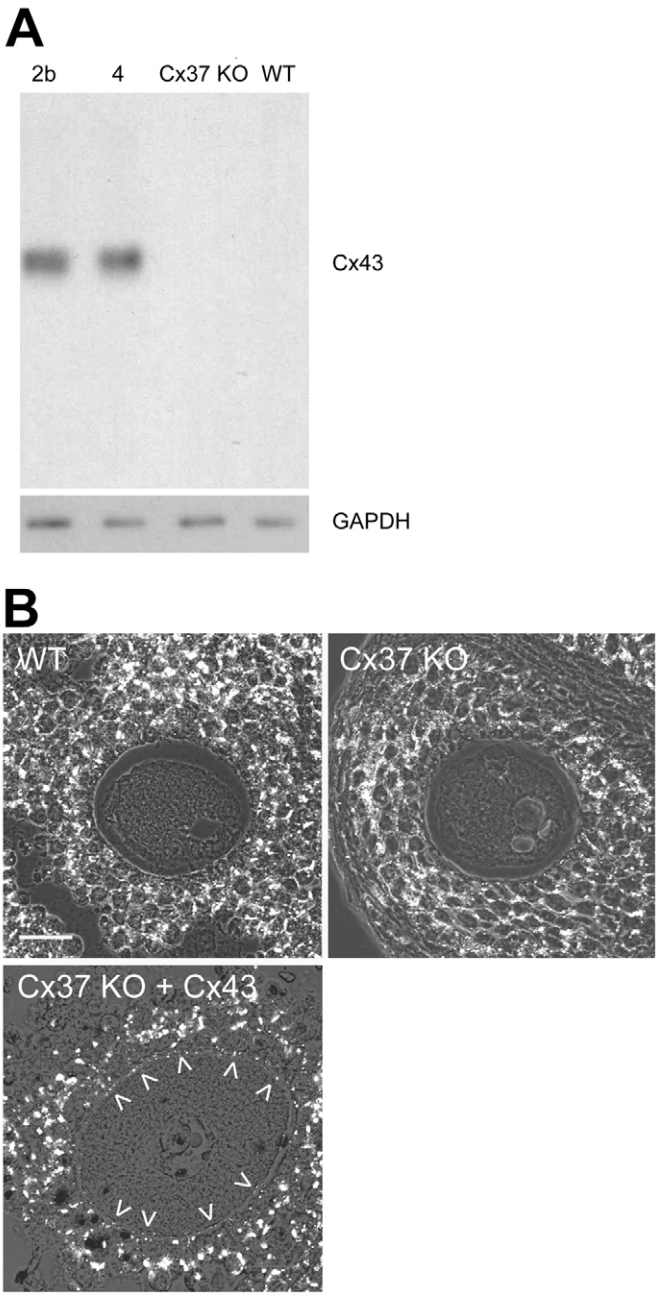

Fig. 3. Expression of Cx43 at the oocyte surface in connexin-replacement follicles. (A) Detection of Cx43 in isolated oocytes by western blot. Oocyte lysates from transgenic lines 2b and 4 along with lysates from Cx37-deficient (Cx37 KO) and wild-type (WT) oocytes were separated electrophoretically, blotted, and the blot probed sequentially with Cx43 antibody (top) and GAPDH antibody (bottom row). (B) Localization of Cx43 in sections of follicles. Whereas no Cx43 was detected at the oocyte surface in wild-type or Cx37-deficient follicles, small foci of Cx43 immunoreactivity typical of gap junction plaques (arrowheads) were detected at the oocyte surface in Cx37-deficient follicles carrying the Cx43-expressing transgene. Bar, 20 μm.

cell immunoblots, although these were seen consistently in granulosa cell blots (our unpublished data) (Flenniken et al., 2005). These results confirmed expression of Cx43 from the transgene in oocytes and are consistent with previous work indicating that Cx43 does not normally contribute to connexons on mouse oocytes (Kidder and Mhawi, 2002; Veitch et al., 2004; Gittens and Kidder, 2005).

Ectopic expression of Cx43 in oocytes of transgenic females, and its incorporation into putative gap junctions, was demonstrated by immunolocalization in ovary sections. As shown in Fig. 3B, confocal immunofluorescence microscopy revealed Cx43 in tiny punctate foci on the surface of transgenic oocytes lacking Cx37 (connexin-replacement oocytes). By contrast, no such Cx43 puncta were detected on the surface of oocytes in sections from Cx37-deficient or wild-type ovaries lacking the transgene.

Restoration of oocyte–granulosa-cell coupling in Cx37-null mutant follicles by replacement with Cx43

To confirm functional expression of Cx43 in the transgenic oocytes, a dye-transfer test was performed. Isolated follicles from Cx37-null mutant ovaries and connexin replacement ovaries were cultured separately for 24 hours before microinjecting 5% Lucifer yellow into the oocytes. In total, 15 microinjections into connexin-replacement oocytes were recorded and 14 of those showed strong dye transfer into the surrounding granulosa cells (Fig. 4C,D), as also occurred with wild-type follicles (Fig. 4E,F). By contrast, none of the seven microinjections into oocytes of the Cx37-knockout line demonstrated dye transfer to the granulosa cells (Fig. 4A,B).

