Patch-clamp study reveals that the importance of connexin43-mediated gap junctional communication for ovarian folliculogenesis is strain specific in the mouse

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Tong, Dan, Joanne E. I. Gittens, Gerald M. Kidder, and Donglin Bai. 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, 2006. First published August 31, 2005; doi:10.1152/ajpcell.00297.2005.—Genetic ablation of connexin37 (Cx37) or connexin43 (Cx43), the two gap junction proteins expressed by mouse ovarian granulosa cells, has been shown to result in impaired follicle development. We used patch-clamp techniques to evaluate quantitatively the contribution of these connexins to gap junctional intercellular communication (GJIC) among granulosa cells. The coupling conductance derived from a voltage step-induced capacitive current transient was used as a measure of GJIC in cultured granulosa cells. Using this method, we determined that the conductance of wild-type (84.1 ± 28.6 nS; n = 6) and Cx37-deficient granulosa cells (83.7 ± 6.4 nS; n = 11) does not differ significantly (P = 0.35), suggesting a limited contribution, if any, of Cx37 to granulosa cell coupling. In contrast, the conductance between granulosa cells of Cx43-deficient mice (2.6 ± 0.8 nS; n = 5) was not significantly different from that of single, isolated wild-type granulosa cells (2.5 ± 0.7 nS, n = 5; P = 0.83), indicating that Cx43-deficient granulosa cells were not electrically coupled. A direct measurement of transjunctional conductance between isolated granulosa cell pairs using a dual patch-clamp technique confirmed this conclusion. Interestingly, a partial rescue of folliculogenesis was observed when the Cx43-null mutation in C57BL/6 mice was crossed into the CD1 strain, and capacitive current measurement demonstrated that this rescue was not due to reestablishment of GJIC. These results demonstrate that folliculogenesis is impaired in the absence of GJIC between granulosa cells, but they also indicate that the severity is dependent on genetic background, a phenomenon that cannot be attributed to the expression of additional connexins.

ovarian follicle; oogenesis; connexin37; intercellular communication

GAP JUNCTIONS ARE CLUSTERS of cell-cell channels that enable neighboring cells to exchange small signaling molecules (less than ~1 kDa) and to synchronize electrical activities (14). Each gap junction channel consists of two multimeric subunits called hemichannels or connexons that reside in the plasma membranes of two closely apposed cells. A gap junction channel is formed when two hemichannels dock with each other through their extracellular domains. Each hemichannel is composed of six transmembrane proteins called connexins (Cx). To date, 19 and 20 connexin genes have been found in the mouse and human genomes, respectively (23). These connexin genes are expressed in different cell types, although many cells express multiple connexins.

The ovarian follicle provides a good example of a multicellular unit that exhibits expression of multiple connexins and is considered to be reliant on gap junctional intercellular communication (GJIC) for proper development (16). GJIC between oocytes and granulosa cells, as well as between granulosa cells, is critical for oocyte growth and follicular development as revealed by studies of connexin-knockout mice (1, 20). As the most abundant connexin in the follicle, Cx43 is expressed in granulosa cells as early as postnatal day 1, when follicles start to form (15). As follicles grow and reach maturity, the expanding granulosa cell population continues to express Cx43. In contrast, Cx37 is localized mainly at the interface between the oocyte and the surrounding granulosa cells from the primary follicle stage onward (20). However, Cx37 mRNA and protein are also detected within the granulosa cell population (22, 24).

The specific roles of Cx37 and Cx43 in oogenesis and folliculogenesis have been elucidated by experiments with the respective knockout lines. In C57BL/6 Cx37-knockout mice (Gja4−/−), folliculogenesis is disrupted approximately at the late preantral stage (20). Dye coupling between oocytes and granulosa cells is abolished but that between granulosa cells is maintained, indicating that Cx37 serves only to couple oocytes with granulosa cells. In contrast, in mice of the same strain lacking Cx43 (Gja1−/−), folliculogenesis proceeds only to the primary stage (1). Dye coupling between granulosa cells is absent, but that between oocytes and granulosa cells is maintained, indicating that the role of Cx43 is restricted to coupling between granulosa cells in early stages of folliculogenesis (12, 22). In the absence of either Cx37 or Cx43, oocyte development stops before competence for meiotic maturation is achieved (1, 5).

