Differential contributions of connexin37 and connexin43 to oogenesis revealed in chimeric reaggregated mouse ovaries

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Summary

The gap junction proteins connexin37 and connexin43 are required for ovarian folliculogenesis in the mouse. To define their respective roles in oogenesis, chimeric ovaries containing either null mutant oocytes and wild-type granulosa cells or the reverse combination were grafted to the renal capsules of immunodeficient female mice. After three weeks, the oocytes were tested for meiotic competence and fertilizability in vitro. Ovaries composed of connexin43-deficient oocytes and wild-type granulosa cells produced antral follicles enclosing oocytes that could develop to at least the two-cell stage, demonstrating that oocytes need not express connexin43 to reach maturity. Conversely, both follicle development and oocyte maturation were impaired in ovaries containing either wild-type oocytes and connexin43-deficient granulosa cells or connexin37-deficient oocytes and wild-type granulosa cells. Thus absence of connexin43 from granulosa cells or connexin37 from oocytes is sufficient to compromise both oocyte and follicle development. Wild-type oocytes paired with connexin37-deficient granulosa cells generated antral follicles containing oocytes that developed to at least the two-cell stage. Therefore, connexin37 absence from granulosa cells need not impair fertility in mice. Dye transfer experiments revealed persistent oocyte-granulosa cell coupling in those follicles, indicating functional compensation by another connexin. The results indicate that mouse oocytes do not need to express connexin43 in order to develop into meiotically competent, fertilizable gametes, but must express connexin37 for communication with granulosa cells, a requirement for oogenesis.

Key words: Gap junctions, Ovarian follicle, Granulosa cells, Oocyte

Introduction

Closely opposed cells communicate directly with one another via intercellular channels called gap junctions. The basic unit of the gap junction channel is the connexon (hemichannel), which is composed of six integral membrane proteins called connexins (reviewed by Sáez et al., 2003). Two connexons from adjacent cells dock end-to-end to form an intercellular channel. There are at least 20 members of the connexin family in mammals. Different connexins can associate to form connexons and gap junction channels composed of two or more different types of connexins (reviewed by Goldberg et al., 2004). Each connexin conveys unique biophysical properties on the gap junction channel, thereby influencing the types of molecules transmitted by the gap junction. Thus, the complexity of gap junctions is determined by the types of connexins expressed by the contributing cells.

Gap junctional communication features prominently in mammalian oogenesis. In the mouse, nutrients, nucleotides and regulatory molecules are transferred from granulosa cells to oocytes to coordinate their timely growth from a diameter of ~15 μm to 80 μm (reviewed by Eppig, 1991). As oocytes grow, the surrounding squamous granulosa cells become cuboidal in shape, increase in number and stratify around the centrally located oocyte. During the growth phase, oocytes remain arrested in prophase of the first meiotic division. They do not achieve competence to resume meiosis until fully grown and a cavity, the antrum, has formed among the granulosa cells to delineate cumulus and mural cell compartments (reviewed by Vanderhyden, 2002). Once this has occurred, a surge of luteinizing hormone (LH) leads to meiotic resumption and disruption of gap junctional communication between granulosa cells, accompanied by cumulus expansion. These events can be triggered, in vitro, by removing fully grown oocytes from follicles and culturing oocytes and associated cumulus cells with follicle stimulating hormone (FSH) (Vanderhyden et al., 1990).

A connexin protein with molecular mass of ~43 kDa, connexin43 (Cx43), forms gap junctions between granulosa cells throughout all stages of folliculogenesis in the mouse (Valdimarsson et al., 1993; Juneja et al., 1999; Gittens et al., 2003). The same is true in the rat, but in that species Cx43 was also demonstrated in oocytes (Granot et al., 2002). The ovaries of mice lacking Cx43 contain follicles that arrest development in preantral stages with oocytes that do not undergo meiotic maturation in vitro (Ackert et al., 2001). In the absence of

Cx43, there is no gap junctional communication between granulosa cells of such early follicles (Gittens et al., 2003). However, oocyte-granulosa cell gap junctional coupling is maintained in Cx43-knockout follicles (Veitch et al., 2004), which indicates that the principal role of Cx43 in the follicle is to couple granulosa cells with each other.

Connexin37 (Cx37) is found in gap junctions between the oocyte and granulosa cells commencing at the primary follicle stage and is required for fertility (Simon et al., 1997). Mice lacking Cx37 do not ovulate (Simon et al., 1997) and oocytes are meiotically incompetent (Carabatsos et al., 2000). Cx37 is expressed in oocytes and without it there is no oocyte-granulosa cell gap junctional communication, whereas coupling among granulosa cells is maintained (Simon et al., 1997). Uncertainty has existed concerning the granulosa cell connexin that docks with oocyte Cx37 forming gap junctions between the two cell types. Using short term in vitro co-culture and a dye preloading test, we recently determined that both oocytes and granulosa cells must express Cx37 to establish gap junctional communication between them (Veitch et al., 2004). Cx37 is normally expressed in granulosa cells but its assembly into gap junctions is dependent on contact with the oocyte (Veitch et al., 2004). Thus, it appears that the principal role of Cx37 in granulosa cells is to mediate communication with the oocyte.

The experiments in this report were designed to define the respective roles of Cx37 and Cx43 through the full course of oocyte growth and follicle development in vivo. The use of connexin knockouts to study the roles of gap junctions during follicle development has provided considerable insight into which compartments of the follicle are most vulnerable to a null mutation. However, they have precluded investigations of the effects of null mutations on either oocytes or granulosa cells alone. In particular, given the dominant role played by oocytes in directing follicle development (Eppig et al., 2002), it was of interest to determine if wild-type oocytes can promote normal folliculogenesis even in the presence of connexin-deficient granulosa cells. This and other questions were explored through the construction of chimeric ovaries, assembled from either mutant oocytes and wild-type granulosa cells, or wild-type oocytes and mutant granulosa cells.