Restoration of folliculogenesis in Cx37-null mutant ovaries by replacement with Cx43

Ovaries from the connexin-replacement mice, from the Cx37-knockout line and from wild-type littermate mice were sectioned and stained with hematoxylin and eosin. All developmental stages of follicles could be seen in the ovaries of connexin-replacement mice (Fig. 5C), and there was no obvious morphological difference between those and wild-type mice (Fig. 5A). Ovaries from comparably aged Cx37-knockout mice (Fig. 5B), by contrast, were devoid of late-antral-stage or preovulatory follicles, as reported previously (Simon et al., 1997). Measurements of follicle diameter confirmed that the rate of follicle growth in the connexin-replacement ovaries was the same as that of wild-type ovaries (P=0.96 by two-way ANOVA; Fig. 6). Furthermore, the oocytes of wild-type and connexin-replacement mice grew at the same rate with no significant effect of genotype (P=0.12; Fig. 7). By contrast, both follicles and oocytes of Cx37-knockout ovaries were reduced in diameter in comparison with wild-type and connexin-replacement ovaries by the early antral stage (Figs 6 and 7). These results indicate that folliculogenesis in Cx37-knockout ovaries had been restored by expression of Cx43 from the transgene.

Complete restoration of folliculogenesis implied that the oocytes of connexin-replacement mice should be developmentally competent. To confirm this, we tested their meiotic competence and ovulation potential. Cumulus-oocyte complexes were obtained from punctured antral follicles of females primed with equine chorionic gonadotropin (eCG), a peptide hormone with follicle-stimulating hormone (FSH) activity. After Hoechst staining, it was apparent that oocytes from connexin-replacement females were equally as competent as wild-type oocytes in undergoing the first meiotic division to produce the first polar body (Table 2). Likewise, after priming with eCG and inducing ovulation with human chorionic

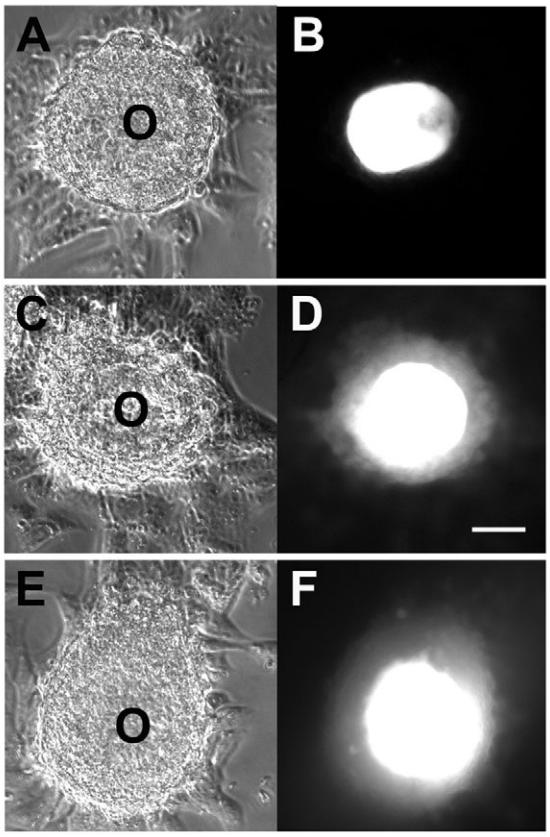

Fig. 4. Restoration of oocyte–granulosa-cell coupling in Cx37-deficient follicles by replacement with Cx43. Injection of Lucifer yellow into oocytes (O) of follicles from Cx37-deficient mice (A,B) did not result in dye transfer to the surrounding granulosa cells. By contrast, dye injected into oocytes of follicles from Cx37-deficient mice carrying the transgene (C,D) and from wild-type mice (E,F) passed readily into the surrounding granulosa cells. Bar, 50 μm.

gonadotropin (hCG) as a source of luteinizing hormone (LH) activity, connexin-replacement females ovulated as many oocytes as wild-type females (Table 3). With respect to both of these tests, Cx37-knockout females proved to be incompetent, as reported previously (Simon et al., 1997; Carabatsos et al., 2000).

To test the fertility of the connexin-replacement mice, females from both transgenic founder lines were mated with Cx37-knockout males. The connexin-replacement females produced litters equivalent to those of females heterozygous for the Cx37-null mutation and carrying the transgene encoding Cx43, whereas Cx37-knockout females were infertile

Table 2. Meiotic competence of oocytes

Stage	Cx37 KO + Cx43	Wild type	Cx37 KO
GV	3	3	65
MI	8	5	1
MII	60	61	0
Total	71	69	66

Test for meiotic competence of oocytes from connexin-replacement females (Cx37 KO + Cx43; *n*=7) in comparison with wild-type (*n*=6) and Cx37-knockout (Cx37 KO; *n*=7) females. GV, germinal vesicle; MI, first meiotic metaphase; MII, second meiotic metaphase.