Despite the growing body of information derived from dye transfer experiments regarding the roles of Cx37 in oocyte-granulosa cell coupling and Cx43 in granulosa cell-to-granulosa cell coupling, little is known about the contributions of these gap junction channels to electrical coupling between granulosa cells. Furthermore, given that Cx37 was detected in granulosa cells (22, 24), it is possible that Cx37 may still play some role in mediating communication between them. Therefore, in the present study, we examined quantitatively the contributions of Cx37 and Cx43 to granulosa cell coupling using patch-clamp techniques. The results confirmed the re-
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spective contributions of these connexins to GJIC within the different compartments of the follicle. In addition, we used the same techniques to explore the previously reported effect of strain background on folliculogenesis in Cx43-knockout ovaries (1). In contrast to the primary follicle arrest observed in C57BL/6 mice, when the Gja1-null mutation was bred into the CD1 background, advanced stages of follicular development were observed, albeit in reduced numbers compared with CD1 wild-type ovaries. We hypothesized that a reestablishment of GJIC between granulosa cells via other connexins, such as Cx37, Cx45, or Cx32, all of which are reported to be present in granulosa cells in the later stages of folliculogenesis (16), could compensate for the loss of Cx43 and thus partially rescue folliculogenesis in CD1 ovaries. To test this hypothesis, single and double patch-clamp methods were used to measure GJIC quantitatively between granulosa cells of antral follicles of wild-type and Cx43-deficient CD1 ovaries. Surprisingly, our results have revealed that the partial rescue of folliculogenesis in CD1 strain Cx43-null mutant ovaries does not involve the reestablishment of GJIC.

MATERIALS AND METHODS

Animals. All animal experiments were approved by and conducted under the guidelines of the Department of Animal Care and Veterinary Services at The University of Western Ontario. Gja4<sup>−/−</sup> (C57BL/6 strain lacking Cx37) and Gja1<sup>−/−</sup> mice (C57BL/6 strain lacking Cx43) were produced by mating heterozygous mice. The Gja4-null mutant line was generously provided by Dr. David Paul (Harvard Medical School, Boston, MA) (20). The Gja1-null mutant line originally was obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently maintained in our own colony. CD1 strain Gja1-null mutant mice were derived by back-crossing Gja1<sup>−/−</sup> males with CD1 strain females (Harlan Sprague Dawley, Indianapolis, IN) for more than 10 generations. Mice were bred and maintained in the John P. Robarts Research Institute Barrier Facility on the campus of the University of Western Ontario. The genotype of the mice was determined by polymerase chain reaction (PCR) as previously described (1, 22). To obtain follicles from mice lacking Cx43 (which die at birth), ovaries from late-gestation fetuses (embryonic days 17 and 18) were grafted into kidney capsules of adult ovarioctomized female hosts as described previously (1, 12). Graft recipients were 18 to 20 g severe combined immunodeficient (SCID) Prkd<sup>−/−</sup>/Prkd<sup>−/−</sup> females obtained from Harlan Sprague Dawley.

Cell culture. Follicles were isolated and cultured as described previously (12, 22). Briefly, 20- to 22-day-old female mice or graft hosts 20–22 days after transplantation were anesthetized with CO<sub>2</sub> and killed by cervical dislocation. The ovaries were removed and placed into culture in Waymouth MB 752/1 medium containing 5% FBS and 100 IU/ml penicillin and 100 µg/ml streptomycin. Supernatant (containing oocytes) was removed, and granulosa cells were then resuspended in culture medium and transferred to 12-mm coverslips to culture for <48 h.