Materials and Methods

Animals

Mice lacking Cx43 were derived from matings of heterozygous (*Gja1*$^{+}$/*Gja1*$^{-}$) CD1or C57Bl/6 mice. Cx37-deficient offspring were obtained by mating heterozygous (*Gja4*$^{+}$/*Gja4*$^{-}$) C57BL/6 mice. *Prkdc*^{scid}/*Prkdc*^{scid} (C.B.-17/IcrHsd-*scid*) females to be used as graft recipients were purchased from Harlan Sprague-Dawley, Indianapolis, IN. To obtain ovaries lacking Cx43, pregnant dams were killed by cervical dislocation following CO_2 anaesthesia on either day 17.5 or 18.5 of gestation. Fetuses were removed from the uteri, decapitated and their ovaries collected (Gittens et al., 2003). To obtain ovaries lacking Cx37, pregnant dams were killed as above on day 18.5 to obtain fetuses, or ovaries were collected directly from neonates or 1-day-old pups. Ovaries were cultured in Waymouth's MB 752/1 medium (Invitrogen Canada, Burlington, ON) containing 10% fetal bovine serum (Qualified, Mexican; Invitrogen) on Millicell-PC (3.0 μm; 30 mm) polycarbonate membrane (Millipore, Bedford, MA) for 1 or 2 days until the genotypes of the ovary donors were determined (Gittens et al., 2003).

Genotyping

The genotype of each ovary donor was determined by polymerase chain reaction (PCR) of proteinase K-digested tail snips. The primer pairs and protocol used to amplify the *Gja1*$^{+}$ (wild-type Cx43) and *Neo* (disrupted Cx43) alleles were described (Ackert et al., 2001). The following primer pair was used to amplify a 707 bp fragment of the *Gja4*$^{+}$ (wild-type Cx37) allele: 5′-TGC TAG ACC AGG TCC AGG AAC-3′ and 5′-GTC CCT TCG TGC CTT TAT CTC-3′. Another set of primers was used to amplify the 800 bp *Neo* (disrupted Cx37) allele in a separate reaction tube. The primer pair was: 5′-TGC TAG ACC AGG TCC AGG AAC-3′ and 5′-GCT TGC CGA ATA TCA TGG TGG A-3′. A primer pair for the *Gjb1*$^{+}$ allele (Cx32; 850 bp fragment) was included in the reaction mixture as an internal control. The primers for *Gjb1*$^{+}$ were: 5′-GAG CAT AAA GAC AGT GAA GAC GG-3′ and 5′-CCA TAA GTC AGG TGT AAA GGA GC-3′. PCR was performed in 10 μl volumes containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.125 U Platinum *Taq* DNA polymerase (all reagents from Invitrogen), 1 μl tail digestate, 0.1 μM *Gjb1*$^{+}$ primers and 0.25 μM *Gja4*$^{+}$ primers and *Neo* primers. PCR was performed using a Perkin Elmer 2400 thermal cycler and commenced with an initial denaturation for 3 minutes at 95°C followed by 45 cycles of denaturation (30 seconds at 94°C), annealing (30 seconds at 61°C) and extension (30 seconds at 72°C). The final step was an extension at 72°C for 7 minutes.

Construction of chimeric ovaries

Ovaries having the same genotype were pooled (four to six per group), washed with 1 mg/ml BSA (Sigma-Aldrich, Oakville, ON) in PBS and dissociated with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA tetrasodium salt, Invitrogen) for 45 minutes as previously described (Eppig and Wigglesworth, 2000). For construction of Cx43 chimeric ovaries, mutant cells derived from pooled 17.5 and 18.5 days post coitus (dpc) ovaries were combined with cells derived from pooled ovaries of the corresponding wild-type littermates. Ovaries from both CD1 and C57BL/6 strains were used for these experiments, but the two strains were never combined. Cx37 chimeric ovaries were constructed using cells obtained from pooled C57BL/6 strain mutant ovaries, 18.5 dpc and 1 day postnatal and cells from pooled ovaries of the corresponding wild-type littermates. Pooling of ovary ages, which preliminary experiments demonstrated did not affect the outcome of experiments, was done to increase the number available for chimera construction. Cell suspensions were centrifuged at 591 ***g*** for 5 minutes, resuspended in Medium 199 (M199) containing 10% FBS (Invitrogen) and transferred to tissue culture dishes (Easy Grip^{TM} 35×10 mm, Falcon). Cells were cultured overnight in 5% CO_2, 5%O_2, 90% N_2 (5/5/90) at 37°C. The following morning, unattached oocytes were removed and transferred to another tissue culture dish. The monolayer of somatic cells was rinsed free of oocytes using 1 mg/ml BSA-PBS, dislodged from the dish with trypsin-EDTA and centrifuged at 591 ***g*** for 5 minutes to pellet the cells. Pelleted cells were resuspended in M199/10% FBS and transferred to a new culture dish. After 6 hours of culture, oocytes and somatic cells were re-collected as described above and centrifuged at 591 ***g*** for 5 minutes. Cells were subjected to two rounds of seeding and trypsin treatment to ensure the purity of the somatic cell preparations. Pellets were resuspended in 100 μl M199/10% FBS with 7 μl/ml PHA lectin (0.05% phytohemagglutinin, Sigma-Aldrich). Oocytes and somatic cells, from either the same or a different mutant strain, were mixed in a suitable ratio and pelleted in a microfuge. Pellets were 'organ' cultured overnight (15-20 hours) in 5% CO_2 in air as described previously for fetal gonads (Ackert et al., 2001). Chimeric ovaries were implanted beneath the renal capsules of bilaterally ovarectomized SCID mice according to procedures outlined (Gittens et al., 2003). As folliculogenesis proceeds more rapidly in CD1 mice, chimeric ovaries were recovered 20-21 days after grafting for those mice but 23-24 days after grafting for C57BL/6 mice.