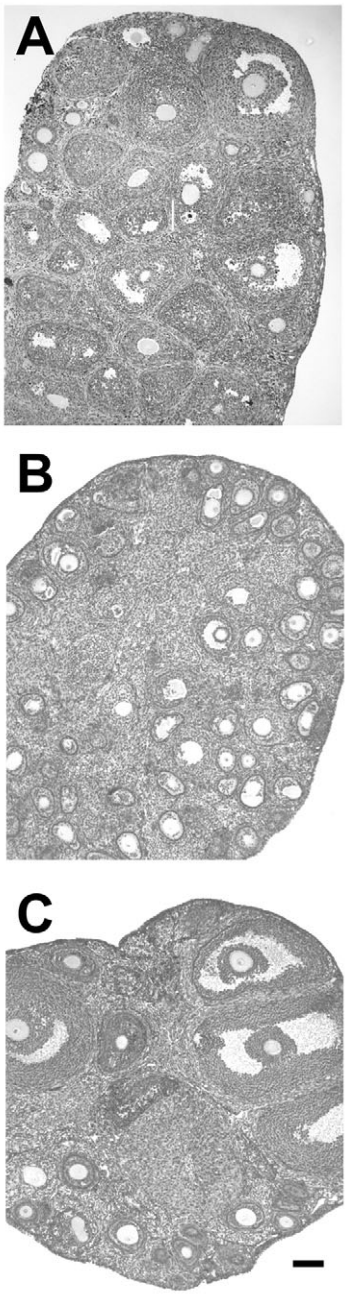

Fig. 5. Restoration of folliculogenesis in Cx37-deficient ovaries by replacement with Cx43. Sections of wild-type ovaries (A) and ovaries from Cx37-deficient mice carrying the transgene (C) revealed all stages of folliculogenesis, including large antral follicles, whereas sections from Cx37-deficient ovaries without the transgene (B) did not contain follicles beyond early antral stages. Bar, 100 μm.

(Table 1). No phenotypic differences have been noted between offspring of wild-type and connexin-replacement females. This rescue of oogenesis in Cx37-deficient females by ectopic expression of Cx43 in the oocytes implies that these two connexins are physiologically equivalent in this context.

Discussion

Based on the different locations of Cx37 and Cx43 gap junctions within developing ovarian follicles and the fact that the two

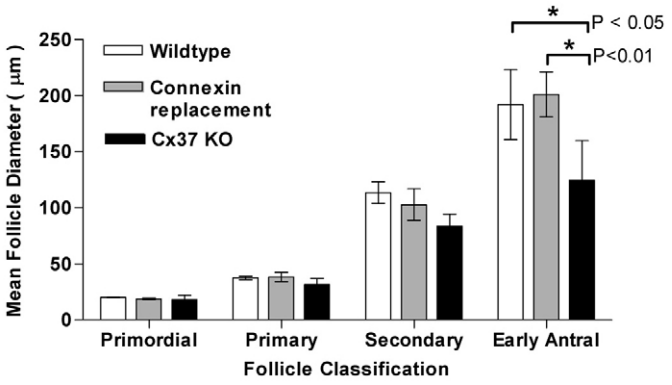

Fig. 6. Rates of follicle growth in wild-type, connexin-replacement and Cx37-knockout ovaries. Mean follicle diameter was determined for samples of 195 follicles of four wild-type mice, 124 follicles of five connexin-replacement mice and 84 follicles of three Cx37-knockout mice. The chi-square test was used to compare the frequency distributions of follicle types between genotypes, whereas two-way ANOVA with Bonferroni test was used to test for interaction between genotype and follicle size. Although connexin-replacement follicles achieved the same mean diameter as wild-type follicles in the early antral stage, mean follicle diameter in Cx37-knockout follicles was significantly less. Bars represent means ± s.e.m.

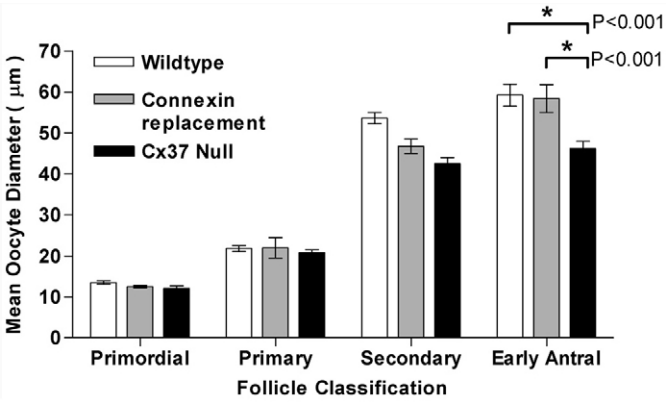

Fig. 7. Rates of oocyte growth in wild-type, connexin-replacement and Cx37-knockout ovaries. Mean oocyte diameter was determined for the same follicles analyzed for Fig. 5. The chi-square test was used to compare the frequency distributions of follicle types between genotypes, whereas two-way ANOVA with Bonferroni test was used to test for interaction between genotype and oocyte size. Although oocytes of connexin-replacement follicles achieved the same mean diameter as oocytes of wild-type follicles in the early antral stage, mean oocyte diameter in Cx37-knockout follicles was significantly less (P<0.001).

types of channel differ in their permeability properties, we hypothesized that they play distinct and non-redundant roles in oogenesis, possibly transmitting different metabolites or second messengers between the different compartments of the follicle. The evidence accumulated to date indicates that the gap junctions coupling growing oocytes with granulosa cells comprise homomeric/homotypic Cx37 channels, whereas those coupling granulosa cells with each other comprise homomeric/homotypic Cx43 channels (Kidder, 2005). The properties of these two types of channel have been characterized in transfected cells and in mRNA-injected *Xenopus* oocytes. Not only do Cx37 and Cx43 channels differ markedly in their highest unitary conductance states (310 versus 100 pS, respectively), they also differ in the limiting diameter of the channel pore, with Cx37 channels being more restrictive to larger test molecules (Harris, 2001; Weber et al., 2004). Cx37 channels have also proven more restrictive than Cx43 channels when permeability to specific anionic and cationic test molecules is concerned (Elfgang et al., 1995; Veenstra et al., 1995; Ek-Vitorín and Burt, 2005). Given these differences in molecular selectivity, it would not have been surprising if Cx43 had failed to functionally replace Cx37 in supporting oogenesis even though it restored gap junctional coupling between oocytes and granulosa cells. On the contrary, ectopic expression of Cx43 in oocytes completely rescued oogenesis in Cx37-knockout females. It might be argued that this rescue occurred only because the amount of Cx43 exceeded that of endogenous Cx37 in wild-type oocytes; however, analysis of replacement mice from transgenic lines having widely divergent Cx43 expression levels did not reveal any difference in outcome.