Histology and follicle counts. Ovaries were fixed in Bouin’s fixative for 2 h, embedded in paraffin, and sectioned at a thickness of 5 µm. Sections were stained with hematoxylin and eosin. Follicles were counted on consecutive sections of each ovary. Data were obtained from four to six grafted ovaries in each group (CD1 wild-type, CD1 Cx43-null, C57BL/6 wild-type, and C57BL/6 Cx43-null mice).

Cumulus expansion and evaluation of oocyte maturation. Five IU of pregnant mare serum gonadotropin (National Hormone and Peptide Program) was administered on day 21 of grafting 24 h before the commencement of oocyte maturation in vitro. Grafted ovaries were removed as described above and placed into Waymouth’s MB 752/1 medium plus 5% FBS and 0.23 mM pyruvic acid (sodium salt; Sigma-Aldrich Canada). Follicles were pierced using 30-gauge needles to liberate COCs. Oocytes enclosed by a complete layer of cumulus cells were washed with culture medium and transferred to a 35-mm petri dish containing 3 ng/ml FSH (100 IU Pregnenolone folliculin-β; Organon Canada, Scarborough, ON, Canada) in 3 ml of Waymouth’s medium-5% FBS. Oocytes were matured for 18 h in a 5% CO<sub>2</sub>-5% O<sub>2</sub>-90% N<sub>2</sub> atmosphere at 37°C and stained with Hoechst 33342 (Molecular Probes, Eugene, OR) diluted 1:1,000 in Waymouth’s medium-5% FBS to evaluate oocyte maturation.

Current transient measurement. Single-electrode whole cell patch-clamp recording was used to measure ovarian granulosa cell membrane capacitance and conductance. This method was developed by de Roos and colleagues (8, 13) and provided a convenient and quantitative estimate of gap junctional conductance between the cell being recorded and its adjacent cells. Briefly, voltage clamping (V<sub>clamp</sub> = −60 mV) was applied to individual granulosa cells from different preparations (single cells, small clusters of cells, confluent cells, and whole follicles) (Fig. 1A). A depolarization voltage pulse (10 mV, 120-ms duration) was used to generate a transient capacitative current. The peak current (I<sub>peak</sub>) and the steady-state current (I<sub>ss</sub>) were measured. Currents were high-cut filtered at 10 kHz and digitized at 100 kHz. Data acquisition and analysis were performed using a Digidata 1200A interface and pClamp6 software (Axon Instruments, Union City, CA). The estimated conductance (G) between the patched cell and its surrounding cells was calculated using the following equation (13):

\[ G = \frac{I_s}{V_p} \times \frac{I_{peak}}{I_{peak} - I_s} \]

where G<sub>series</sub> = I<sub>peak</sub>/V<sub>p</sub> is the series conductance between the patch pipette and the patched cell. It is noted that the estimated conductance (G) is an underestimation of actual conductance (13). Pipettes were made from borosilicate glass capillaries using a two-stage pipette puller (PP-83; Narishige, Tokyo, Japan). The intracellular pipette solution contained (in mM) 70 KCl, 70 CsCl, 2 EGTA, 4 MgCl<sub>2</sub>, 5 HEPES, pH 7.3, and pipettes had a resistance of 3–5 MΩ. Cells grown on 12-mm coverslips were transferred to a 2-ml recording chamber mounted on the stage of an inverted microscope (Olympus IMT-2). They were bathed in solution containing (in mM) 140 NaCl, 5.4 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, and 20 HEPES, pH 7.4. In experiments performed to test gap junction blockers, the cells in the chamber were under constant perfusion at a rate of 4 ml/min. The estimated solution exchange time was ~30 s.