Histology and testing for cell separation purity

Chimeric ovaries were fixed in Bouin's solution, embedded in paraffin and sectioned at a thickness of 5 μm. Sections were stained with hematoxylin and eosin. Measurements of oocyte and follicle diameter were taken using Northern Eclipse image analysis software (Empix Imaging). Only follicles sectioned through the nucleus of the oocyte were measured. Follicles were counted and classified according to previously described stages (Pederson and Peters, 1968): type 3 follicles contained one layer of granulosa cells; type 4 follicles contained two layers of granulosa cells; type 5 follicles had 3 or more layers of granulosa cells and multilaminar follicles containing an antrum of varying size were scored in the type 6-8 follicle category. A minimum of four chimeric ovaries was examined for each combination of cell types and two to three sections from each ovary were evaluated. One-way ANOVA followed by Tukey's test was used to judge the significance of differences in measurements between ovaries. To confirm that the methodology did not allow for wild-type cells to contaminate knockout cell preparations and vice versa, chimeric ovaries lacking Cx43 in somatic cells were constructed and ovarian sections or fixed follicle cultures were treated with a polyclonal antibody raised against the C-terminal tail of Cx43 as described previously (Gittens et al., 2005). Images were captured on either a Zeiss LSM 510 Meta inverted confocal microscope or Zeiss Axioskop 2 microscope equipped with a Retiga 1300 CCD digital camera (Q imaging).

Cumulus expansion and oocyte maturation

Pregnant mare serum gonadotrophin (5 i.u.; PMSG; National Hormone and Peptide Program) was administered on day 22 of grafting, 24 hours prior to the commencement of 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% FBS 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 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 Waymouth's medium/5% FBS. Oocytes were matured for 18 hours in a 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.

Fertilization

Mature oocytes were washed through three dishes of MEM (Eppig et al., 2002) with 3 mg/ml BSA and transferred to 500 μl droplets of MEM/BSA under mineral oil (Sigma-Aldrich) containing approximately 2×10^5 sperm/ml. Sperm were collected from the cauda epididymi of mice 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 MEM/BSA and cultured for 24 hours in 1 ml MEM/BSA in four-well plates (Nunc) in 5/5/90 at 37°C to yield two-cell stage embryos (Eppig et al., 2002).

Oocyte microinjection

Small secondary follicles from each of the three classes of reaggregated ovaries were isolated and cultured for 1 day on 12 mm coverslips in Petri dishes as described (Veitch et al., 2004). Oocytes were impaled with a microinjection needle containing 5% Lucifer Yellow CH dilithium salt (FW 457; Sigma-Aldrich) in H_2O for 10 minutes. Injections were recorded if they resulted in rapid filling of the oocyte within 1 minute and showed no evidence of a granulosa cell having been injected. Images were captured on a Leica DM inverted microscope equipped with a Hamamatsu Photonic Systems digital camera.

Results

Our first step was to demonstrate that the chimeric reaggregated ovary procedure could produce ovaries lacking a connexin in the desired compartment of the follicle. To do this, chimeric ovaries derived from wild-type oocytes and Cx43-deficient somatic cells (CD1 strain) were immunostained for Cx43. Cx43 is an ideal marker in this context because it is highly expressed by granulosa cells through all stages of follicle growth, allowing for easy detection of contaminating wild-type granulosa cells in chimeric ovaries constructed to contain wild-type oocytes and Cx43-deficient granulosa cells (hereafter referred to as $43^{+/+}$oo/$43^{-/-}$gc). Ovaries of $43^{+/+}$oo/$43^{-/-}$gc constitution did not demonstrate any Cx43 immunofluorescence in ovarian sections (Fig. 1B,D,F). Punctate staining, typical of gap junctions, was readily detectable between the granulosa cells of chimeric ovaries containing Cx43-deficient oocytes and wild-type granulosa cells ($43^{-/-}$oo/$43^{+/+}$gc, not shown) and control chimeric ovaries, containing wild-type or heterozygous oocytes and granulosa cells (Fig. 1A,C,E). Thus the chimeric reaggregated ovaries we constructed could be expected to comprise the desired combinations of genotypes with little or no contamination from the opposite genotype.

