Interplay between paracrine signaling and gap junctional communication in ovarian follicles

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Summary

Intercellular communication is required for ovarian folliculogenesis. This is apparent in mice lacking connexin43 (Cx43, a gap junction protein strongly expressed in granulosa cells), or growth/differentiation factor-9 (GDF9, an oocyte-specific growth factor that stimulates granulosa cell proliferation and differentiation), or in mice expressing a mutant form of Kit ligand (KITL, a paracrine factor that, in the ovary, is secreted by granulosa cells to stimulate oocyte growth). In all of these mutant lines, follicle growth is impaired suggesting a possible interaction between paracrine signaling and gap junctional communication. To assess this possibility, we analyzed gene expression in mutant ovaries. Despite the lack of gap junctional coupling between granulosa cells of Cx43 null mutant ovaries, expression of the genes encoding KITL and its receptor, KIT, is maintained. Furthermore, GDF9 expression is maintained. In GDF9 null mutant ovaries, there is no apparent change in Cx43 expression and, correspondingly, the granulosa cells remain coupled. There is also no increase in granulosa cell apoptosis in ovaries lacking Cx43 or GDF9. Staining for proliferating cell nuclear antigen (PCNA) revealed that the granulosa cells of Cx43 null mutant ovaries have a reduced frequency of DNA synthesis. Using both radiolabeled thymidine incorporation and PCNA staining in vitro, we showed that recombinant GDF9 could restore the proliferation of coupling-deficient granulosa cells to the level of control cells. These results indicate that impaired folliculogenesis in mice lacking Cx43 is due at least in part to reduced responsiveness of granulosa cells to oocyte-derived GDF9, indicating an interaction between these two modes of intercellular communication.

Key words: Gap junctions, Connexin43, Ovarian follicle, Granulosa cell, GDF9, Kit ligand

Introduction

Ovarian folliculogenesis requires complex regulatory mechanisms involving both extrinsic (endocrine) and intrinsic signaling pathways. In the latter case, numerous peptides, which are members of several growth factor families, have been implicated as influencing follicle growth and maturation through paracrine or autocrine signaling (reviewed by Udoff and Adashi, 1999). Analysis of knockout mice has highlighted the roles of two particular paracrine factors, KITL (Kit ligand) and GDF9 (growth/differentiation factor-9), in folliculogenesis. KITL (also known as mast cell growth factor, MGF, stem cell factor, SCF and Steel factor, SF) is produced by granulosa cells surrounding growing oocytes whereas its receptor, KIT, is expressed on the surface of those oocytes; in both cell types, expression can be detected at least from the primordial follicle stage onward (Manova et al., 1990; Manova et al., 1993). The *Steel-panda* allele ($Kitl^{Sl\text{-}pan}$) at the locus encoding KITL contains a DNA rearrangement upstream of the coding region that severely reduces expression (Huang et al., 1993; Bedell et al., 1995). In $Kitl^{Sl\text{-}pan}/Kitl^{Sl\text{-}pan}$ female mice, postnatal folliculogenesis arrests in the primary follicle stage and the oocytes do not grow above 22 μm in diameter (Huang et al., 1993). GDF9 is a TGFβ family member that, within the mouse ovary, is expressed exclusively in growing oocytes at least from the primary follicle stage onward (McGrath et al., 1995; Fitzpatrick et al., 1998; Elvin et al., 1999a). In vitro studies have shown that GDF9 promotes both proliferation and differentiation of granulosa cells (Hayashi et al., 1999; Elvin et al., 1999a). Female mice lacking GDF9 are sterile, as folliculogenesis arrests in the primary follicle stage (Dong et al., 1996). GDF9 null mutant oocytes grow at an accelerated rate that is correlated with increased expression of KITL by the granulosa cells (Elvin et al., 1999b). Thus, the GDF9 and KITL signaling pathways interact to control oocyte growth. Despite their faster growth, GDF9 null mutant oocytes are morphologically abnormal, exhibit reduced meiotic competence and eventually degenerate (Dong et al., 1996; Carabatsos et al., 1998).

Another intraovarian communication pathway: that mediated by gap junctions, has recently come under scrutiny. Gap junctions occur at sites of close cell apposition; they are arrays of intercellular membrane channels that allow inorganic ions, second messengers and small metabolites (less that about 1000 Da) to pass from cell to cell (reviewed by Harris, 2001). The fundamental unit of the gap junction is the connexon, a cylindrical organelle that forms a hemichannel in the plasma

