

Oogenesis defects in a mutant mouse model of oculodentodigital dysplasia

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SUMMARY

The essential role of connexin43 (Cx43) during oogenesis has been demonstrated by the severe germ cell deficiency and arrested folliculogenesis observed in Cx43 knockout mice. Recently, another mutant mouse strain became available (*Gja1^{Jrt/+}*) that carries the dominant loss-of-function Cx43 mutation, Cx43^{G60S}. *Gja1^{Jrt/+}* mice display features of the human disease oculodentodigital dysplasia (ODDD), which is caused by mutations in the *GJA1* gene. We used this new mutant strain to study how a disease-linked Cx43 mutant affects oogenesis. We found that female mutant mice are subfertile with significantly reduced mating success and small litters. The phosphorylated species of the Cx43 protein are reduced in the mutant ovaries in association with impaired trafficking and assembly of gap junctions in the membranes of granulosa cells, confirming that the mutant protein acts dominantly on its wild-type counterpart. Correspondingly, although starting with a normal abundance of germ cells, ovaries of the mutant mice contain significantly fewer pre-ovulatory follicles and do not respond to superovulation by gonadotropins, which is at least partially the result of reduced proliferation and increased apoptosis of granulosa cells. We conclude that the *Gja1^{Jrt}* mutation has a dominant negative effect on Cx43 function in the ovary, rendering the females subfertile. Given these findings, closer examination of reproductive function in ODDD human females is warranted.

INTRODUCTION

Connexins are the protein subunits of gap junction channels, which enable neighboring cells to exchange small signaling molecules (less than ~1 kDa) and to synchronize electrical activities. Six identical or different connexins hexamerize to form a homomeric or heteromeric connexon (hemichannel), respectively. Two connexons (hemichannels) from adjacent cells dock with each other to form an intercellular gap junction channel (reviewed by Laird, 2006). So far, 20 and 21 connexin genes have been found in the mouse and the human genomes, respectively, which have distinct but overlapping patterns of expression (Laird, 2006).

The ovarian follicle provides a good example of a multicellular unit that exhibits expression of multiple connexins and is considered to be reliant upon gap junctional intercellular communication (GJIC) for proper development (reviewed by Kidder, 2005). As the most abundant connexin in the ovary, connexin43 (Cx43) is continuously expressed in the mouse fetal ovary from the onset of ovarian differentiation (Perez-Armendariz et al., 2003). After birth, Cx43 forms numerous large gap junctions among granulosa cells in primordial, primary, secondary and antral follicles with the expression level increasing in parallel with follicle development (reviewed by Granot and Dekel, 2002). The essential role of Cx43 during oogenesis and folliculogenesis has been clearly demonstrated by the Cx43 knockout mice (*Gja1^{-/-}*). Offspring (both male and female) homozygous for the null mutation have very few primordial germ cells (PGCs) (Juneja et al., 1999). The

marked loss of PGCs is at least partially because of increased apoptosis associated with abnormal P53 activation in the germ cells, demonstrating that Cx43 is required for PGC survival in the genital ridges (Francis and Lo, 2006). However, the gonads of Cx43-null fetuses do contain about 10% of the normal number of germ cells (Juneja et al., 1999). Owing to the neonatal death of Cx43-null mice, late fetal ovaries were grafted into the kidney capsule of ovariectomized adult mice to allow postnatal follicular development. In contrast to the full range of follicles, from primordial through to large preovulatory follicles, that are observed in grafted wild-type ovaries, folliculogenesis in mutant ovaries of the C57BL/6 strain did not proceed beyond the primary unilaminar stage (Ackert et al., 2001). Further studies demonstrated that intercellular coupling among granulosa cells was totally abolished in Cx43-null mice, indicating that cell coupling through Cx43 channels is required to sustain granulosa cell proliferation (Gittens et al., 2003; Gittens et al., 2005; Tong et al., 2006). Failure of the Cx43-null follicles to develop multiple layers of granulosa cells was correlated with reduced growth of the oocytes, which were morphologically abnormal, meiotically incompetent and could not be fertilized (Ackert et al., 2001).

Oculodentodigital dysplasia (ODDD) is a rare, human autosomal dominant disorder that is caused by mutations in the *GJA1* gene encoding Cx43. Common symptoms include syndactyly of hands and feet, enamel hypoplasia, craniofacial abnormalities, ophthalmic defects and occasionally heart and neurological dysfunction (Loddenkemper et al., 2002; Paznekas et al., 2003). More than 39 different ODDD-causing mutations have been identified in the *GJA1* gene so far (reviewed by Laird, 2006). In vitro studies of the ODDD-linked Cx43 mutants have shown that, to varying degrees, most mutants will assemble into gap junction plaque-like structures at the cell surface, however, all of the mutants have severely reduced GJIC compared with wild-type Cx43. Furthermore, when co-

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expressed with wild-type Cx43, the mutants often act in a dominant negative fashion (Roscoe et al., 2005; Shibayama et al., 2005; McLachlan et al., 2005).

In 2005, a mouse model of ODDD became available when an *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen resulted in the generation of a mouse that exhibits many classic symptoms of ODDD including syndactyly, enamel hypoplasia and craniofacial bone anomalies (Flenniken et al., 2005). This mouse carries a G60S point mutation (the product of the *Gja1*^{Jrt} allele) in the first extracellular loop of Cx43, which is one residue away from the P59H mutation identified in human ODDD patients (Vasconcellos et al., 2005). Our previous work demonstrated that the granulosa cells isolated from these mice (*Gja1*^{Jrt/+}) exhibit either very weak coupling (10–20% versus wild type) or a complete lack of coupling, indicating that the G60S mutant dominantly inhibits the function of co-expressed wild-type Cx43 in vivo (Flenniken et al., 2005). Given the defects observed in Cx43-null ovaries, we hypothesized that Cx43^{G60S} affects oogenesis by inhibiting the function of wild-type Cx43 during germ cell migration and folliculogenesis. To test this hypothesis, we compared the fertility, germ cell numbers and follicular development of *Gja1*^{Jrt/+} females with their wild-type littermates. We found that the mutant mice are subfertile with reduced mating success and have smaller litter sizes compared with wild-type siblings. Although they have normal germ cell numbers, the ovaries of the mutant mice contain significantly fewer pre-ovulatory follicles and are impaired in their response to gonadotropins. This was associated with impaired phosphorylation of the Cx43 protein and reduced GJIC among granulosa cells.

RESULTS

Female *Gja1*^{Jrt/+} mice have reduced fertility

After mating with wild-type males, the pregnancy rate of female *Gja1*^{Jrt/+} mice (23.5%, *n*=34 matings) was much lower than their wild-type littermates (83.9%, *n*=31) (Fig. 1A). Furthermore, when *Gja1*^{Jrt/+} females were mated with wild-type males, their mean litter size (3.0±0.2, *n*=46 litters) was significantly smaller (*P*<0.001) compared with those resulting from matings between wild-type females and wild-type males (7.9±0.3, *n*=53). Surprisingly, litter sizes were normal (7.0±0.2, *n*=172; *P*>0.05 compared with wild type) if only the males were carrying the mutation (Fig. 1B), indicating that the mutation selectively affects female fertility. In addition, if the mother carried the mutation, litter sizes were further reduced given the fact that only around 16% of the pups survived beyond postnatal day 1 (Fig. 1C), which was at least partially because of a co-existent lactation problem that has been described elsewhere (Plante and Laird, 2008).