The importance of gap junctional coupling between oocytes and granulosa cells, both in supplying the oocyte with essential nutrients and in transmitting signaling molecules between the two compartments, has been amply demonstrated (Eppig, 1994; Kidder, 2005). Therefore, we have assumed that gap-junction-channel permeability was the crucial feature determining the ability of Cx43 to replace Cx37 in oocytes, as opposed to connexin functions not related to gap junctions (Stout et al., 2004). Although the permeability of Cx37 channels to naturally occurring molecules has not been directly examined, a variety of molecules have been demonstrated or inferred to pass through the gap junctions connecting oocytes with granulosa cells in the mouse. These include pyruvate, alanine and histidine, cAMP, Ca^{2+} and/or inositol trisphosphate (IP_3), and inorganic ions involved in oocyte pH regulation (Webb et al., 2002a; Webb et al., 2002b; Sugiura and Eppig, 2005; Eppig et al., 2005; FitzHarris and Baltz, 2006). Of this list of molecules, cAMP and IP_3 have been demonstrated experimentally to pass through Cx43 channels along with other nucleoside phosphates, nucleosides, glutamate, glucose and glutathione (Kam et al., 1998) (reviewed by Goldberg et al., 2004). We propose that it is the permeability of Cx43 channels to such a broad range of molecules that accounts for its ability to replace the more restrictive Cx37 channels in coupling oocytes with granulosa cells. This property could also account for the fact that Cx43 is the most widely expressed of all the connexins.

In addition to demonstrating the physiological equivalence of Cx37 and Cx43 channels in coupling oocytes with granulosa cells, our results help to clarify uncertainty about the involvement of Cx43 at the oocyte surface. Previous evidence from microscopical analyses of mouse follicles is conflicting, with several reports indicating failure to detect Cx43 at the

Table 3. Ovulation competence

	Number of mice	Number of oocytes	Oocytes/mouse
Cx37 KO + Cx43	5	108	21.6±9.1
Wild type	5	113	22.6±9.5
Cx37 KO	3	0	0

Test for ovulation competence (oocytes/mouse ± s.d.) of connexin-replacement females (Cx37 KO + Cx43) in comparison with wild-type and Cx37-knockout (Cx37 KO) females.

oocyte surface and at least one report indicating its presence there (reviewed by Kidder, 2005). Dye-transfer tests demonstrated that oocyte–granulosa-cell coupling does not depend on Cx43 but requires Cx37 in both cell types (Veitch et al., 2004), but the relative insensitivity of that technique left open the possibility of a minor contribution from Cx43 channels. Our western blot analysis, however, indicated that this is not likely: no Cx43 was detected in denuded oocytes from either wild-type or Cx37-knockout follicles. This finding also rules out the possibility that Cx43 expression might be activated in oocytes lacking Cx37. If Cx43 plays any role in oocyte–granulosa-cell coupling, it must be via a small number of channels formed by the docking of Cx43 connexons on granulosa cells with Cx37 connexons on the oocyte, forming heterotypic gap junctions. Such junctions have been demonstrated by co-culturing Cx37- and Cx43-transfected HeLa cells (Elfgang et al., 1995).

In summary, we have used transgenesis applied to a knockout mouse strain to effect a connexin substitution in a specific cell type, the developing oocyte. The results indicate that the role of Cx37 in forming gap junctions at the oocyte–granulosa-cell interface is not unique in that the same role can be played by a connexin with very different biophysical and permeability properties. The only requirement for gap junction channels coupling oocytes with granulosa cells might be that they are permeable to a specific set of key molecules involved in oocyte growth, meiotic control and regulation of granulosa cell function. If this is the case, then Cx37 might be one of several connexins whose permeability properties make it suitable for this purpose. A similar lack of stringent permeability requirements might pertain to the gap junctions coupling granulosa cells with each other. Plum et al. generated mice in which the genomic *Gja1* coding sequence was replaced by cDNA encoding either Cx32 or Cx40 (Plum et al., 2000). Because Cx43 is the only connexin that contributes significantly to gap junctional coupling among mouse granulosa cells (Gittens et al., 2003; Tong et al., 2006), the knock-in females would have been expected to express Cx32 or Cx40 in their granulosa cells exclusively. Folliculogenesis in females of both knock-in lines was reported to be ‘histologically normal’, indicating that both Cx32 and Cx40 can serve the function of Cx43 during follicular growth (Plum et al., 2000). However, the Cx32 knock-in females failed to deliver any live offspring and the Cx40 knock-in females did not become pregnant, probably reflecting physiological disturbances in other components of the reproductive system. The strategy employed in the present study avoids this problem by restricting the replacement of one connexin by another to a single cell type.