Double whole cell patch-clamp recording technique. Isolated granulosa cell pairs were selected for double patch-clamp whole cell recordings with each connected to an amplifier (Axopatch 200B; Axon Instruments). At the end of each experiment, we often mechanically separated the assumed pair into component cells by moving the recording pipettes away from each other. The electrical coupling of paired cells was always eliminated, indicating that the coupling had been established at cell-cell junctions. Voltage-clamp protocol.
generation and data acquisition were controlled by a computer using an analog-to-digital converter interface (Axon Instruments). Currents were low-pass filtered at 2 kHz and recorded at a sampling frequency of 5 kHz.

Voltage clamping was used to determine macroscopic transjunctional conductance \((G_j)\) between paired cells. Initially, the holding potentials for cell 1, \(V_1\), and cell 2, \(V_2\), were both set at 0 mV. \(V_1\) was then stepped to −20 mV to establish a transjunctional voltage \((V_j)\). The test pulse duration was 5 s with an interpulse interval of 15 s. Then the transjunctional current \((I_j)\) was measured in cell 2. The patch pipette’s tip size and the internal solution were identical to those used for single-cell patch-clamp recording as described above. \(G_j\) was calculated using the following equation:

\[
G_j = \frac{I_j}{V_j}
\]

Most of the data are expressed as means ± SE. A paired Student’s t-test, unpaired t-test, and one-way ANOVA were used to test statistical significance (*\(P<0.05\), **\(P<0.01\), and ***\(P<0.001\)). Concentration-inhibition relationships and IC50 were determined using Prism software (GraphPad Software, San Diego, CA).

**RESULTS**

Capacitive current transient provides a quantitative measure of electrical coupling between granulosa cells. To extend our previous work on GJIC between granulosa cells (12, 22), we wanted to use a more sensitive method that would allow for accurate quantitative measurements. We adopted a well-developed method to measure various parameters of transient capacitive current (8, 13). A 10-mV depolarizing voltage pulse in a voltage-clamped single isolated granulosa cell resulted in a current transient characterized by a rapid onset to reach \(I_{peak}\), followed by a rapid decay to a \(I_{ss}\) that was almost identical to the holding current. With increasing granulosa cell numbers in a cluster, the decay phase of the capacitive current was prolonged. In addition, the \(I_{ss}\) amplitude also increased (Fig. 1B). Recordings obtained from a granulosa cell on a confluent coverslip or within a follicle revealed a substantially increased \(I_{ss}\) with varied decay time (Fig. 1B). The changes in decay time constant and the steady-state current in a cluster of interconnected cells have been shown to be due to gap junctional coupling between these cells (8, 13). The estimated conductance increased with the increasing size of cell clusters. The conductance recorded from a single, isolated cell \((2.3 ± 0.8 \text{nS}; n = 12)\) was significantly and substantially less than that obtained from a granulosa cell in a cluster of 20–40 cells \((13.3 ± 4.1 \text{nS}; n = 14; P = 0.015)\). With cell cluster size continuously increasing in confluent and whole preantral follicles, the conductance increased to even higher levels \((48.9 ± 7.0 \text{nS}; n = 10, \text{and } 55.1 ± 8.5 \text{nS}; n = 10, \text{respectively})\).

The data from C57BL/6 and CD1 granulosa cells were statistically not different \((P = 0.36)\) and showed the same trend in that the conductance increased with the increase in the number of cells in a cluster (data not shown). These results indicate that the capacitive current transient is a reliable estimate for electrical conductance between the patched granulosa cell and the surrounding cells.