Gja1^{Jrt/+} ovaries contain normal numbers of germ cells

To determine whether the abundance of germ cells might be affected by the *Gja1*^{Jrt} mutation, we counted germ cell numbers in the postnatal day 1 ovaries using germ cell nuclear antigen (GCNA) as a specific germ cell marker (Juneja et al., 1999). In contrast to the severe germ cell deficiency observed in *Gja1*^{-/-} ovaries, there was no significant difference (*P*=0.71) in germ cell numbers between *Gja1*^{Jrt/+} females (*n*=3639±988) and their wild-type littermates (*n*=4123±227) (Fig. 2B). Three ovaries from three different mice were counted for each genotype. As shown in Fig. 2A, germ cells in the ovaries of both genotypes showed similar peripheral

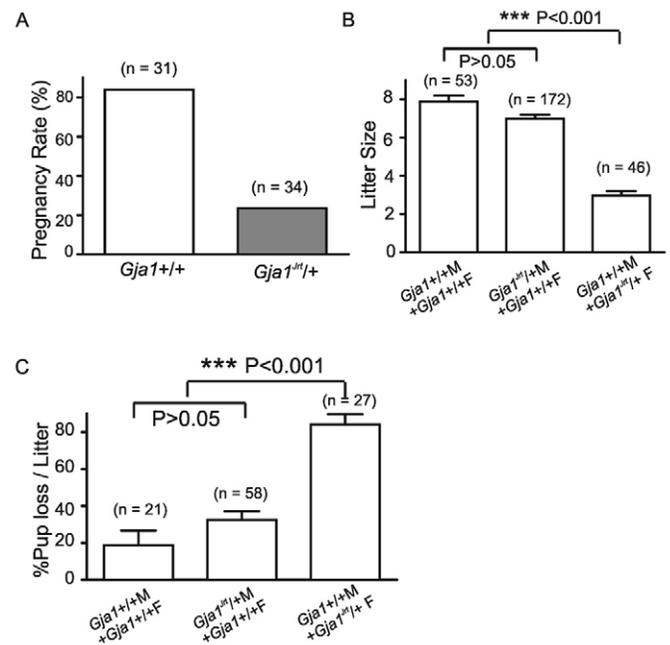


Fig. 1. Female *Gja1*^{Jrt/+} mice have reduced fertility. (A) After mating with wild-type males, the pregnancy rate of female *Gja1*^{Jrt/+} mice was much lower than that of their wild-type littermates. (B) Litters resulting from mating between *Gja1*^{Jrt/+} females and wild-type males were significantly smaller (****P*<0.001) compared with those from mating between wild-type females and wild-type males, and between wild-type females and *Gja1*^{Jrt/+} males. (C) The loss of pups was significantly higher (****P*<0.001) when *Gja1*^{Jrt/+} females were mated with wild-type males compared with mating between wild-type females and wild-type males, and between wild-type females and *Gja1*^{Jrt/+} males. There was no significant difference between the latter two groups (*P*>0.05).

distribution in the ovarian cortex, however, the numerous Cx43 gap junction ‘plaques’ present in the somatic cells were barely observed in the mutant ovaries.

Gja1^{Jrt/+} ovaries contain fewer pre-ovulatory follicles and have reduced response to gonadotropins

Although starting with similar numbers of germ cells, ovaries of sexually mature *Gja1*^{Jrt/+} females contain significantly fewer pre-ovulatory follicles at proestrus (Fig. 3B) (*P*<0.05 versus wild-type littermates, two-way ANOVA and Bonferroni post hoc test, *n*=4 ovaries from four mice). However, there was no significant difference in the distribution of the earlier follicle stages (Fig. 3A) (*P*>0.05, chi-square test, *n*=3 ovaries from three mice for each group), indicating that the mutation mainly affected the final maturation of the follicles. Morphologically atretic follicles were rarely seen in ovaries of both groups. To test their response to gonadotropins, mice from both genotypes were primed with equine chorionic gonadotropin (eCG), a peptide hormone with follicle-stimulating hormone (FSH) activity in the mouse, and induced to ovulate with human chorionic gonadotropin (hCG) as a source of luteinizing hormone (LH) activity. Significantly fewer oocytes were collected from the oviducts of the *Gja1*^{Jrt/+} females (19.6±9.5, *n*=5 mice versus 60.0±3.8, *n*=4 wild-type mice, unpaired *t*-test, *P*<0.01), indicating that the response of the mutant mice to gonadotropins is reduced (Fig. 3C).

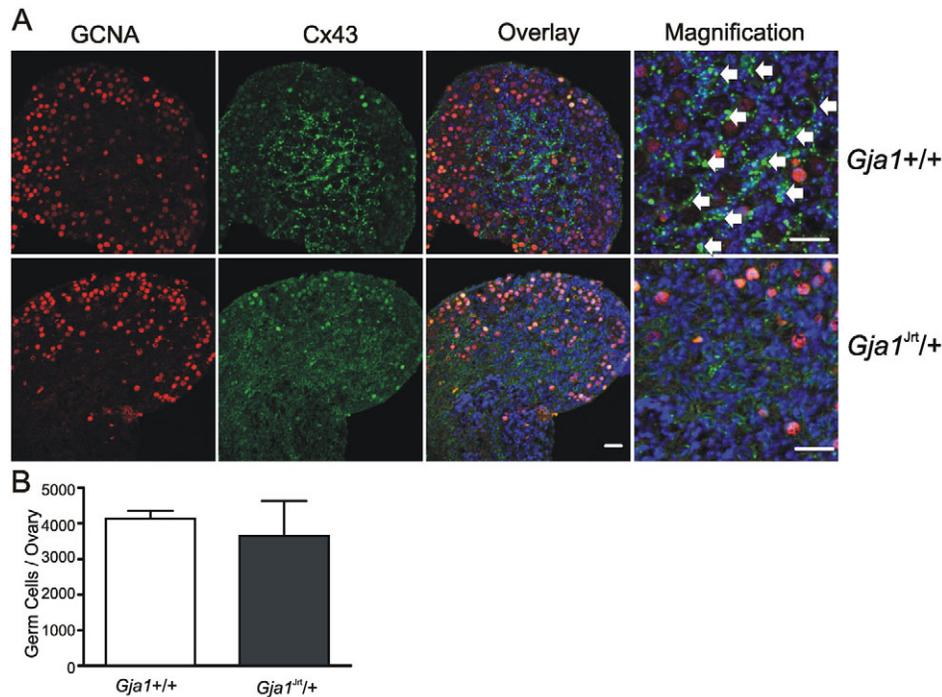


Fig. 2. *Gja1^{Jrt}/+* ovaries contain normal numbers of germ cells. (A) Ovaries from postnatal day 1 wild-type (top row) and *Gja1^{Jrt}/+* (bottom row) females were co-immunolabeled for the germ cell marker GCNA (red) and Cx43 (green). Nuclei were stained with Hoechst (blue). The higher magnification picture (far right column) shows that the gap junction 'plaques' (white arrows), which formed in wild-type ovaries, are rarely present in *Gja1^{Jrt}/+* ovaries. Bars, 20 μ m. (B) Quantification of germ cell numbers per ovary from wild-type and *Gja1^{Jrt}/+* females ($P=0.71$).