Materials and Methods

Generation of transgenic and connexin-replacement mice

Rat *Gja1* cDNA (supplied by David Paul, Harvard Medical School, MA) in Bluescript M13+ phagemid (antisense orientation with respect to the *T7* promoter) was modified by adding an artificial *Xho*I restriction site at the 3′ end to replace the stop codon. This *Xho*I site was used to attach a cassette encoding the MYC epitope and containing the bovine growth hormone (*GH*) gene polyadenylation signal. Prior to attaching the promoter of the transgene, the ability of the construct to encode functional Cx43 was tested by subcloning into an AP2 replication-defective retroviral vector (Galipeau et al., 1999), which was then transfected into 293PGP packaging cells. The resultant virus was used to infect communication-deficient HeLa cells (Veitch et al., 2000), which were then tested for intercellular coupling by Lucifer yellow dye injection. To attach the promoter of the transgene, the Cx43-encoding construct was subcloned into the *Eco*RV-*Not*I restriction sites of the pBluescript II KS vector (provided by Jurrien Dean, National Institutes of Health, MD) downstream of the 6-kb mouse *Zp3* (encoding zona pellucida protein 3) promoter (Rankin et al., 1998). The 7.75 kb transgene (Fig. 1A) containing the *Zp3* promoter, *Gja1* cDNA and MYC-encoding cassette was released from the vector by digestion with meganuclease-I–*Sce*I and *Not*I restriction enzymes, separated from the vector by agarose gel electrophoresis, purified using an Elutip-D mini-column (Schleicher and Schuell, Keene, NH), and dissolved at a concentration of 1-2 ng/μl in injection buffer (10 mM Tris-HCl, pH 7.5, 0.25 mM EDTA). The transgene was injected into the male pronuclei of C57BL/6 × CBA F_1 mouse zygotes. Injected embryos were implanted into the oviducts of pseudopregnant recipient females using a standard protocol approved by the Animal Use Subcommittee of the University Council on Animal Care, The University of Western Ontario. Offspring were screened for the presence of the transgene by Southern blotting of tail-snip DNA using *Ban*I to digest the DNA and using PCR-amplified *Gja1* cDNA fragment as probe.

Transgenic founder lines were maintained by backcrossing with C57BL/6J mice. Genotyping was performed by PCR using primers CX43XB (5′-ATGTCTC-CTCCTGGGTACAAG-3′) and TAGR (5′-GGCAACTAGAAGGCACAGTC-3′) situated in the Cx43 and MYC-encoding sequences, respectively. These primers specifically amplified a 420 bp segment of the transgene. The PCR mixture contained 2.5 μl 10× PCR buffer, 1.5 μl 50 mM $MgCl_2$, 3 μl 2 mM dNTP, 0.75 U Platinum Taq DNA polymerase (Invitrogen Canada, Burlington, ON), 1.5 μl of each primer with concentration 10 μM, 1 μl proteinase K-digested ear punch DNA taken at weaning, and 14 μl dH_2O in a total volume of 25 μl. PCR commenced with a 5 minute incubation at 94°C, followed by 30 cycles of 94°C for 30 seconds, 66°C for 30 seconds and 72°C for 30 seconds. The final extension phase was 5 minutes at 72°C. PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

To generate mice lacking Cx37 but carrying the *Zp3-Gja1* transgene (connexin-replacement mice), male Cx37-knockout mice ($Gja4^{-/-}$, C57BL/6 strain) were first crossed with female mice [C57BL/6-*Tg(Zp3-Gja1)Kdr*] carrying the transgene encoding Cx43. Resultant offspring of genotype $Gja4^{+/-}$; $Tg(Zp3\text{-}Gja1)^{+/}$ were intercrossed to produce connexin-replacement mice [$Gja4^{-/-}$; $Tg(Zp3\text{-}Gja1)^{+/}$]. Offspring from these crosses were genotyped by Southern blotting to identify the transgene and PCR to confirm the presence or absence of the *Gja4* wild-type and null alleles. The primers and amplification conditions were as described previously (Gittens and Kidder, 2005).

Follicle and oocyte isolation

Female mice (2- to 4-weeks old) were killed by cervical dislocation following CO_2 anesthesia. Ovaries were transferred to Waymouth MB 752/1 medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.6% Omnipur HEPES free acid (EM Science, Gibbstown, NJ), 0.5% penicillin-streptomycin (Invitrogen) and 1 mg/ml collagenase type 1 (Sigma-Aldrich Canada, Oakville, ON). Follicles were liberated by repeated pipetting every 5 minutes with a 1 ml pipettor two to three times. Follicles were collected and transferred to another dish containing the above culture medium without collagenase. For oocyte isolation, the follicles were centrifuged for 4 minutes at 250 ***g***, re-suspended in 0.05% Trypsin/EDTA in a Petri dish, incubated in cell culture incubator for 2 minutes at 37°C, pipetted repeatedly to dissociate the oocytes from the granulosa cells, then diluted in more culture medium, centrifuged again, and resuspended in the culture medium. Oocytes were picked individually and placed in another dish with culture medium for one more wash before being collected for further analysis.