**Gap junction channel blockers inhibit the coupling between granulosa cells.** To confirm that the recorded coupling between granulosa cells was mediated by gap junctions, the effects of the gap junction channel blockers flufenamic acid (FFA), heptanol, carbamoxolone (CBX), and mefloquine (MFQ) were tested on the evoked capacitive current. The calculated conductance was rapidly and reversibly reduced to a level identical to that obtained from single cells when FFA (50 \(\mu\text{M}\)) was perfused into confluent ovarian granulosa cells (Fig. 2, A and B), indicating that FFA treatment resulted in a complete func-
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Fig. 2. Gap junction blockers inhibit the capacitive current response. A: effect of 50 μM flufenamic acid (FFA) on the capacitive current of confluent monolayers of ovarian granulosa cells. B: time course was plotted by calculating the value of estimated conductance from each capacitive current at each time point. Addition of 50 μM FFA is indicated by bar. C: effect of 50 μM FFA, 50 μM heptanol, and 50 and 200 μM carbenoxolone (CBX) on the conductance of confluent granulosa cells (remaining % of control conductance was 2.1 ± 0.5%, 2.3 ± 0.2%, 68.3 ± 6.5%, and 8.9 ± 1.0% respectively). Number of experiments performed is shown above each condition in parentheses. D: concentration-inhibition curve showing the effect of mefloquine (MFQ) on the estimated coupling conductance of granulosa cells. Each point represents the mean ± SE of conductance values (normalized to initial conductance) obtained from 5 cells. Estimated IC50 was 5.2 ± 1.3 μM.

Optional uncoupling of granulosa cell gap junctions. A similar reduction in the conductance was observed after perfusion of heptanol (50 μM) and CBX (50 and 200 μM) (Fig. 2C). A concentration inhibition curve was constructed to show the actions of MFQ on the estimated coupling conductance (Fig. 2D). The estimated IC50 was 5.2 ± 1.3 μM, a value close to that reported for Cx43 gap junction channels in N2A cell pairs transfected with rat Cx43 (7). However, application of gap junction blockers to isolated single granulosa cells did not result in a significant effect on the evoked capacitive transients and the conductance (data not shown), indicating that the observed blocking effects were exerted on the gap junctional conductance between cells and not on the nonjunctional membrane conductance.

Cx43, but not Cx37, contributes to the coupling between granulosa cells. Both Cx43 and Cx37 are found in granulosa cells, although to date, only a role for coupling with the oocyte has been demonstrated for Cx37 (22, 24). To investigate the relative contributions of Cx43 and Cx37 to electrical coupling between granulosa cells, we studied confluent granulosa cells from Cx43- and Cx37-knockout mice. The granulosa cells obtained from Cx37-deficient C57BL/6 mice showed a capacitive transient response similar to that of the wild-type cells (Fig. 3A), and the conductance also increased with the increase in the number of cells in a cluster (data not shown). The conductance of confluent Cx37-deficient granulosa cells on the same genetic background (83.7 ± 6.4 nS; n = 11) was not statistically different from that of confluent granulosa cells from wild-type C57BL/6 mice (84.1 ± 28.6 nS; n = 6; P = 0.35) (Fig. 3B). Similar blocking effects were observed for the gap junction blockers FFA, CBX, and MFQ (Fig. 3E). These data indicate that Cx37-deficient granulosa cells are still coupled via gap junctions and that the conductance and sensitivity to gap junction channel blockers are not different from those of wild-type granulosa cells. In contrast, the conductance between Cx43-deficient granulosa cells from grafted ovaries of both CD1 (2.6 ± 0.8 nS; n = 5) and C57BL/6 mice (2.3 ± 0.8 nS; n = 5) was significantly lower than that obtained from the corresponding wild-type or Cx37-deficient granulosa cells (P < 0.001 for each case) (Fig. 3, A–C). The conductance between confluent Cx43-deficient granulosa cells was the same as the conductance obtained from single, isolated wild-type or Cx43-deficient granulosa cells (Fig. 3D), indicating that Cx43-deficient granulosa cells are not electrically coupled in the early stages (preantral) of folliculogenesis in either strain. To demonstrate electrical coupling between granulosa cells directly, we used the traditional double patch-clamp method applied to isolated granulosa cell pairs. Figure 4A shows Ij recorded from one cell in response to a voltage step of −20 mV applied to the other cell in the pair. The Gj values of the wild-type granulosa cell pairs were 17.3 ± 4.3 nS and 13.8 ± 3.7 nS in C57BL/6 and CD1 mice, respectively (n = 6 and 7, respectively), whereas the Gj values of the Cx37-deficient granulosa cell pairs were 15.1 ± 3.9 nS (n = 6; P = 0.71 vs. WT C57BL/6) (Fig. 4B). However, in Cx43-deficient cell pairs, no transjunctional current flow was detected in either strain (n = 10 for each strain).