Oocytes from *Gja1^{Jrt}/+* females are developmentally competent

To test the meiotic competence of the oocytes, cumulus-oocyte complexes (COCs) were obtained from punctured antral follicles of females primed with eCG. After Hoechst nuclear staining, it was apparent that oocytes from the *Gja1^{Jrt}/+* females are equally as competent as wild-type oocytes in undergoing the first meiotic division to produce the first polar body (Table 1). Although fewer oocytes can be collected from the oviducts of *Gja1^{Jrt}/+* females that have been primed with eCG and hCG, after fertilization these oocytes were able to develop to the two-cell stage at a frequency similar to that observed with wild-type oocytes (Fig. 3D) ($n=50$ wild-type and $n=20$ mutant oocytes from three mice of each genotype). This indicates that female *Gja1^{Jrt}/+* mice produce competent oocytes.

Phosphorylation and trafficking of Cx43 are aberrant in *Gja1^{Jrt}/+* granulosa cells

Western blot analysis demonstrated that the abundance of total Cx43 protein in the ovaries from *Gja1^{Jrt}/+* mice is not significantly different from that of wild-type mice (1.5 ± 0.2 versus 1.1 ± 0.1 , $P>0.05$) (Fig. 4A). However, the phosphorylated P1 and P2 forms of Cx43 were significantly reduced in the mutant ovaries (1.3 ± 0.2 for wild type versus 0.7 ± 0.1 for *Gja1^{Jrt}/+*, $P<0.01$, two-way ANOVA, $n=9$ blots from six mice for each group), suggesting that the mutation impairs phosphorylation of Cx43 in the ovaries. This result is slightly different from that of our previous study (Flenniken et al., 2005), which showed that the level of both total Cx43 and its phosphorylated forms are significantly downregulated in the mutant ovaries. This is probably because of differences in the strain backgrounds and ages of the mice used in the two studies. To confirm this further, the ovary and heart from the same mice were loaded on gels for western blotting. As shown in Fig. 4A, the abundance of Cx43 in the heart is significantly reduced in terms of both total Cx43 and its phosphorylated forms ($P<0.001$ for both, two-way ANOVA, $n=3$ blots

from three mice for each group), which is consistent with previous studies (Flenniken et al., 2005; Manias et al., 2008). The consequence of aberrant phosphorylation on gap junction formation in the ovaries was evaluated by immunostaining. As shown in Fig. 4B, there was a marked reduction in the accumulation of Cx43 at the junctional membrane compared with wild-type controls, as visualized by Cx43 gap junction 'plaques' in the follicles. To further visualize the trafficking of Cx43 on a cellular level, we isolated granulosa cells from the ovaries of wild-type and *Gja1^{Jrt}/+* mice and examined the Cx43 expression profile. Consistent with tissue immunostaining, very few gap junction plaques were evident in the *Gja1^{Jrt}/+* granulosa cells, whereas a much larger population of Cx43 was localized to the Golgi apparatus, indicating that the *Gja1^{Jrt}* mutation affected the normal trafficking of Cx43 to the cell membrane (Fig. 4C).

Proliferation of granulosa cells is reduced in *Gja1^{Jrt}/+* ovaries

The absence of Cx43 has been associated with reduced granulosa cell proliferation (Gittens et al., 2005). Therefore, we evaluated the effect of the *Gja1^{Jrt}* mutation on granulosa cell growth. As demonstrated by using a BrdU uptake assay, only $14.7\pm 3.1\%$ of granulosa cells isolated from *Gja1^{Jrt}/+* ovaries took up BrdU after a 4-hour incubation, compared with $26.5\pm 3.3\%$ of the wild-type granulosa cells ($P<0.05$, t -test, $n=15$ and $n=13$ fields from six mice in the wild-type and mutant groups, respectively) (Fig. 5A). To confirm this further, we quantified the expression of a cell proliferation marker, proliferating cell nuclear antigen (PCNA), and found that PCNA expression is significantly reduced in the mutant cells (0.7 ± 0.1 versus 1.0 ± 0.04 for wild-type cells, $P<0.001$, t -test, $n=52$ fields from six mice for each group) (Fig. 5B).

Apoptosis rate is elevated in *Gja1^{Jrt}/+* antral follicles

To determine whether the reduced proportion of mature follicles observed in the mutant ovaries was also the result of an elevated

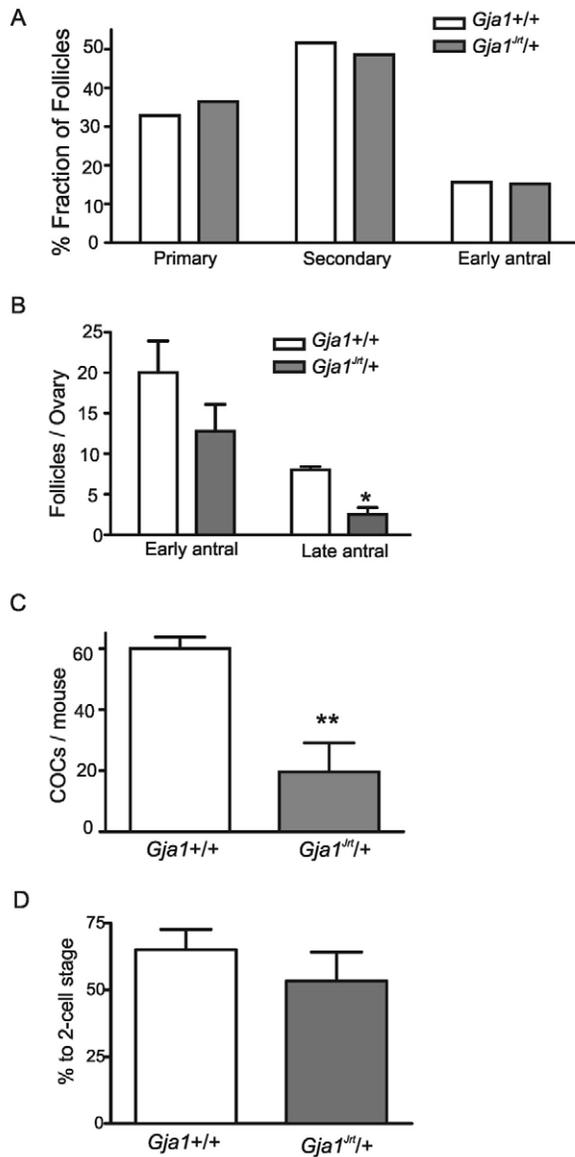


Fig. 3. *Gja1^{Jrt/+}* ovaries contain fewer pre-ovulatory follicles and have reduced response to gonadotropins. (A) Distribution of follicles at different developmental stages in the wild-type and *Gja1^{Jrt/+}* ovaries ($P>0.05$). (B) Quantification of early and late antral follicles in the wild-type and *Gja1^{Jrt/+}* ovaries during proestrus (wild-type vs *Gja1^{Jrt/+}*, $P>0.05$ for early antral, $*P<0.05$ for late antral). (C) Quantification of cumulus-oocyte complexes (COCs) collected from the oviducts of wild-type and *Gja1^{Jrt/+}* mice after priming with eCG and hCG ($**P<0.01$). (D) Percentage of oocytes that cleaved to the 2-cell stage after in vitro fertilization ($P>0.05$).

apoptosis rate in granulosa cells, we used the TUNEL assay to fluorescently label fragmented DNA from apoptotic cells. *Gja1^{Jrt/+}* and wild-type ovaries were examined in parallel. As shown in Fig. 6A, apoptotic granulosa cells were prominent in the early antral follicles of *Gja1^{Jrt/+}* ovaries but were infrequent in wild-type ovaries. The fluorescent intensities of TUNEL staining in antral follicles are quantified in Fig. 6B ($n=14$ wild-type follicles from five mice versus $n=17$ *Gja1^{Jrt/+}* follicles from five mice, $P<0.05$, unpaired

Table 1. In vitro maturation of oocytes isolated from wild-type and *Gja1^{Jrt/+}* females

	<i>Gja1^{+/+}</i>	<i>Gja1^{Jrt/+}</i>
GV	1	2
MI	3	5
MII	40	28

GV, germinal vesicle intact; MI, metaphase I; MII, metaphase II (first polar body).

t-test). It appears that granulosa cell apoptosis is significantly elevated in the antral follicles of *Gja1^{Jrt/+}* ovaries.