Chimeric ovaries composed of wild-type oocytes and Cx43-

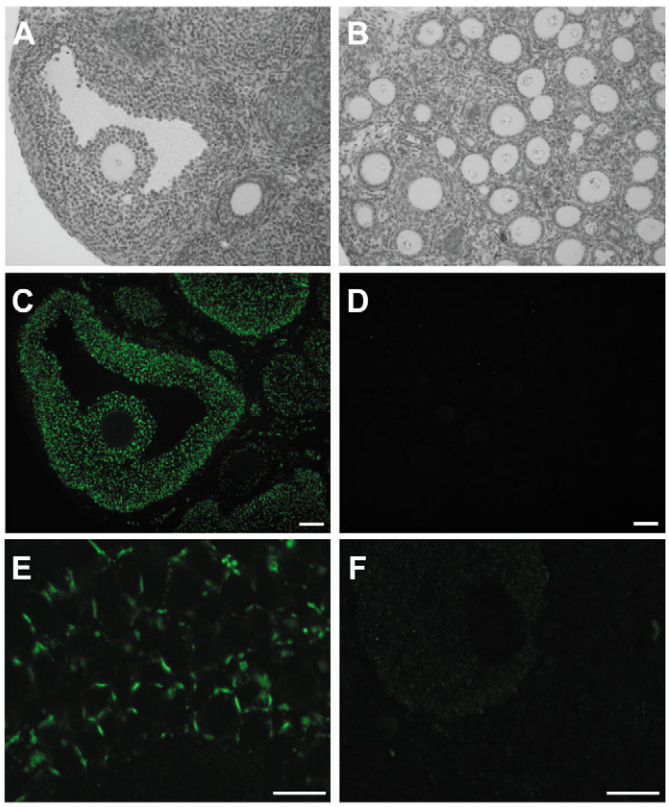

Fig. 1. Absence of wild-type granulosa cell contamination in ovaries constructed from wild-type oocytes and Cx43-deficient somatic cells. $43^{+/+}$oo/$43^{+/+}$gc (A,C,E) and $43^{+/+}$oo/$43^{-/-}$gc (B,D,F) ovary sections were immunostained for Cx43. Punctate immunoreactivity characteristic of gap junctions is evident between the granulosa cells of $43^{+/+}$oo/$43^{+/+}$gc chimeric ovaries (C,E) but not $43^{+/+}$oo/$43^{-/-}$gc chimeric ovaries (D,F). E and F are magnifications of C and D, respectively. Bar, 50 μm (C,D); 10 μm (E,F).

Table 1. Classification of follicles in 23- to 24-day-old Cx37 (C57BL/6 strain) and 20- to 21-day-old Cx43 (CD1 strain) chimeric ovaries into developmental stages

		Number (percentage) of follicles			
Genotype	*n*	Type 3 (primary)	Type 4 (early secondary)	Type 5 (secondary)	Type 6-8 (tertiary)
$37^{+/+}$oo/$37^{+/+}$gc	116	11 (9%)	47 (41%)	43 (37%)	15 (13%)
$37^{-/-}$oo/$37^{+/+}$gc	240	73 (30%)	165 (70%)	2 (1%)	0 (0%)
$37^{+/+}$oo/$37^{-/-}$gc	182	9 (5%)	66 (36%)	82 (45%)	25 (14%)
$43^{+/-}$oo/$43^{+/-}$gc	212	17 (8%)	108 (51%)	67 (32%)	20 (9%)
$43^{-/-}$oo/$43^{+/+}$gc	120	16 (13%)	46 (38%)	36 (30%)	22 (18%)
$43^{+/+}$oo/$43^{-/-}$gc	273	236 (86%)	34 (12%)	3 (1%)	0 (0%)

deficient granulosa cells (CD1 strain) contained follicles at early stages of development, whereas control chimeric follicles contained follicles at all stages (Table 1, Figs 1 and 2). Even when the ratio of granulosa cells to oocytes was increased, the majority of $43^{+/+}$oo/$43^{-/-}$gc follicles were significantly smaller (P<0.01) (Fig. 2) and contained only a single layer of granulosa cells around each growing oocyte. On occasion, follicles with five to seven granulosa cell layers contained what appeared to be a small antrum (Fig. 2B) as previously reported for ovaries from Cx43 knockout mice of the CD1 strain (Ackert et al., 2001). The largest oocyte and follicle from $43^{+/+}$oo/$43^{-/-}$gc ovaries spanned 69 μm and 200 μm, respectively. In contrast, chimeric $43^{-/-}$oo/$43^{+/+}$gc ovaries contained antral follicles that were not morphologically different from those of control chimeric follicles. Moreover, the growth of the Cx43-deficient oocytes was not compromised and the mutant oocytes did not limit the growth of the follicles (Fig. 2C,D). These results make it clear that the impairment of follicle development in Cx43 knockout ovaries is due to defects in the granulosa cells, not the oocytes.

Chimeric ovaries composed of wild-type oocytes and Cx37-deficient granulosa cells (C57BL/6 strain) contained antral stage follicles that were not different in size from control ovaries ($37^{+/-}$oo/$37^{+/-}$gc) (Fig. 3B). The diameters of the largest oocyte and follicle from $37^{+/+}$oo/$37^{-/-}$gc ovaries were 77 μm and 473 μm, respectively. Conversely, ovaries of $37^{-/-}$oo/$37^{+/+}$gc constitution did not contain follicles with more than three layers of granulosa cells (Fig. 3A). The majority of $37^{-/-}$oo/$37^{+/+}$gc follicles had only progressed to the type 4 stage (Table 1) and oocytes and follicles were significantly smaller (P<0.01) than control chimeric and $37^{+/+}$oo/$37^{-/-}$gc ovaries