membrane. The end-to-end docking of connexons from two adjacent cells creates an intercellular channel. Each connexon is a hexamer of protein subunits, members of a family of homologous proteins called connexins (Cx). Several connexins are expressed in ovarian follicles and two in particular, Cx37 and Cx43, have been demonstrated to play essential roles in folliculogenesis (Veitch et al., 2004) (reviewed by Kidder and Mhawi, 2002). Cx37 is localized at the interface between the oocyte and surrounding granulosa cells from the primary follicle stage onward (Simon et al., 1997). In C57BL/6 knockout mice lacking Cx37, ovarian folliculogenesis proceeds in apparently normal fashion until a late preantral stage. Mature Graafian follicles never develop, however, and oocyte growth arrests at 74% of normal size (Simon et al., 1997; Carabatsos et al., 2000). Eventually, the mutant ovaries become filled with structures resembling corpora lutea, as though the granulosa cells had differentiated prematurely as luteal cells (Simon et al., 1997). In contrast, in mice of the same strain lacking Cx43, folliculogenesis proceeds only to the unilaminar stage (Ackert et al., 2001). Cx43 is expressed in the granulosa cells throughout folliculogenesis (Juneja et al., 1999) and its absence results in complete ablation of gap junctional coupling (Gittens et al., 2003). The failure of Cx43-deficient follicles to develop multiple layers of granulosa cells is correlated with reduced growth of the oocytes, which are morphologically abnormal, meiotically incompetent and cannot be fertilized (Ackert et al., 2001). Thus, loss of gap junctional coupling from the granulosa cells has severe consequences for the growing oocyte.

The fact that loss of signaling via KITL, GDF9 and Cx43 gap junctions all cause folliculogenesis to arrest in the primary follicle stage in C57BL/6 mice suggests that paracrine and gap junctional signaling pathways may interact within the developing follicle: paracrine factor expression might depend on gap junctional coupling (or vice-versa), or gap junctional coupling might mediate the effects of paracrine action. To explore these possibilities, we have analyzed the expression of KITL, KIT and GDF9 in Cx43 null mutant ovaries as well as Cx43 expression and gap junctional coupling in GDF9 null mutant ovaries. The results demonstrate that gap junctional coupling among the granulosa cells is not required to sustain expression of these paracrine factors and that GDF9 is not required to sustain gap junctional coupling among granulosa cells. On the other hand, our evidence indicates that granulosa cells must be coupled via Cx43 gap junctions in order to respond optimally to GDF9.

Materials and Methods

Experimental animals

All mice were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Offspring lacking Cx43 were obtained by mating heterozygous (*Gja1*$^+$/*Gja1*$^-$) mice, maintained on either the CD1 or C57BL/6 genetic background. As with our previous studies with this mutant strain (Ackert et al., 2001), fetal ovaries were grafted into the kidney capsules of adult female hosts to avoid the neonatal lethality associated with the loss of Cx43 (Reaume et al., 1995). Follicular development proceeds at the normal rate in ovaries grafted into the kidney capsule, and the oocytes derived from such follicles can be fertilized and will develop into viable, fertile mice, confirming that this procedure can be used to explore normal processes of folliculogenesis (Eppig and Wigglesworth, 2000). On day 17.5 (CD1 background) or 18.5 (C57BL/6 background) of gestation, pregnant dams were killed by cervical dislocation following CO_2 anesthesia. Fetuses were dissected from the uteri and decapitated. Ovaries were removed, freed of the ovarian bursa and cultured for 24-48 hours in culture medium (Waymouth medium plus 10% fetal bovine serum) while genotypes were being determined by PCR as previously described (Ackert et al., 2001). Mice used as graft recipients were 20-22 g *Prkdc*^{scid}/*Prkdc*^{scid} females (C.B-17/IcrHsd-*scid*) purchased from Harlan Sprague-Dawley (Indianapolis, IN). The ovaries of either a wild-type or a homozygous mutant donor were transplanted to the right kidney capsule of an ovariectomized recipient as previously described (Ackert et al., 2001). In brief, host ovaries were removed through small incisions on both the left and right dorsolateral surfaces caudal to the last ribs. The right kidney was brought to the surface through the right incision, a small hole was made in its capsule, and a pair of ovaries was inserted into the hole beneath the capsule. The body wall was sutured and the skin was closed with wound clips.

GDF9-deficient mice were obtained by mating heterozygous (*Gdf9*$^+$/*Gdf9*$^-$) and homozygous (*Gdf9*$^-$/*Gdf9*$^-$) C57BL/6 mice. Ovaries were recovered from offspring killed by cervical dislocation 20 days, 40 days or 50 days post parturition. The genotypes of the ovary donors were determined by PCR applied to proteinase K-digested tail snips. The primer pair, designed to amplify a 369 bp fragment of the *Gdf9*$^+$ allele, was 5′-TCCTTCAACCTCAGCGA-ATA-3′ and 5′-GCCCCCATGCTAACGAC-3′. A second set of primers was used as a positive control for each PCR; these primers were designed to amplify a 206 bp segment of the T-cell receptor gene. The sequences were 5′-CAAATGTTGCTTGTCTGGTG-3′ and 5′-GTCAGTCGAGTGCACAGTTT-3′. The PCR mixture consisted of 1× PCR buffer (Invitrogen Canada, Burlington, ON), 2 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTP (Invitrogen), 5′ and 3′ T-cell receptor primers (1 μM each), 0.125 U Platinum *Taq* DNA polymerase (Invitrogen), 1 μl tail digestate and 5′ and 3′ primers for the *Gdf9*$^+$ allele (1 μM each) in a total volume of 10 μl. PCR commenced with a 3 minute incubation at 94°C, followed by cycles of 94°C for 20 seconds, 56°C for 30 seconds and 72°C for 35 seconds until the 45th cycle was reached. The final step was a 7 minute extension at 72°C.