Oocyte-granulosa cell coupling and GDF9 expression are not altered in *Gja1^{Jrt/+}* ovaries

Previous work had indicated that the gap junctions coupling the oocyte with the surrounding granulosa cells are composed of Cx37, with little or no contribution from Cx43 (Veitch et al., 2004) (reviewed by Kidder, 2005). To test whether the *Gja1^{Jrt/+}* mutation might also affect oocyte-granulosa cell coupling, a gap junction channel-permeant fluorescent dye, Lucifer Yellow, was injected into granulosa cell-enclosed oocytes. As shown in Fig. 7A, in both wild-type and *Gja1^{Jrt/+}* follicles, the injected dye passed from oocytes to the surrounding granulosa cells ($n=10$ for wild type versus $n=13$ for *Gja1^{Jrt/+}*), indicating that oocyte-granulosa cell coupling is still intact in *Gja1^{Jrt/+}* ovaries. There was no obvious difference between genotypes in the rate of dye passage. However, less extensive dye spreading among the granulosa cells was observed in the *Gja1^{Jrt/+}* follicles, confirming that the *Gja1^{Jrt/+}* mutation preferentially impairs coupling among the granulosa cells.

Previous work with the Cx43-null mutant line (*Gja1^{-/-}*) had indicated an interaction between Cx43-mediated gap junctional communication and intra-ovarian paracrine signaling, with the mutant granulosa cells having reduced responsiveness to the oocyte-derived mitogen, GDF9 (Gittens et al., 2005). GDF9 expression, however, was not reduced. Likewise, the expression of GDF9 in the *Gja1^{Jrt/+}* ovaries did not differ from that in wild-type littermates (0.20 ± 0.01 for wild type, $n=6$ versus 0.23 ± 0.02 for *Gja1^{Jrt/+}*, $n=6$, $P>0.05$, unpaired *t*-test) (Fig. 7B). Thus, it is unlikely that the impaired oogenesis observed in *Gja1^{Jrt/+}* ovaries is the result of altered GDF9 expression.

DISCUSSION

The essential role of Cx43 during oogenesis has been demonstrated in previous studies using Cx43 knockout mice in which the absence of Cx43 caused severe germ cell deficiency and arrested folliculogenesis (Juneja et al., 1999; Ackert et al., 2001). Germ cells are known to express Cx43 and are well-coupled to surrounding cells during their development (Francis and Lo, 2006). Cx43 is also present at all stages of ovary development, where it contributes to coupling between the somatic cells. Therefore, it is believed that the intercellular communication mediated by Cx43 gap junctions is crucial for germ cell survival and ovarian folliculogenesis. However, the knockout mouse model does not mimic any human condition since complete loss of Cx43 is lethal (Reaume et al., 1995). The *Gja1^{Jrt/+}* mice, which have a phenotype resembling the human disease ODDD, provided us with a good model to study how a disease-linked Cx43 mutation can affect oogenesis.

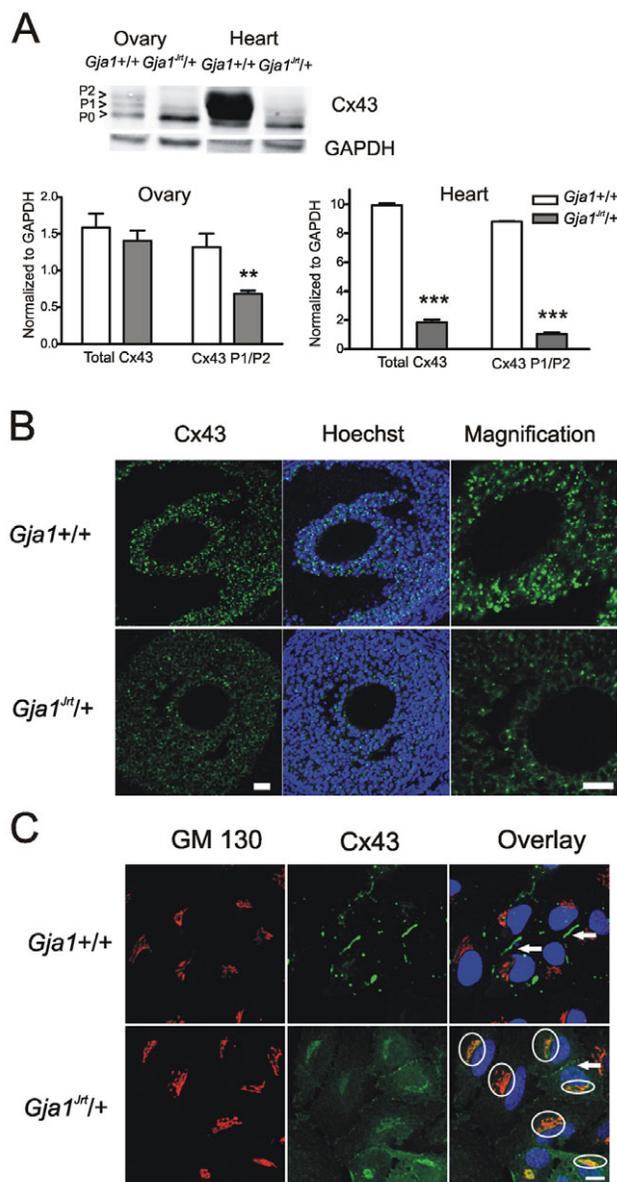


Fig. 4. Aberrant phosphorylation and trafficking of Cx43 in *Gja1*^{Jrt/+} granulosa cells. (A) Western blot analysis demonstrated that the abundance of total Cx43 protein in the ovaries from *Gja1*^{Jrt/+} mice is not significantly different ($P > 0.05$) from wild-type littermates. However, the phosphorylated P1 and P2 forms of Cx43 are significantly reduced in the mutant ovaries (** $P < 0.01$). In contrast, the abundance of Cx43 in the hearts from *Gja1*^{Jrt/+} mice is significantly lower than in wild-type hearts in terms of both total Cx43 and its phosphorylated forms (*** $P < 0.001$). Cx43 abundance is expressed relative to GAPDH. (B) Representative micrographs showing ovaries from 6–8-week-old wild-type (top row) and *Gja1*^{Jrt/+} (bottom row) females immunolabeled for Cx43 (green). Nuclei were stained with Hoechst (blue). The magnified micrograph (far right column) shows that there are numerous gap junction ‘plaques’ in wild-type ovarian follicles, whereas very few similar structures are seen in *Gja1*^{Jrt/+} follicles. Bars, 20 μ m. (C) Primary granulosa cells isolated from the ovaries of wild-type (top row) or *Gja1*^{Jrt/+} (bottom row) mice were co-immunolabeled for Cx43 (green) and the Golgi marker GM130 (red). Nuclei were stained with Hoechst (blue). The merged image shows co-localization (yellow within white circles) in *Gja1*^{Jrt/+} cells. White arrows indicate the gap junction plaques that have formed between neighboring cells in both cell populations. Bar, 20 μ m.