Partial rescue of folliculogenesis in Cx43-null ovaries by crossing the mutation into the CD1 background. In grafted ovaries of Cx43-knockout (Gja1−/−) C57BL/6 mice, folliculogenesis arrests in the primary or early secondary stage and oocytes fail to acquire competence for meiotic maturation (Fig. 5) (1). However, when the Gja1-null allele was bred into the CD1 background, the follicles of all developmental stages were detected in the ovaries after 21–22 days of development in kidney grafts (see Ref. 1 and Fig. 5A), although the number of follicles that developed to antral stages was reduced compared with those in wild-type littermates (Fig. 5B). To assess the meiotic competence of the oocytes from CD1 antral follicles, we examined oocyte maturation in these groups. As summarized in Table 1, 42% of oocytes from Cx43-deficient CD1 antral follicles were able to progress to metaphase II (first polar body), although an equal proportion remained at the intact germinal vesicle stage. This finding is in contrast to the findings in Cx37- or Cx43-deficient oocytes of the C57BL/6 strain, which are meiotically incompetent (1, 5). This result suggests that strain-specific modifier genes, possibly encoding other connexins, were able to compensate partially for the loss of Cx43 in CD1 ovaries.

Partial rescue of folliculogenesis in Cx43-deficient CD1 ovaries is not due to reestablishment of gap junction channels between granulosa cells. To test the hypothesis that intercellular coupling is restored in Cx43-deficient antral stage gran-
ulosa cells of CD1 mouse follicles, we obtained conductance measurements using the capacitive transient method. In wild-type CD1 granulosa cells, there was a trend toward increasing conductance from the preantral stage (41.3 ± 7.6 nS; n = 16) to the antral stage (54.5 ± 10.1 nS; n = 15), but the difference was not significant (P > 0.05; one-way ANOVA). More importantly, in both preantral and antral stages, the conductance measured in CD1 Cx43-deficient whole follicles was close to that obtained from single wild-type granulosa cells (P = 0.8 and 0.4, respectively), indicating a complete lack of coupling in both stages when Cx43 is absent. This result was confirmed using double patch-clamp measurement applied to isolated granulosa cell pairs (n = 8 and 10 for preantral and antral stages, respectively; Fig. 6B). Thus Cx43 is solely responsible for providing intercellular coupling in both preantral and antral stages, and rescue of folliculogenesis in Cx43-deficient CD1 ovaries is not due to restoration of GJIC by other connexins forming functional gap junction channels.

DISCUSSION

Studies have demonstrated that the penetrance and/or expressivity of a given mutation can be genetic background dependent, suggesting the interaction of modifier genes (18, 21). The phenotypic differences could be qualitative or quantitative. In our study, the more extensive folliculogenesis in Cx43-deficient ovaries of the CD1 strain compared with C57BL/6 inspired us to compare gap junctional coupling levels quantitatively between granulosa cells of the two mutant