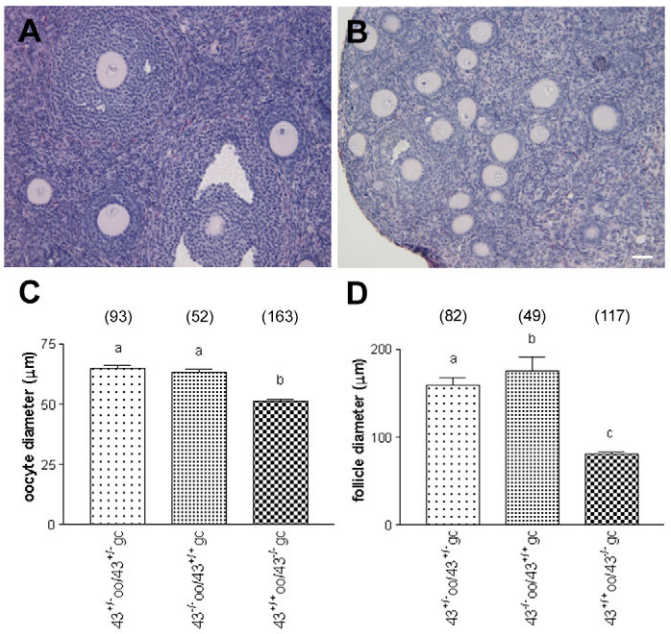

Fig. 2. Aberrant development in Cx43-deficient ovaries is due to defects in granulosa cells, not oocytes. (A) A $43^{-/-}$oo/$43^{+/+}$gc ovary containing follicles at all developmental stages, including antral stages. (B) A $43^{+/+}$oo/$43^{-/-}$gc ovary at the same magnification as (A) showing a preponderance of small follicles at the primary and early secondary follicle stages. The graphs show mean oocyte (C) and follicle (D) diameters across chimeric ovary combinations. Bars with different letters above them are significantly different (P<0.01). The numbers in parentheses indicate the numbers of oocytes or follicles measured. Bar, 50 μm.

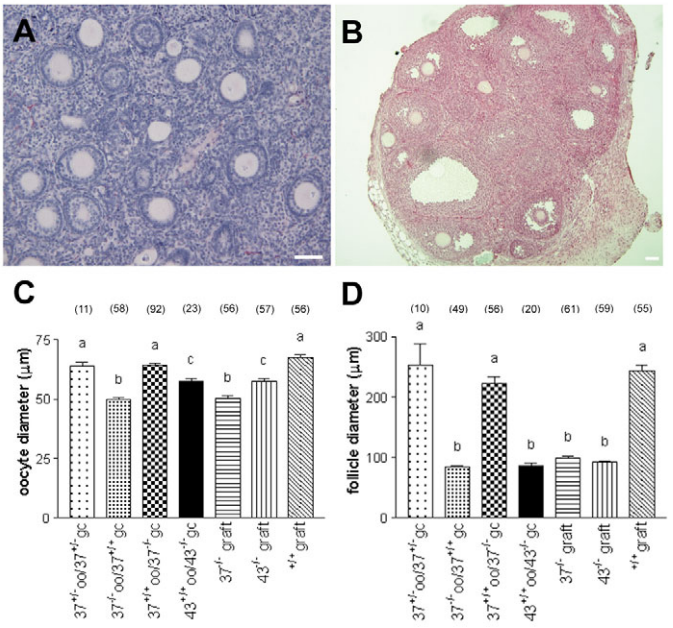

Fig. 3. Aberrant development in Cx37-deficient ovaries is due to defects in oocytes, not granulosa cells. (A) A $37^{-/-}$oo/$37^{+/+}$gc ovary containing only type 4 secondary follicles. (B) A $37^{+/+}$oo/$37^{-/-}$gc ovary showing antral follicles. The graphs show mean oocyte (C) and follicle (D) diameters for chimeric ovary combinations in parallel with grafted wild-type, Cx37 and Cx43 knockout ovaries (BL/6 background). Measurements from $43^{+/+}$oo/$43^{-/-}$gc ovaries on the same background are included in both C and D for comparison. Bars with different letters above them are significantly different (P<0.01). The numbers in parentheses indicate the numbers of oocytes or follicles measured. Bar, 50 μm.

Table 2. Oocyte developmental competence indicated by in vitro maturation and fertilization

	Wild-type not grafted	Wild-type grafted	$37^{+/-}$oo/$37^{+/-}$gc	$37^{+/+}$oo/$37^{-/-}$gc	$43^{+/-}$oo/$43^{+/-}$gc	$43^{-/-}$oo/$43^{+/+}$gc	$37^{-/-}$oo/$37^{+/+}$gc	$43^{+/+}$oo/$43^{-/-}$gc
Total	108	83	59	74	59	57	43	37
GV	3	11	8	5	15	23	43	37
MI	6	8	10	25	16	4	0	0
MII	0	1	15	23	6	8	0	0
Two-cell	99	63	26	21	22	22	0	0
% Two-cell	92	76	44	28	37	39	0	0

GV, germinal vesicle intact; MI, metaphase of meiosis I; MII, metaphase of meiosis II (first polar body); Two-cell, cleavage to the two-cell stage.

(Fig. 3C,D). The sizes of oocytes and follicles in $37^{-/-}$oo/$37^{+/+}$gc ovaries were not different from those of Cx37 knockout ovaries grafted for 24 days (Fig. 3C,D). Therefore, impaired follicle development in Cx37-deficient mice is caused exclusively by defects in the oocytes.

It was also of interest to compare the reduction of oocyte and follicle growth caused by the absence of Cx43 with that caused by the absence of Cx37. When directly compared on the C57BL/6 genetic background, the loss of Cx37 from the oocytes or the entire follicle had a greater impact on oocyte growth than did the loss of Cx43 from the granulosa cells or the entire follicle (Fig. 3C,D). For both connexins, loss from the critical compartment of the follicle had the same effect as loss from the entire follicle. For follicle growth, the effect of Cx43 loss from the granulosa cells or the entire follicle was not significantly different from the effect of Cx37 loss from the oocytes or the entire follicle.