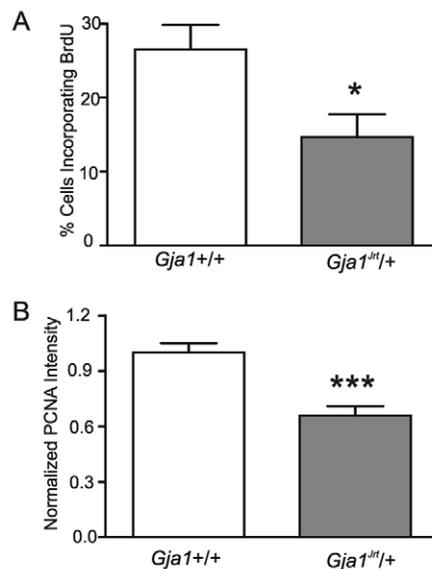


Fig. 5. Reduced proliferation of granulosa cells. (A) Data are expressed as the percentage of granulosa cells that incorporated BrdU relative to the total number of cells in a field of view, counted after Hoechst staining (* $P < 0.05$). (B) Primary cultured granulosa cells were immunolabeled for PCNA and the signal intensity was quantified and normalized to one of the wild-type granulosa cell readings from the same experiment (*** $P < 0.001$).

Several in vitro studies have demonstrated that ODDD-linked Cx43 mutations exert dominant negative effects on wild-type Cx43, severely limiting Cx43-mediated intercellular communication (Laird, 2006). Our data indicate that the mutation causes aberrant Cx43 phosphorylation, as demonstrated by marked reduction in the phosphorylated forms P1 and P2 and an increased amount of the P0 form in the ovaries. This is consistent with the immunostaining results showing that most of the Cx43 is retained in the intracellular compartment, with very few gap junction plaques being visible in the junctional membranes. This finding indicates that the *Gja1*^{Jrt} mutation impairs the normal trafficking and assembly of Cx43 gap junction channels in the granulosa cells, presumably through the oligomerization of mutant and wild-type connexins and the impaired assembly of the resulting complex. However, the detailed mechanism underlying this anomaly awaits further investigation. Hypophosphorylation and reduced junctional plaque formation are consistent with our previous work showing that granulosa cells isolated from *Gja1*^{Jrt/+} mice are very weakly coupled (10–20% versus wild type), or exhibit a complete lack of coupling (Flenniken et al., 2005), indicating a dominant negative effect of the *Gja1*^{Jrt/+} mutation. A similarly severe reduction of intercellular coupling has been observed in other cell types from the same *Gja1*^{Jrt/+} mice including osteoblasts, mammary epithelium and cardiomyocytes (McLachlan et al., 2008; Plante and Laird, 2008; Manias et al., 2008) (see Table 2).

Given these findings, it is surprising that germ cell abundance was not affected in the *Gja1*^{Jrt/+} mice. It is known that Cx43 is expressed in germ cells and plays an essential role in maintaining their survival, since increased germ cell apoptosis was observed in Cx43-null genital ridges starting from embryonic day (E)11.5 (Francis and Lo, 2006). It is possible that the residual low level of

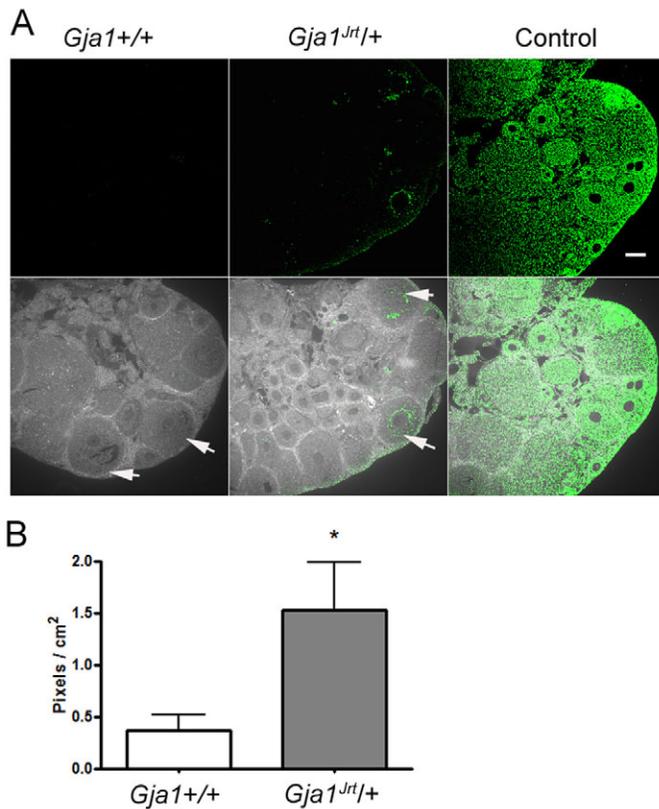


Fig. 6. Elevated apoptosis in *Gja1*^{Jrt/+} antral follicles indicated by the TUNEL assay. (A) Representative pictures showing apoptotic cells in the ovaries from 6–8-week-old wild-type (left column) and *Gja1*^{Jrt/+} (middle column) females. The right column shows the positive control pretreated with DNase. Fluorescent images are shown in the top row, whereas the corresponding phase contrast pictures are merged in the bottom row to show the tissue structure. White arrows indicate antral follicles. Bar, 100 μ m. (B) The fluorescence intensities in the antral follicles were quantified relative to the follicle area and found to differ significantly between genotypes (* P <0.05).

coupling found in the mutant granulosa cells from *Gja1*^{Jrt/+} mice is also present in migrating primordial germ cells, and that it is sufficient for maintaining the survival of those cells. Another possibility is that the role of Cx43 in migrating germ cells does not involve GJIC, but is based on other functions of the connexin molecule (reviewed by Jiang and Gu, 2005). For example, it was demonstrated that Cx43 can interact with the growth factor NOV/CCN3 (nephroblastoma overexpressed gene/connective tissue growth factor) through its C-terminal (CT) domain and thereby regulate cell proliferation (Fu et al., 2004; Gellhaus et al., 2004). Since the mutated Cx43 still has an intact CT domain, the functions of the molecule that depend on binding to other cellular proteins via this segment may well remain intact. Indeed, it was recently reported that some ODDD-linked mutations, including G21R, G138R and G60S, still interact with caveolin, whereas fs260, a frameshift mutation in which the CT region is altered and truncated, did not co-immunoprecipitate with caveolin (Langlois et al., 2008). Therefore, it will be interesting to examine oogenesis in a mouse model carrying the ODDD fs260 mutation. The same explanation might also apply to the presence of follicles of all