RT-PCR

Total RNA from pooled oocytes was isolated using the RNeasy Mini kit (Qiagen, Mississauga, ON), whereas total RNA from tissues was isolated using the Trizol method (Invitrogen). The RNA was reverse transcribed using oligo(dT) primer, SuperScript reverse transcriptase, reaction buffer and dNTPs, all supplied by Invitrogen. PCRs were performed using the primers described above for the transgene and a second set of primers, 5′-GAACGGATTTGGCCGTATTG-3′ (GAPDH-forward) and 5′-CCTTCTCCATGGTGGTGAAG-3′ (GAPDH-reverse), designed to amplify transcripts encoding the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH; amplicon size 299 bp), as an internal control. The PCR parameters were the same as described above except the number of cycles ranged between 26 and 30. Semi-quantitative RT-PCR was performed as previously described (Gittens et al., 2005), using transcripts encoding the housekeeping enzyme adenosine phosphoribosyl transferase (APRT) as an internal standard.

Ovarian histology and immunolocalization

Ovaries from 2- to 3-month-old females were fixed in either Bouin’s solution (5 ml glacial acetic acid, 25 ml 40% formalin and 75 ml picric acid) for 2 hours for histological examination, or zinc fixative (0.05 g calcium acetate, 0.5 g zinc acetate and 0.5 g zinc chloride dissolved in 100 ml 0.1 M Tris, pH 7.4) overnight for immunofluorescence, embedded in paraffin and sectioned at a thickness of 5 μm. For histological examination, sections were deparaffinized in xylene and rehydrated in graduated ethanol solutions followed by staining with hematoxylin alone or hematoxylin and eosin. For diameter measurements, follicles were classified as primordial (with a single layer of flattened granulosa cells), primary (a single layer of cuboidal granulosa cells), secondary (multiple layers of granulosa cells but lacking

any sign of an antral cavity) or early antral (with one or more small fluid-filled cavities). Oocyte and follicle diameters were determined for those follicles in which the section passed through the oocyte nucleus; the recorded diameter was taken to be the mean of the smallest and largest measured diameters for each oocyte/follicle. For immunofluorescence, sections were deparaffinized before blocking with 2% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 1 hour. Cx43 was detected using a rabbit polyclonal antibody (CT360; provided by Stephen Lye, Samuel Lunenfeld Research Institute, Toronto, ON) raised against a synthetic peptide corresponding to residues 360-382 of the C-terminal portion of Cx43. The CT360 primary antibody was diluted 1:250 in blocking solution and applied to slides for 1 hour. After washing two times over 10 minutes in PBS, a goat anti-rabbit Alexa-Fluor-488-conjugated secondary antibody (Molecular Probes, Eugene, OR) diluted 1:300 in blocking solution was applied for 1 hour and washed three times over 15 minutes in PBS. A drop of Airvol (Air Products and Chemicals, Allentown, PA) was added to each slide before applying coverslips. These immunofluorescently labeled sections were analyzed on a Zeiss LSM 510 META confocal microscope based on an Axiovert 200M platform with a 63× oil, 1.4 numerical aperture objective. Fluorescent signals were imaged by excitation with a 488 nm laser line from an argon laser. Images were captured using Zeiss LSM software. They were not further enhanced or modified prior to constructing the final figures, when brightness and contrast adjustments were made.

For immunolocalization of MYC on ovarian sections, deparaffinized slides were immersed into antigen-retrieval solution (0.01 M sodium citrate buffer, pH 6.0), heated to boiling, maintained at close to boiling for 15 minutes, then cooled to room temperature for 20 minutes. The slides were then treated for 10 minutes with 1% hydrogen peroxide in PBS to quench endogenous peroxidase activity and were washed in PBS twice for 5 minutes each. MYC was detected using the ABC staining system (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-MYC monoclonal antibody (Genscript Corporation, Piscataway, NJ) diluted 1:200. Images were captured on a Zeiss Axioskop 2 microscope equipped with a Retiga 1300 CCD digital camera (Q Imaging Alliance, Surrey, BC).

Western blotting

Oocytes were suspended in 20 μl of 2× SDS-PAGE sample buffer containing protease inhibitor cocktail (Sigma-Aldrich) and frozen at –70°C. Before loading the samples onto 10% SDS-PAGE gels, they were thawed on ice and heated to 100°C for 5 minutes. The proteins were transferred to PVDF membrane (Invitrogen) and were then probed using rabbit anti-Cx43 polyclonal antibody (Sigma) diluted 1:1000 and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody diluted to 1:5000. The specific immunoreactive protein signals were visualized by enhanced chemiluminescence using high-performance chemiluminescence film (Amersham Biosciences). To assure equal gel loading, the PVDF membrane was stripped with Restore Western Blot Stripping Buffer (Pierce) and re-probed using 1:10,000 anti-GAPDH monoclonal antibody (Cedarlane Laboratories, Hornby, ON). For detection of the MYC tag, whole ovaries were homogenized in single-detergent lysis buffer (Sambrook et al., 1989) (150 μl per ovary). Protein (20 μg) was loaded onto each lane and the blot was probed using mouse anti-human c-MYC monoclonal antibody (Chemicon International, Temecula, CA) diluted 1:400.

Oocyte microinjection

Isolated follicles were placed on 12-mm-diameter coverslips in Petri dishes containing Waymouth medium and were incubated at 37°C in an atmosphere of 5% CO_2 in air for 24 hours to allow the follicles to attach to the coverslips. The oocytes were impaled for 10 minutes with a 1 mm thin-wall glass capillary (World Precision Instruments, Sarasota, FL), tip diameter 1 μm, which was backfilled with 5% Lucifer yellow in ddH_2O (Molecular Probes, Eugene, OR) via capillary action. Dye was injected using an Eppendorf microinjection system and a Zeiss Axiovert S100 inverted epifluorescence microscope with a 40×, 1.3 numerical aperture objective. Images were captured using a PCO Sensicam SVGA camera with Sensicam software and were not further enhanced or modified prior to constructing the final figures, when brightness and contrast adjustments were made. Only those injections were recorded in which the dye filled the oocyte within 1 minute, as evidenced by bright fluorescence.