Fig. 3. Connexin43 (Cx43), but not connexin37 (Cx37), contributes to the coupling between granulosa cells. A: capacitive current response of confluent granulosa cells isolated from wild-type, Cx37-deficient (Cx37-KO), and Cx43-deficient (Cx43-KO) C57BL/6 mouse ovaries. Dotted line shows the initial holding current. B: calculated conductance of confluent granulosa cells isolated from wild-type, Cx37-deficient, and Cx43-deficient C57BL/6 mouse ovaries. Number of experiments for each condition is indicated in parentheses above bars. **P < 0.001 vs. control. C: calculated conductance of confluent granulosa cells isolated from wild-type and Cx43-knockout CD1 mice. Number of experiments for each condition is indicated in parentheses above bars. ***P < 0.001 vs. control. D: conductance values of single wild-type granulosa cells and different preparations of Cx43-deficient granulosa cells (single cells, confluent cells, and whole follicles). Number of experiments for each condition is indicated in parentheses above bars. P > 0.05; one-way ANOVA. E: effect of gap junction blockers 50 μM FFA, 200 μM CBX, and 30 μM MFQ on the conductance of confluent wild-type and Cx37-deficient ovarian granulosa cells (P = 0.36, 0.91, and 0.71, respectively). Each bar represents the mean ± SE of 5 or 6 cells. Dashed line indicates control level.

Fig. 4. Transjunctional current (Ij) and conductance (Gj) measured using dual patch-clamp recording in isolated granulosa cell pairs from Cx43- and Cx37-deficient ovaries. A: Ij traces recorded from cell 2 of isolated granulosa cell pairs after −20-mV test voltage of 5-s duration was applied to cell 1. B: Gj measured in isolated granulosa cell pairs from control, Cx37-deficient (Cx37-KO), and Cx43-deficient (Cx43-KO) C57BL/6 mice. Number of experiments for each condition is indicated in parentheses above bars. **P < 0.001 vs. control. C: Gj measured in isolated granulosa cell pairs from control and Cx43-deficient (Cx43-KO) CD1 mice. Number of experiments for each condition is indicated in parentheses above bars. ***P < 0.001 vs. control.
strains. Given that multiple connexins are expressed in mouse granulosa cells both before and after antrum formation (16, 22), patch-clamp methods were used to evaluate the specific roles of individual connexins.

Electrical coupling between cells can be measured using a double patch-clamp method, but this technique is not suitable for multicellular preparations because of the voltage-clamp escape. Furthermore, previous studies have shown that gap junctions can be internalized when cells are isolated, leading to a reduction in conductance between isolated cell pairs that may not reflect the actual conductance in vivo (3, 17). Therefore, we used a single patch-clamp method introduced by de Roos and colleagues (8, 13) to measure the current transient evoked in clusters of cells by a small voltage step. The basic principle of this assay is that if cells are electrically coupled, the total membrane surface and thus the capacitance and conductance will increase with increasing numbers of cells in a cluster. In our experiments, the responses observed in granulosa cells isolated from both strains were similar to those reported in normal rat kidney fibroblasts and Cx43-transfected human hepatoma SKHep1 cells (8, 13), and the conductance increased with cell numbers, indicating that granulosa cells are electrically coupled. The results also demonstrate that this method can be used to measure coupling between granulosa cells.

The effect of gap junction blockers was also assessed using this method to confirm the gap junction channel dependence of the measured current transients. Because to date no single drug has been found to be specific for gap junction channels, previously reported gap junction blockers from four different families, FFA, heptanol, CBX, and MFQ, were used. All of the blockers reduced the transient response and conductance of cell clusters to the single-cell level, demonstrating a complete uncoupling of cells. Furthermore, some gap junction uncoupling drugs such as MFQ displayed selectivity to gap junction channels composed of different connexins (7). The sensitivity of ovarian granulosa cells to different gap junction channel blockers is consistent with previous pharmacological studies of Cx43 gap junctions (2, 13). For MFQ, our IC50 value was close to that reported using a double patch-clamp technique in N2A cells transfected with rat Cx43 (7). These pharmacological similarities, as well as the fact that the capacitive current dropped to the single-cell level in the absence of Cx43, confirm that gap junctional communication between granulosa cells is mediated exclusively by connexin43 as previously indicated in studies in which investigators used other, less sensitive methods (12). This conclusion applies to both genetic backgrounds studied. In contrast, the absence of Cx37 did not change the coupling of granulosa cells and their sensitivity to gap junction blockers.