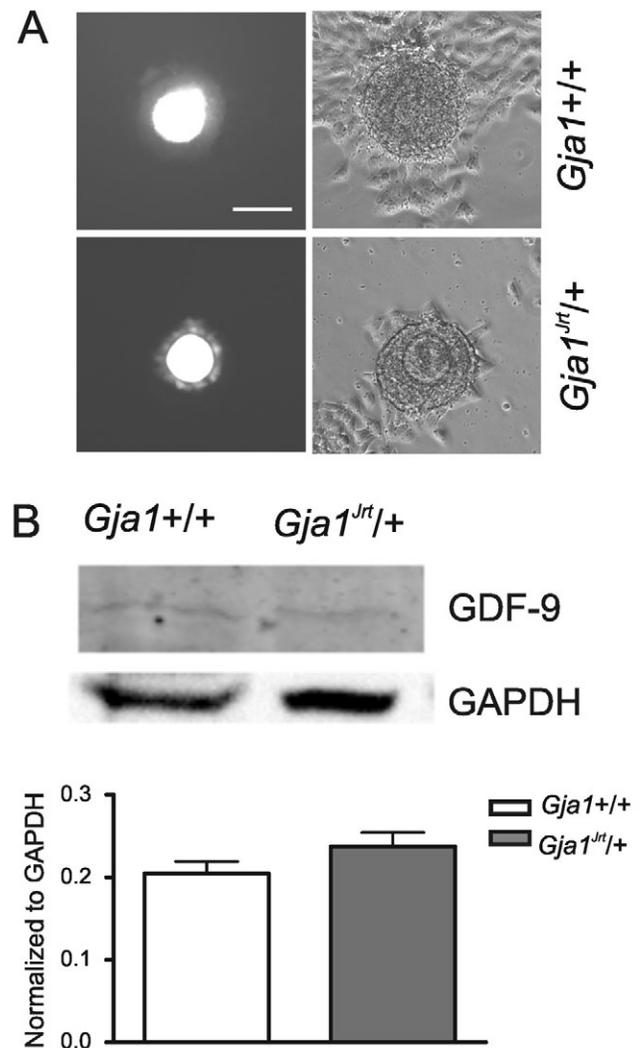


Fig. 7. Unchanged oocyte-granulosa cell coupling and GDF9 expression in *Gja1*^{Jrt/+} ovaries. (A) Microinjection of Lucifer Yellow into cumulus-enclosed oocytes demonstrated that the injected dye can pass readily from the oocyte to the surrounding cumulus cells in both wild-type and *Gja1*^{Jrt/+} follicles. Bar, 100 μ m. (B) Western blot analysis demonstrated that the abundance of GDF9 in ovaries from *Gja1*^{Jrt/+} mice is not significantly different from wild-type littermates (P >0.05). GDF9 abundance is expressed relative to GAPDH.

developmental stages in *Gja1*^{Jrt/+} ovaries, in contrast to the arrested folliculogenesis observed in Cx43 knockout females (Ackert et al., 2001).

In any case, *Gja1*^{Jrt/+} females are subfertile with significantly reduced mating success and smaller litter sizes compared with their wild-type siblings. Although the ovaries of the mutant mice start with normal numbers of germ cells, they contain significantly fewer pre-ovulatory follicles and have a reduced response to gonadotropins, which can at least partially explain the observed reduction of fertility. As with Cx43-null granulosa cells (Gittens et al., 2005), granulosa cells isolated from *Gja1*^{Jrt/+} females have a reduced proliferation rate. However, unlike Cx43-null granulosa cells, granulosa cells in *Gja1*^{Jrt/+} antral follicles demonstrated a

Table 2. Features of the three current ODD mouse models

		G60S¹	G138R²	I130T³
ODDD syndrome	Craniofacial abnormalities	Yes	Yes	NR
	Microphthalmia	Yes	Yes	NR
	Enamel hypoplasia	Yes	Yes	NR
	Syndactyly	Yes	Yes	Yes
Cx43 expression (western blot)	Total	↓ in heart, epidermis, mammary gland; NC in ovary*, calvaria	↓ in heart	↓ in heart
	P0 band	NC in heart, mammary gland; ↑ in ovary*, calvaria	IC in heart	NC in heart
	P1/2 bands	↓ in heart, mammary gland, ovary*, calvaria	↓ in heart	↓ in heart
Cx43 subcellular localization		↓ in membrane plaques, ↑ intracellularly (granulosa cells, cardiomyocytes, osteoblasts, keratinocytes, mammary myoepithelium)	↓ in intercalated disks; partial redistribution to lateral cardiomyocyte membranes	↓ in intercalated disks of cardiomyocytes
GJ conductance	Transfected cell line	No electrical coupling (N2A)	No electrical coupling (HeLa)	Weak electrical coupling (N2A)
	Cultured primary cells or intact hearts	↓ in granulosa cells, cardiomyocytes, osteoblasts	↓ in cardiomyocytes	↓ in cardiomyocytes
Heart pathology	Gross morphology	Mostly normal; patent foramen ovale in some individuals	Normal	Normal
	Function	Prolonged electrical wave in some individuals, bradycardia, AV block	Prolonged electrical wave, reduced R-wave amplitude, spontaneous ventricular arrhythmia	Reduced conduction velocity, reduced QRS amplitude, spontaneous or induced ventricular arrhythmia
Reproductive capacity	Pregnancy rate (mutant female)	↓*	NR	NR
	Litter size (mutant female)	↓*	↓	↓
	Ovulation rate	↓*	NR	NR
Mortality	Prenatal death	Yes	Yes	IC
	Premature postnatal death	Yes	Yes	NR

¹Flenniken et al., 2005; Langlois et al., 2007; McLachlan et al., 2008; Plante and Laird, 2008; Manias et al., 2008; *this study.

²Dobrowolski et al., 2008.

³Kalcheva et al., 2007; Shibayama et al., 2005.

NC, no change; IC, inconclusive from the available data; NR, not reported.

significantly higher apoptosis frequency. This difference is possibly associated with the additional dominant negative effect of the mutant Cx43 protein on wild-type Cx43 function in granulosa cells. Another possibility is that the role of Cx43 in preventing granulosa cells from undergoing apoptosis is follicle-stage-dependent since few, if any, follicles develop to antral stages in Cx43-null ovaries (Ackert et al., 2001; Tong et al., 2006). This too must await further investigation. These factors might, collectively, contribute to the reduced number of mature follicles observed in the *Gja1^{Int}/+* ovaries. Previous work demonstrated that Cx43 is required for granulosa cells to fully respond to the oocyte-derived mitogen, GDF9 (Gittens et al., 2005), and another study showed that GDF9 protects granulosa cells from apoptosis and is required for the maintenance of FSH receptor expression (Orisaka et al., 2006). The present study confirmed that the expression of GDF9 is not altered in *Gja1^{Int}/+* ovaries and is the same as that observed in *Gja1^{-/-}* ovaries (Gittens et al., 2005). Therefore, the *Gja1^{Int}* mutation might affect the normal response of granulosa cells to endocrine and paracrine signals, thus restricting final maturation of the follicles.

Our finding that the *Gja1^{Int}* mutation preferentially affects coupling among the granulosa cells, and leaves oocyte-cumulus cell coupling intact, is consistent with the accumulated evidence that Cx43 is preferentially assembled into the gap junctions that connect granulosa cells, whereas Cx37 forms the gap junctions connecting oocytes with granulosa cells (reviewed by Kidder, 2005). This view was supported recently by Gershon et al. (Gershon et al., 2008), who studied conditional knockout mice in which Cx43 was depleted from oocytes by using the oocyte-specific *Zp3* promoter to drive Cre recombinase expression in growing oocytes that carried floxed *Gja1* alleles (there was also some reduction of Cx43 expression in the granulosa cells). Gershon et al. reported that the Cx43-deficient oocytes retained gap junctional coupling with the granulosa cells, underwent meiotic maturation in response to LH and, after fertilization, developed normally up to the blastocyst stage. However, the females producing Cx43-deficient oocytes showed a decrease in litter size, an effect that was traced to a reduction in the ability of their blastocysts to implant in the uterus. Thus, it remains possible that the Cx43 deficiency in developing oocytes

has an impact on oocyte/embryo developmental competence, which does not manifest itself until implantation. Further experiments will be needed to explore the possibility that implantation failure contributes to the reduced litter sizes of *Gja1^{ht}/+* females.