To evaluate the quality of oocyte development in chimeric ovaries, oocytes from chimeric ovaries (C57BL/6 strain) were tested for their ability to induce cumulus expansion, progress through meiosis, be fertilized and undergo first cleavage. The results of these studies are summarized in Table 2. Overnight treatment in vitro with recombinant FSH caused all cumulus-oocyte complexes from wild-type 24-day-old mice, grafted wild-type ovaries, control chimeric ovaries, as well as $43^{-/-}$oo/$43^{+/+}$gc and $37^{+/+}$oo/$37^{-/-}$gc ovaries to undergo cumulus expansion (example in Fig. 4A). Follicles with $43^{+/+}$oo/$43^{-/-}$gc or $37^{-/-}$oo/$37^{+/+}$gc constitution did not show evidence of cumulus expansion (example in Fig. 4B). In other words, oocytes from chimeric ovaries exhibiting impaired follicle development were themselves impaired in their ability to induce cumulus expansion. The frequency of development to the two-cell stage varied with the category of experiment. When oocytes were taken directly from wild-type mice the frequency was 92%. Oocytes obtained from wild-type grafted ovaries demonstrated reduced cleavage competence with 76% of oocytes developing into two-cell embryos. The frequency of cleavage to the two-cell stage was 28-44% in oocytes from chimeric ovaries, except those containing Cx37-deficient oocytes or Cx43-deficient granulosa cells. These latter two categories of chimeric ovaries yielded oocytes that were not meiotically competent and could not be fertilized.

Chimeric ovaries (C57BL/6 strain) were assayed for oocyte-granulosa cell gap junctional coupling by dye microinjection using the gap junction permeable dye, Lucifer Yellow. Dye was injected into each oocyte for 10 minutes to evaluate dye transfer to surrounding granulosa cells. Lucifer Yellow injected into the oocytes of $37^{-/-}$oo/$37^{+/+}$gc follicles failed to pass to companion granulosa cells in ten trials. In contrast, oocytes from $37^{+/+}$oo/$37^{-/-}$gc follicles passed Lucifer Yellow to granulosa cells in eight of twelve trials, to the same extent (six of nine trials) as control chimeric follicles ($37^{+/+}$oo/$37^{+/+}$gc) (Fig. 5). This suggests that another granulosa cell connexin compensates for the absence of Cx37 to sustain gap junctional communication with the oocyte. Given the strong expression of Cx43 in Cx37-deficient granulosa cells, it was of interest to determine if Cx43 was replacing Cx37 in $37^{+/+}$oo/$37^{-/-}$gc ovaries to maintain follicle development. To test this, chimeric follicles were immunostained for Cx43. Follicles from $37^{+/+}$oo/$37^{-/-}$gc ovaries displayed sparse Cx43 staining around oocytes that was not different from the pattern seen in control chimeric follicles (Fig. 6).

Discussion

The experiments presented here were designed to evaluate the specific functional contributions of Cx37 and Cx43 to two cell types of developing follicles, the oocytes and granulosa cells. Previous studies with homozygous null mutant ovaries showed that both of these connexins are required for oogenesis and folliculogenesis (Simon et al., 1997; Carabatsos et al., 2000;

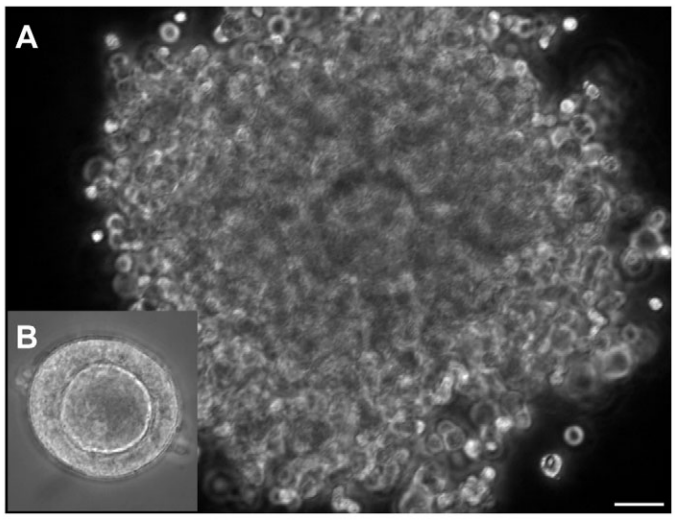

Fig. 4. Cumulus-oocyte complexes from chimeric ovaries composed of either $43^{+/+}$oo/$43^{-/-}$gc or $37^{-/-}$oo/$37^{+/+}$gc do not undergo cumulus expansion. (A) Example of a chimeric follicle with mucified cumulus. (B) Example showing the lack of cumulus expansion. Follicles in A and B were cultured overnight in the same maturation medium and photographed at the same magnification. Bar, 50 μm.