Table 1. *In vitro maturation of oocytes isolated from antral follicles (CD1 strain)*

<table>
<thead>
<tr>
<th>Stage</th>
<th>Wild type (n = 32)</th>
<th>Cx43 deficient (n = 31)</th>
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<tbody>
<tr>
<td>GV</td>
<td>3</td>
<td>42</td>
</tr>
<tr>
<td>MI</td>
<td>6</td>
<td>16</td>
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<tr>
<td>MII</td>
<td>91</td>
<td>42</td>
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Cx43, connexin43; GV, germinal vesicle intact; MI, metaphase I; MII, metaphase II (presence of first polar body).

Fig. 5. Partial rescue of folliculogenesis in Cx43-deficient C57BL/6 mouse ovaries by back-crossing into the CD1 strain. Neonatal ovaries were grafted into the kidney capsules of adult females for 3 wk. A: follicles of all developmental stages were observed in wild-type (WT) ovaries (left). Follicles of only the primary (unilaminar) stage were observed in C57BL/6 strain ovaries lacking Cx43 (Cx43-KO; middle). Follicles of all developmental stages were also detected in CD1 strain Cx43-deficient ovaries (Cx43-KO; right), although the proportion of antral follicles was reduced compared with wild-type ovaries. Scale bar, 50 μm. B: distribution of follicle stages in Cx43-deficient (Cx43-KO) ovaries compared with that of wild-type ovaries for both strains. A total of 115–149 follicles from 4–6 grafted ovaries were categorized for each genotype.
deficient follicles. Detected in isolated granulosa cell pairs from both preantral and antral Cx43 among the granulosa cell population to maximize their re-

of Cx43 may be to propagate signals downstream of GDF9 of growing oocytes, stimulates granulosa cells to proliferate, an

difference. One obvious possibility is that paracrine signaling than gap junctional communication is responsible for the strain difference caused by mutations in the GJA1 gene encoding Cx43 (19). Recently, a mouse model of ODDD was generated in a mutagenesis screen for dominant mutations affecting morphogenesis (10). The mutant mice carry a single nucleotide substitution in Gja1 (designated Gja1<sup>ΔN</sup>), causing serine to replace glycine at residue 60, and exhibit many of the symptoms of ODDD, including syndactyly, enamel hypoplasia, craniofacial anomalies, and cardiac dysfunction. Gap junctional coupling among granulosa cells of the mutant females is reduced to ~10% of the wild-type level, yet the females are not infertile. Histological analysis of the ovaries revealed a reduction in the proportion of advanced follicle stages compared with wild-type littermates (Colley D, Barr KJ, and Kidder GM, unpublished results), however, similar to the situation in Gja1<sup>ΔN</sup> null mutant ovaries on the CD1 background. Thus the Gja1<sup>ΔN</sup>-mutant females substantiate our finding that oogenesis can proceed to completion, apparently with fewer mature oocytes being produced, despite severe impairment of gap junctional coupling among granulosa cells. Further insight into the roles of Cx43 in human physiology will undoubtedly come to light on the basis of careful pathophysiological comparisons, including measures of female fertility, of patients with ODDD.

In summary, for the first time, we have used the patch-clamp technique to evaluate gap junctional coupling between ovarian granulosa cells. Our study has revealed that granulosa cells are electrically coupled via gap junctions and that Cx43 is essential for this intercellular communication in all stages of follicular development. Folliculogenesis is impaired in the absence of communication between granulosa cells. However, the severity is dependent on genetic background, a phenomenon that cannot be attributed to the presence of other connexins.

Furthermore, the consistency of the results obtained by measurement of capacitive current and dual patch-clamp recording indicates that the former method is a useful and reliable method for the functional analysis of gap junctions, especially in multicellular preparations and for pharmacological studies.

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GRANTS
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