To date, two mutant mouse models have been generated that carry human *GJA1* mutations: I130T and G138R (Kalcheva et al., 2007; Dobrowolski et al., 2008); it is of interest to compare the phenotype of these two mutants with that of the *Gja1^{ht}/+* phenotype. Overall, the G138R and I130T mutants demonstrated ODDD-like phenotypes that are similar to the *Gja1^{ht}/+* mice (summarized in Table 2). Studies of G138R and I130T mice have so far focused on the heart, where the same negative effects that were found in G60S mice have been reported (Flenniken et al., 2005; Manias et al., 2008), including effects on Cx43 expression level, phosphorylation state, subcellular localization and function in cardiomyocytes. However, the cardiac electrical waves described in the three mutant strains were not identical; the differences will need to be examined more carefully within a single laboratory to confirm that they represent different effects of the three mutant alleles. A reduction in litter size was reported for I130T and G138R females, but in both cases it was attributed to prenatal death; ovulation rate was not investigated (Kalcheva et al., 2007; Dobrowolski et al., 2008). It will be important to investigate whether defects in oogenesis, similar to those observed in G60S females, are also present in the I130T and G138R mutants.

Finally, we note that phenotypic descriptions of ODDD patients to date have not included the reproductive system, which has presumably not been investigated since neither men nor women with the ODDD syndrome have been reported to be infertile. The reduced fertility observed in *Gja1^{ht}/+* female mice raises a concern that some human ODDD females may have unrecognized reproductive deficiencies that are related to *GJA1* mutations. Given this uncertainty and the prominent expression of Cx43 in human ovarian follicles (Wang et al., 2008), we believe that further clinical investigations of ODDD females are warranted.

METHODS

Mouse breeding and genotyping

All animal experiments were approved by the Animal Use Subcommittee of the University Council on Animal Care at the University of Western Ontario. The *Gja1^{ht}/+* mice were generated at the Centre for Modeling Human Disease, University of Toronto, and were kindly provided by Dr Janet Rossant. The original mice were on a mixed C57BL/6J and C3H/HeJ background (Flenniken et al., 2005) and were backcrossed to C57BL/6J for up to four generations. The genotypes were determined by polymerase chain reaction (PCR), as described previously (Flenniken et al., 2005).

Fertility testing

Mice were housed under controlled lighting (12 hours of light, 12 hours of dark) and temperature (21–24°C) conditions. To evaluate the fertility of mice with different genotypes, three mating groups were set up separately: wild-type male with wild-type female mice; *Gja1^{ht}/+* male with wild-type female mice; and wild-type male with *Gja1^{ht}/+* female mice. All of the mice were sexually mature by at least 6 weeks of age. One male and one female mouse were housed

together for up to 7 days for mating. Offspring were counted on postnatal day 1, including both living and identifiable dead pups. The survival rate was calculated by comparing pup numbers on postnatal days 1 and 7.

Superovulation

To test the ability to ovulate in response to gonadotropins, 3–4-week-old female mice were injected intraperitoneally with 5 IU of eCG (Sigma-Aldrich, Canada) at around 18.00 h, followed 48 hours later with 5 IU of hCG (Sigma-Aldrich). The females were killed 14 to 15 hours later by cervical dislocation following CO₂ anesthesia. The COCs were collected from the oviductal ampullae and counted.

In vitro oocyte maturation

Female mice (3–4 weeks old) were killed 24 hours after eCG (5 IU) injection. Ovaries were removed and placed in Waymouth's MB 752/1 medium plus 5% fetal bovine serum (FBS) and 0.23 mM pyruvic acid (sodium salt; Sigma-Aldrich). Follicles were pierced with 25-gauge needles to liberate COCs. Oocytes enclosed by a complete layer of cumulus cells were washed through culture medium and transferred to a 35 mm Petri dish containing 3 ng/ml FSH (Puregon 100 i.u. follitropin β; Organon Canada, Scarborough, ON) in 3 ml of Waymouth's medium/5% FBS. Oocytes were matured for 18 hours in a 5% CO₂/5% O₂/90% N₂ (5/5/90) atmosphere at 37°C and stained with Hoechst 33342 (Molecular Probes, Eugene, OR), diluted 1:1000 in Waymouth's medium/5% FBS, to evaluate oocyte maturation.

In vitro fertilization

Female mice (3–4 weeks old) were superovulated as described above. The COCs were collected from the oviductal ampullae in pre-gassed human tubal fluid (HTF) medium (Jackson Laboratory) with 4 mg/ml bovine serum albumin (BSA), and transferred to 500 μl droplets of HTF/BSA under mineral oil (Sigma-Aldrich) containing approximately 2 × 10⁵ sperm/ml. Sperm were collected from the cauda epididymi of mice at 3–5 months of age. Inseminated oocytes were incubated for 4 hours at 37°C in a 5/5/90 atmosphere. They were then washed through three dishes of HTF/BSA and cultured for 24 hours in 1 ml of HTF/BSA in four-well plates (Nunc) in a 5/5/90 atmosphere at 37°C to yield two-cell stage embryos.

Ovarian histology and follicle counts

Ovaries were collected from 6–8-week-old females to determine follicle distribution. Ovaries were fixed in Bouin's solution, embedded in paraffin and sectioned serially 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 and eosin. Follicles were classified as primary (a single layer of cuboidal granulosa cells), secondary (multiple layers of granulosa cells but lacking any sign of an antral cavity), early antral (with one or more small fluid-filled cavities) and late antral (having a single large cavity with the oocyte against one side of the follicle). Care was taken to ensure that only follicles sectioned through the nucleus of the oocyte were counted. For early stage (primary and secondary) follicle counts, 3–4 representative sections from each ovary were examined. For antral (early and late) follicle

counts, ovaries were collected from mice selected at proestrus based on vaginal smears (Fox and Laird, 1970), and all sections from each ovary were examined. One ovary from each mouse was counted, with 3-5 ovaries from each genotype being examined.

Granulosa cell culture

Follicles were isolated and cultured as described previously (Tong et al., 2006). Briefly, the ovaries of 6–8-week-old female mice were placed in culture in Waymouth MB 752/1 medium containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Canada). The surrounding fat and connective tissue was removed by using fine 30-gauge needles. The ovaries were then digested in medium containing 2 mg/ml type I collagenase (Sigma-Aldrich) to facilitate follicle release. Follicles were liberated by repeated aspiration and expulsion with a 1 ml pipettor. Follicles were then washed with culture medium and transferred to another dish where oocytes and granulosa cells were separated by treatment with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA tetrasodium salt; Invitrogen) for 5 minutes followed by centrifugation at 780 *g* for 5 minutes. The supernatant (containing oocytes) was removed and the granulosa cells resuspended in culture medium. They were then transferred to 12 mm glass coverslips and cultured at 37°C, in 5% CO₂/95% air, for less than 48 hours.