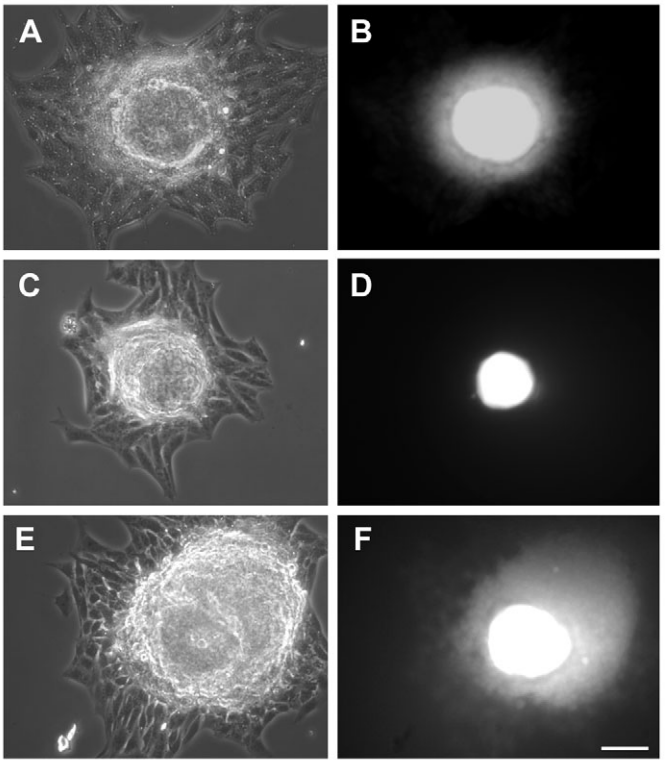

Fig. 5. Cx37 deficiency in granulosa cells does not impair oocyte-granulosa cell gap junctional coupling. $37^{+/+}$oo/$37^{+/+}$gc follicles (A,B), $37^{-/-}$oo/$37^{+/+}$gc follicles (C,D) and $37^{+/+}$oo/$37^{-/-}$gc follicles (E,F) were microinjected with Lucifer Yellow for 10 minutes. Dye transfer from oocytes to granulosa cells was evident when both components were wild-type (B) and when the absence of Cx37 was restricted to the granulosa cells (F). Cx37-deficient oocytes did not pass dye to wild-type granulosa cells (D). Bar, 50 μm.

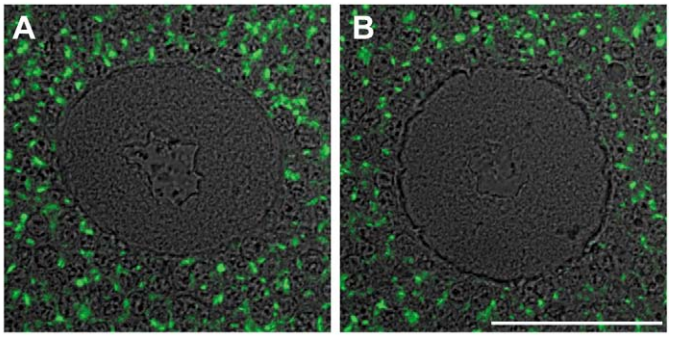

Fig. 6. Cx43 does not appear to replace Cx37 at the oocyte surface in $37^{+/+}$oo/$37^{-/-}$gc ovaries. $37^{+/+}$oo/$37^{+/+}$gc (A) and $37^{+/+}$oo/$37^{-/-}$gc (B) ovary sections were immunostained for Cx43. Punctate staining for Cx43 was seen between granulosa cells, but very little Cx43 was detected between oocytes and granulosa cells in ovaries of either constitution. Bar, 50 μm.

Ackert et al., 2001; Gittens et al., 2003). The present results, obtained using chimeric ovaries, make it clear that each connexin plays an essential role in a specific cell type: Cx37 is required in oocytes but not in granulosa cells whereas Cx43 is required in granulosa cells but not in oocytes, in order for oocytes and follicles to progress through development.

Chimeric ovaries lacking Cx43 only in the granulosa cell compartment contained follicles at early preantral stages of development following 3 weeks of postnatal growth, similar to ovaries lacking Cx43 in all cell types (Ackert et al., 2001). Wild-type oocytes were ~20% smaller when they developed in association with Cx43-deficient granulosa cells and proved themselves to be immature based on their inability to induce cumulus expansion or complete meiosis I in vitro. In contrast, chimeric ovaries lacking Cx43 only in oocytes contained preantral as well as large antral follicles, enclosing developmentally competent oocytes. Once fertilized, Cx43-deficient oocytes from $43^{-/-}$oo/$43^{+/+}$gc ovaries could develop to at least the two-cell stage. Thus, three important conclusions can be drawn from these experiments: (1) folliculogenesis defects in Cx43 knockout ovaries are not due to deficiencies inherent to oocytes at the onset of folliculogenesis, but to deficiencies in the granulosa cells with which they associate postnatally; (2) Cx43 expression by growing oocytes is dispensable for oocyte and follicle development; and (3) Cx43 absence from granulosa cells alone is sufficient to compromise both follicle development and, at least indirectly, oocyte development. As the oocyte was shown to regulate the progression of follicle development using this same technique (Eppig et al., 2002) and as Cx43 has been identified in rat oocytes (Granot et al., 2002), we expected to see folliculogenesis disruptions in $43^{-/-}$oo/$43^{+/+}$gc ovaries. Instead, the results demonstrated that granulosa cells can influence both oocyte and follicle development. Cx43 forms gap junction channels between the granulosa cells of quiescent and growing follicles (Juneja et al., 1999; Valdimarsson et al., 1993; Wiesen and Midgley, 1993) to enable granulosa cell intercommunication (Gittens et al., 2003). Without Cx43, granulosa cell proliferation is hampered because the cells do not respond optimally to mitogenic signals evoked by an oocyte-secreted paracrine factor, growth differentiation factor 9 (GDF9) (Gittens et al., 2005). The fact that female sterility ensues in mice expressing C-terminal truncated Cx43 ($Cx43^{K258stop}$) that gives rise to functional gap junction channels (Maass et al., 2004) leaves open the possibility that Cx43 mediates folliculogenesis via both gap junctional coupling-dependent and -independent mechanisms.

The fact that Cx43-deficient oocytes are developmentally competent sheds light on another aspect of the Cx43 null mutant phenotype: a severe reduction of germ cell numbers. *Gja1*$^{-/-}$ fetuses contain ~10% of the wild-type number of germ cells regardless of strain background (Juneja et al., 1999; Ackert et al., 2001). This deficiency can be seen as early as gestational day 11.5, indicating that it arises early in germline development (Juneja et al., 1999). Our present results make it clear that the deficiency is not due to intrinsic developmental incompetence of the germline, but must arise either from a defect in the process that induces primordial germ cells within the embryonic ectoderm, or through a reduction in their survival and/or motility while migrating to and populating the genital ridges (reviewed by McLaren, 2003).