Testing for meiotic competence and ovulation potential

Both transgenic lines were used to generate connexin-replacement mice for these experiments. Equine chorionic gonadotropin (eCG, National Hormone and Peptide Program) (5 IU) was injected intraperitoneally into 4-week-old mice 24 hours prior to testing for oocyte maturation in vitro. Mice were anesthetized with CO_2 and killed by cervical dislocation. Ovaries were removed and placed in Waymouth's MB 752/1 medium plus 5% fetal bovine serum (FBS; Invitrogen) and 0.23 mM pyruvic acid (sodium salt; Sigma-Aldrich). Follicles were pierced with 25-gauge needles to liberate cumulus-oocyte complexes. Oocytes enclosed by at least one layer of cumulus cells were washed through fresh medium and transferred to a 35 mm Petri dish containing 3 ng/ml FSH (Puregon 100 IU follitropin beta; Organon Canada, Scarborough, ON) in 3 ml of Waymouth's medium/5% FBS. Oocytes were matured for 18 hours in 5% CO_2, 5% O_2, 90% N_2 atmosphere at 37°C. Before microscopic analysis, they were treated with 300 μg/ml hyaluronidase (Sigma Aldrich) to remove the cumulus cells and then stained with Hoechst 33342 (Molecular Probes, Eugene, OR) diluted 1:1000 in Waymouth's medium/5% FBS.

To test their ability to ovulate in response to gonadotropin, 4- to 5-week-old females were injected with eCG (as above) around 6 pm followed 48 hours later with 5 IU of hCG. Thirteen hours later, the females were killed (as above) and oocytes were collected from the oviduct ampullae and counted. The significance of the difference between connexin-replacement mice and wild-type mice was assessed using Student's *t*-test.

Fertility testing

Female connexin-replacement mice (lacking Cx37 but carrying the *Zp3-Gja1* transgene) and their siblings (lacking Cx37 and not carrying the *Zp3-Gja1* transgene) along with females heterozygous for the Cx37-null mutation and hemizygous for the *Zp3-Gja1* transgene were naturally mated with Cx37-knockout males. The females ranged in age from 2 to 6 months. The number of pups delivered by each female was recorded and their health status was monitored.

The authors do not have any commercial affiliations or consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding this work. We thank Jurrien Dean for supplying the *Zp3* promoter construct; David Paul for supplying the Cx37-knockout mouse strain; Stephen Lye for supplying Cx43 antibody; Dale Laird for a critical reading of the manuscript; Dan Tong for assistance with the meiotic and ovulation competence experiments; and the staff of the Department of Animal Care and Veterinary Services, The University of Western Ontario, for their diligence in caring for the mice. This work was supported by a grant from the Canadian Institutes of Health Research.

References

Carabatsos, M. J., Sellitto, C., Goodenough, D. A. and Albertini, D. F. (2000). Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Dev. Biol.* **226**, 67-179.

Ek-Vitorín, J. F. and Burt, J. M. (2005). Quantification of gap junction selectivity. *Am. J. Physiol. Cell Physiol.* **289**, C1535-C1546.

Elfgang, C., Eckert, R., Lichtenberg-Frate, H., Butterweck, A., Traub, O., Klein, R. A., Hülser, D. F. and Willecke, K. (1995). Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J. Cell Biol.* **129**, 805-817.

Epifano, O., Liang, L., Familiari, M., Moos, M. C., Jr and Dean, J. (1995). Coordinate expression of the three zona pellucida genes during mouse oogenesis. *Development* **121**, 1947-1956.

Eppig, J. J. (1994). Oocyte-somatic cell communication in the ovarian follicles of mammals. *Semin. Dev. Biol.* **5**, 51-59.

Eppig, J. J., Pendola, F. L., Wigglesworth, K. and Pendola, J. K. (2005). Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: amino acid transport. *Biol. Reprod.* **73**, 351-357.

FitzHarris, G. and Baltz, J. M. (2006). Granulosa cells regulate intracellular pH of the murine growing oocyte via gap junctions: development of independent homeostasis during oocyte growth. *Development* **133**, 591-599.

Flenniken, A. M., Osborne, L. R., Anderson, N., Ciliberti, N., Fleming, C., Gittens, J. E. I., Gong, X.-Q., Lounsbury, C., Moreno, L., Nieman, B. J. et al. (2005). A *Gja1* missense mutation in a mouse model for oculodentodigital dysplasia (ODDD). *Development* **132**, 4375-4386.

Galipeau, J., Li, H., Paquin, A., Sicilia, F., Karpati, G. and Nalbantoglu, J. (1999). Vesicular stomatitis virus G pseudotyped retrovector mediates effective in vivo suicide gene delivery in experimental brain cancer. *Cancer Res.* **59**, 2384-2394.

Gittens, J. E. I. and Kidder, G. M. (2005). Differential contributions of connexin37 and connexin43 to oogenesis revealed in chimeric reaggregated mouse ovaries. *J. Cell Sci.* **118**, 5071-5078.