Immunofluorescent staining

Granulosa cells grown on glass coverslips were fixed with ice-cold 80% methanol/20% acetone for 20 minutes before blocking with 2% BSA (Sigma-Aldrich) in PBS for 1 hour. Cx43 was detected using a rabbit polyclonal antibody (1:500; Sigma-Aldrich). The Golgi apparatus was labeled with a mouse antibody directed against a resident protein, GM130 (1:100; BD Transduction Laboratories, Mississauga, ON). Appropriate Alexa 594- or Alexa 488-conjugated anti-mouse and anti-rabbit secondary antibodies (Molecular Probes) were used at a 1:200 dilution. Nuclei were labeled with Hoechst 33342 (1:1000; Molecular Probes). Slides were imaged on a Zeiss (Thornwood, NY) LSM 510 META confocal microscope.

For germ cell counting, ovaries were collected from postnatal day 1 female littermates and fixed, embedded and sectioned as described above. Ovary sections were deparaffinized and washed three times with PBS before immunolabeling. GCNA, a specific marker for germ cells, was detected with undiluted rat anti-GCNA antibody (a gift from Dr George Enders, University of Kansas Medical Center) and Texas Red-conjugated anti-rat secondary antibody (1:200; Molecular Probes). To determine germ cell numbers, GCNA-positive cells were counted on consecutive sections for each ovary. Care was taken to ensure that only cells sectioned through the largest diameter of the nucleus were counted. Ovary sections from 6–8-week-old females were also immunolabeled with Cx43 antibody as described above.

Proliferation assays

Granulosa cells were grown on glass coverslips at a confluency of 50-70%. For the bromodeoxyuridine (BrdU) uptake assay, BrdU (Sigma-Aldrich) was added into the culture medium to reach a final concentration of 10 µM. The cells were then incubated for 4 hours at 37°C in 5% CO₂/95% air. They were washed three times with PBS

before being fixed with ice-cold 80% methanol/20% acetone for 20 minutes. Following incubation with 2 M HCl for 20 minutes to denature DNA, granulosa cells were immunolabeled as described above. BrdU detection was carried out using a mouse anti-BrdU antibody (1:100; Sigma-Aldrich) and Texas Red-conjugated anti-mouse secondary antibody (1:200). Nuclei were labeled with Hoechst 33342 (1:1000). Cell counts were performed after Hoechst staining and expressed as the percentage of cells that were positive for BrdU staining relative to the total number of cells in view. The proliferation rate of granulosa cells was also evaluated by immunostaining with mouse anti-PCNA antibody (1:200; Sigma-Aldrich) and Texas Red-conjugated anti-mouse secondary antibody (1:200). The densities of the PCNA signals were quantified using Zeiss LSM software (Thornwood, NY). To minimize variation between experiments, each measurement was normalized to one of the wild-type granulosa cell readings from the same experiment.

Apoptosis assay

Ovary sections were stained for apoptotic cells by incorporating fluorescein-12-dUTP at the 3'-OH ends of fragmented DNA using the TUNEL (TdT-mediated dUTP nick-end labeling) assay kit (Promega, Madison, WI), according to the manufacturer's instructions. Sections treated with DNase (1 mg/ml, 10 minutes) (Sigma-Aldrich) were used as positive controls. To quantify the TUNEL staining in antral follicles, the average pixel intensity for the area of antral follicles was determined using ImageJ 1.38x software (National Institutes of Health, USA).

Western blot analysis

Ovaries or hearts were homogenized in single-detergent lysis buffer supplemented with 1 mM NaF, 1 mM Na₃VO₄ and a protease inhibitor cocktail (1 tablet per 10 ml of buffer) (Sigma-Aldrich). Protein concentrations were determined by using the bicinchonic acid (BCA) assay (Pierce Biotechnology Inc., Rockford IL). 50 µg of protein was separated by electrophoresis on 12% SDS-PAGE gels and transferred to a nitrocellulose membrane. The membrane was incubated with either a rabbit anti-Cx43 antibody (1:1000; Sigma-Aldrich), a goat anti-GDF9 antibody (c-20) (1:400; Santa Cruz, CA) or a mouse anti-GAPDH antibody (1:10,000; Chemicon, Temecula, CA) overnight at 4°C. Following three washes with Tris-buffered saline containing Tween 20 (5 minutes each at room temperature), infra-red fluorescent-labeled secondary antibodies (IRDye 800 anti-rabbit, Rockland Immunochemicals, Gilbertsville, PA; Alexa 680-conjugated anti-mouse, Molecular Probes) were incubated at room temperature for 1 hour and immunoblots were processed and quantified using the Odyssey infrared-imaging system (Li-Cor).

Oocyte microinjection

Ovarian follicles were isolated as described above and transferred to 12 mm glass coverslips then cultured at 37°C in 5% CO₂/95% air for 24 hours. 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 (Molecular Probes) in ddH₂O through capillary action. The dye was injected using an Eppendorf InjectMan NI 2 microinjection system and a Leica DMIRE2 inverted microscope with a 20×, 0.5 numerical aperture objective. Images were captured

TRANSLATIONAL IMPACT

Clinical issue

Oculodentodigital dysplasia (ODDD) is a rare genetic abnormality that affects multiple organs, most obviously the eyes (microphthalmia), teeth (enamel hypoplasia) and digits (oligosyndactyly of the fingers and toes). In some affected individuals, variable neurological problems and heart and skin defects have also been described. The diverse features that characterize this inherited syndrome reflect the widespread expression of its affected gene, *GJA1*, which encodes connexin43 (Cx43). Connexins form the intercellular membrane channels of gap junctions – structures that allow cells to pass ions and small molecules between them to coordinate their functions. Studies in knockout mice indicate that connexins, including Cx43, play essential developmental and physiological roles.

Results

Cx43 is necessary for mouse oogenesis and is widely expressed in human ovaries. The role for Cx43 in reproduction is not well understood in mice and has not been studied in humans. This study examines Cx43 and its influence on reproductive health using mice carrying a Cx43 mutation that causes a phenotype similar to ODDD in humans. Their results show that mutant mice are subfertile; they have smaller litters and their follicles do not respond properly to hormone stimulation.

Implications and future directions

Connexins play an important role in the female reproductive system and the research presented here demonstrates that Cx43 contributes to the normal process of oogenesis and fertility. This study encourages a closer examination of the reproductive history of ODDD women as it may reveal previously undiagnosed problems. Furthermore, using this mouse model to study the role of Cx43 in oogenesis might lead to new treatments for human reproductive problems, including infertility.

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using a Hamamatsu C4742-95ER camera with Openlab software. Only those injections in which the dye filled the oocyte within 1 minute, as evidenced by bright fluorescence, were recorded.

Data analysis and statistics

The data are expressed as mean±s.e.m., with ‘*n*’ denoting the number of independent experiments. Comparisons of two groups were carried out using a two-tailed unpaired *t*-test, with a *P* value below 0.05 indicating significance. Comparisons of more than two groups were carried out with one-way ANOVA followed by a Tukey test. Two-way ANOVA and the Bonferroni test were used to compare two groups under different experimental conditions. Statistical significance is indicated in the graphs with a single asterisk (*) for *P*<0.05, double asterisk (**) for *P*<0.01 and triple asterisk (***) for *P*<0.001.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

This work was a collaboration between the laboratories of D.B., G.M.K. and D.W.L. who jointly provided supervision and technical advice. D.T. performed most of the

experiments and drafted the manuscript; D.C. contributed the data for Fig. 3A; R.T. contributed to the results of Fig. 2B and Fig. 5; T.Y.L. contributed the data for Fig. 7A; and I.P. contributed data to Fig. 1C and part of Fig. 1A.

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