In a recent report, we demonstrated that Cx37 is the predominant connexin mediating communication between oocytes and granulosa cells as transfer of a gap junction-permeable dye between oocytes and granulosa cells co-cultured for 4 hours was not possible unless Cx37 was present

in both cell types (Veitch et al., 2004). The results presented here demonstrate that, during a prolonged period of development in vivo, folliculogenesis can proceed to completion in the absence of Cx37 in granulosa cells. Wild-type oocytes combined with Cx37-deficient granulosa cells supported complete folliculogenesis, attained a normal size and developed to at least the two-cell stage once fertilized. In contrast, oocytes from $37^{-/-}$oo/$37^{+/+}$gc ovaries failed to support folliculogenesis, were ~25% smaller, meiotically incompetent and could not be fertilized. These two observations demonstrate that oogenesis only requires expression of Cx37 by the oocyte. Age-matched Cx37 knockout ovaries contained follicles that were morphologically identical to those of $37^{-/-}$oo/$37^{+/+}$gc ovaries, reinforcing the finding that sterility in Cx37-deficient female mice is due to connexin defects restricted to the oocytes. These results are consistent with those of Carabatsos et al. (Carabasatos et al., 2000), who reported that Cx37-deficient oocytes and follicles cease growth at 52 μm and 150 μm, respectively. We interpret the discrepancy between the results reported here and those of Veitch et al. (Veitch et al., 2004), indicating a requirement for Cx37 in granulosa cells to maintain oocyte-granulosa cell coupling in a transient co-culture situation, as due to the different designs of the two experiments. Whereas Cx37 may be the connexin that normally couples the two cell types, during the 3 week period of follicle development in vivo in the present study, another connexin was able to replace Cx37 in granulosa cells to re-establish gap junctional communication with the oocyte. The granulosa cells of antral follicles incorporate additional connexins into their gap junctions (reviewed by Kidder and Mhawi, 2002) yet Cx43 is still the most abundant (Wright et al., 2001), making it the prime compensatory candidate. Nevertheless, we were unable to show a change in Cx43 distribution within chimeric follicles suggestive of recruitment to the oocyte/granulosa cell interface. It is worth mentioning that gap junctions between oocytes and granulosa cells are very small relative to those between granulosa cells: gap junctions on the oocyte surface viewed by freeze-fracture electron microscopy ranged in size from 0.05 μm to 0.22 μm (Anderson and Albertini, 1976), whereas granulosa cell gap junctions in our immunofluorescence images ranged in size from 1 to 5 μm, consistent with other published data from freeze-fracture (Kidder and Mhawi, 2002). Thus, it is possible that oocyte-granulosa cell coupling in $37^{+/+}$oo/$37^{-/-}$gc ovaries is maintained by heterotypic channels utilizing Cx43 connexons supplied by the granulosa cells, but the gap junctions were too small to detect. An alternative explanation would be that a different connexin was compensating for the lack of Cx37 in granulosa cells to sustain intercellular communication and follicle development.

Despite the distinct requirements for Cx37 and Cx43 in different compartments of the follicle, both null mutations limit follicle development to an early preantral stage within the first three weeks of postnatal development. A direct comparison of follicle development in Cx37-deficient and Cx43-deficient ovaries of C57BL/6 mice following grafting to the kidney capsule for 24 days (Fig. 3) revealed that oocytes of Cx43 knockout ovaries are significantly larger than those of Cx37 knockout ovaries whereas follicle diameters are the same, owing to an extra layer of granulosa cells around Cx37 knockout oocytes. Oocytes of $43^{+/+}$oo/$43^{-/-}$gc ovaries are also significantly larger than those of $37^{-/-}$oo/$37^{+/+}$gc ovaries. It is well established that gap junctional coupling between oocytes and granulosa cells facilitates nutrient uptake by oocytes, promoting oocyte growth (reviewed by Eppig, 1991). Thus it is not surprising that the block in coupling between oocytes and granulosa cells imposed by the Cx37 null mutation retards oocyte growth. Oocytes can autonomously take up nutrients from the extracellular environment, but those that are coupled to granulosa cells do better, relative to the number of granulosa cell contacts (Eppig, 1991). In contrast to Cx37-deficient ovaries, gap junctional communication between oocytes and granulosa cells persists in the absence of Cx43 (Veitch et al., 2004). For this reason, it is understandable that oocytes from Cx43 knockout ovaries are larger than oocytes from Cx37 knockout ovaries: the granulosa cell layer surrounding the Cx43-deficient oocyte can nourish it via persisting oocyte-granulosa cell gap junctions. Accordingly, the lack of oocyte-granulosa cell coupling in $37^{-/-}$oo/$37^{+/+}$gc ovaries, as assessed by Lucifer Yellow dye transfer, impairs oocyte growth and follicle development. This implies that Cx37 in oocytes is obligatory for intercellular communication between these two compartments, because either it is the only connexin available to meet coupling requirements, or it performs a unique function unmatched by other connexins.

The different phenotypes caused by loss of gap junctions from different compartments of developing follicles reinforce the concept that gap junctions that couple the oocyte with surrounding granulosa cells have a different function from those coupling granulosa cells with each other. In part, the function of oocyte-granulosa cell coupling is to support oocyte growth, regulate meiotic maturation and regulate granulosa cell differentiation (Eppig, 1991), whereas the function of granulosa cell coupling is to optimize the proliferative response to paracrine signals emanating from the oocyte (Gittens et al., 2005). Given that Cx37 and Cx43 gap junction channels have different permeability properties (Elfgang et al., 1995), one might predict that different signals pass between cells of different compartments of the developing follicle. Confirmation of this hypothesis must come from identification of the molecules passing through those gap junctions.

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