Gittens, J. E. I., Mhawi, A. A., Lidington, D., Ouellette, Y. and Kidder, G. M. (2003). Functional analysis of gap junctions in ovarian granulosa cells: a distinct role for connexin43 in early stages of folliculogenesis. *Am. J. Physiol. Cell Physiol.* **284**, C880-C887.

Gittens, J. E. I., Barr, K. J., Vanderhyden, B. C. and Kidder, G. M. (2005). Interplay between paracrine signaling and gap junctional communication in ovarian follicles. *J. Cell Sci.* **118**, 113-122.

Goldberg, G. S., Valiunas, V. and Brink, P. R. (2004). Selective permeability of gap junction channels. *Biochim. Biophys. Acta* **1662**, 96-101.

Guerrier, A., Fonlupt, P., Morand, I., Rabilloud, R., Audebet, C., Krutovskikh, V., Gros, D., Rousset, B. and Munari-Silem, Y. (1995). Gap junctions and cell polarity: connexin32 and connexin43 expressed in polarized thyroid epithelial cells assemble into separate gap junctions, which are located in distinct regions of the lateral plasma membrane domain. *J. Cell Sci.* **108**, 2609-2617.

Harris, A. L. (2001). Emerging issues of connexin channels: biophysics fills the gap. *Q. Rev. Biophys.* **34**, 325-472.

Kam, Y., Kim, D. Y., Koo, S. K. and Joe, C. O. (1998). Transfer of second messengers through gap junction connexin43 channels reconstituted in liposomes. *Biochim. Biophys. Acta* **1372**, 384-388.

Kidder, G. M. (2005). Roles of gap junctions in ovarian folliculogenesis: implications for female infertility. In *Gap Junctions in Development and Disease* (ed. E. Winterhager), pp. 223-237. Heidelberg: Springer Verlag.

Kidder, G. M. and Mhawi, A. A. (2002). Gap junctions and ovarian folliculogenesis. *Reproduction* **123**, 613-620.
Plum, A., Hallas, G., Magin, T., Dombrowski, F., Hagendorff, A., Schumacher, B., Wolpert, C., Kim, J.-S., Lamers, W. H., Evert, M. et al. (2000). Unique and shared functions of different connexins in mice. *Curr. Biol.* **10**, 1083-1091.
Rankin, T. L., Tong, Z. B., Castle, P. E., Lee, E., Gore-Langton, R., Nelson, L. M. and Dean, J. (1998). Human ZP3 restores fertility in Zp3 null mice without affecting order-specific sperm binding. *Development* **125**, 2415-2424.
Reaume, A. G., De Sousa, P. A., Kulkarni, S., Langille, B. L., Zhu, D., Davies, T. C., Juneja, S. C., Kidder, G. M. and Rossant, J. (1995). Cardiac malformation in neonatal mice lacking connexin43. *Science* **267**, 1831-1834.
Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning, A Laboratory Manual* (2nd edn). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Simon, A. M., Goodenough, D. A., Li, E. and Paul, D. L. (1997). Female infertility in mice lacking connexin 37. *Nature* **385**, 525-529.
Söhl, G. and Willecke, K. (2003). An update on connexin genes and their nomenclature in mouse and man. *Cell Commun. Adhes.* **10**, 173-180.
Stout, C., Goodenough, D. A. and Paul, D. L. (2004). Connexins: functions without junctions. *Curr. Opin. Cell Biol.* **16**, 507-512.
Sugiura, K. and Eppig, J. J. (2005). Control of metabolic cooperativity between oocytes and their companion granulosa cells by mouse oocytes. *Reprod. Fertil. Dev.* **17**, 667-674.
Tong, D., Gittens, J. E. I., Kidder, G. M. and Bai, D. (2006). Patch clamp study reveals that the importance of connexin43-mediated gap junctional communication for ovarian folliculogenesis is strain-specific in the mouse. *Am. J. Physiol. Cell Physiol.* **290**, C290-C297.
Veenstra, R. D., Wang, H.-Z., Beblo, D. A., Chilton, M. G., Harris, A. L., Beyer, E. C. and Brink, P. R. (1995). Selectivity of connexin-specific gap junctions does not correlate with channel conductance. *Circ. Res.* **77**, 1156-1165.
Veitch, G. I., Gittens, J. E. I., Shao, Q., Laird, D. W. and Kidder, G. M. (2004). Selective assembly of connexin37 into gap junctions at the oocyte/granulosa cell interface. *J. Cell Sci.* **117**, 2699-2707.
Webb, R. J., Bains, H., Cruttwell, C. and Carroll, J. (2002a). Gap-junctional communication in mouse cumulus-oocyte complexes: implications for the mechanism of meiotic maturation. *Reproduction* **123**, 41-52.
Webb, R. J., Marshall, F., Swann, K. and Carroll, J. (2002b). Follicle-stimulating hormone induces a gap junction-dependent dynamic change in [cAMP] and protein kinase A in mammalian oocytes. *Dev. Biol.* **246**, 441-454.
Weber, P. A., Chang, H. C., Spaeth, K. E., Nitsche, J. M. and Nicholson, B. J. (2004). The permeability of gap junction channels to probes of different size is dependent on connexin composition and permeant-pore affinities. *Biophys. J.* **87**, 958-973.
White, T. W. (2002). Unique and redundant connexin contributions to lens development. *Science* **295**, 319-320.