Semi-quantitative RT-PCR

The levels of mRNAs encoding KITL, KIT and GDF9 were compared between the ovaries of wild-type and mutant fetuses (17.5 dpc) using the semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) method (Davies et al., 1996). Briefly, the optimal number of PCR cycles avoiding plateau was determined in advance for each primer pair using wild-type cDNA as template. Subsequently, the reactions were run at the optimum number of cycles for each primer pair using serial twofold dilutions of template, comparing mutant and wild-type samples. The consistency between amplicon ratios for several template inputs of a given sample gave assurance that the reactions were not reaching plateau, and these data were averaged to determine the final amplicon ratio for each sample. Primers are listed in Table 1. The KITL primers were designed to amplify both of the two alternate transcripts derived from the *Kitl* gene (Besmer et al., 1993). As an internal standard for comparing mRNA levels between genotypes we used endogenous transcripts encoding adenosine phosphoribosyl transferase (APRT), a housekeeping enzyme that has been shown to remain constant on a per-cell basis in both germ cells and somatic cells of developing ovaries (Monk and McLaren, 1981). RNA was extracted using the Trizol® method (Invitrogen) and was then DNase treated for 15 minutes at room temperature by the addition of 1 U RQ-DNase I (Promega, Madison, WI) in 20 mM Tris-Cl, pH 8.4, 50 mM KCl and 2 mM $MgCl_2$. The reaction was terminated by the addition of EDTA, pH 8, to a final concentration of 2.5 mM followed by heating for 10 minutes at 65°C. 1 μl (0.5 μg) oligo(dT) primer was then added to the RNA and incubated for 10 minutes at

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Table 1. Primers used for RT-PCR

Gene	GenBank accession no.	Primer sequences (upstream/downstream)	Optimal annealing temperature	Amplicon size (bp)	Amplified segment (codons)	Diagnostic restriction enzyme	Fragment sizes (bp)
Aprt	M11310	5′-GTCTTCCCCGACTTCCCAATC-3′ 5′-CCGGGTTCCAAGGCATCTTT-3′	61°C	317	15-119	*Xba*I	171, 146
Kit	Y00864	5′-ACTCGCACGGGCACATACA-3′ 5′-ATGGCTTCCCGCACTTTGAG-3′	60°C	356	91-216	*Dde*I	60, 296
Kitl	S40364, S40534	5′-ATCGCGGGAATCCTGT-3′ 5′-GTCAGATGCCACCATAAAGTC-3′	53°C	396	28-167	*Hae*III	92, 304
Gdf9	L06444	5′-TCCTTCAACCTCAGCGAATA-3′ 5′-GCCCCCATGCTAACGAC-3′	56°C	369	323-445	*Hinc*II	282, 87

70°C. After snap chilling on ice, 200 U Superscript II® reverse transcriptase (Invitrogen) was added to a final reaction volume of 20 µl containing 50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol and the four deoxynucleotide triphosphates (dNTPs, 0.5 mM). The reaction proceeded at 43°C for 1 hour, followed by 10 minutes at 95°C. The cDNA was aliquoted for storage at –80°C. An amount of cDNA corresponding to 0.05 ovary equivalents was used for each PCR reaction. The absence of genomic DNA in each cDNA preparation was confirmed by PCR (35 cycles) using intron-spanning primers for the mouse β-actin gene (De Sousa et al., 1993).

PCRs were performed in 25 µl volumes containing 20 mM Tris-Cl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 200 µM dNTPs and 3 U Platinum™ *Taq* DNA polymerase (Invitrogen). Thermal cycling was conducted with a Perkin Elmer 2400 thermal cycler as follows: initial denaturation for 3 minutes at 95°C followed by 27-35 cycles of denaturation (45 seconds at 95°C), annealing (30 seconds at optimum temperature) and extension (30 seconds at 72°C). The final extension phase was 7 minutes at 72°C. Because of differences in optimal annealing temperatures between the *Kitl* and *Gdf9* primer pairs and the *Aprt* primer pair (Table 1), the *Aprt* amplifications were conducted in separate tubes with the amplicons recombined in equal portions for electrophoretic separation. For the *Kit* measurements, the *Kit* and *Aprt* primers were combined in the same tubes for amplification at 60°C. Amplicons were separated and quantified using a Beckman P/ACE capillary electrophoresis system equipped with a laser-induced fluorescence detector. The two-tailed Student's *t*-test was used to judge the significance of differences between amplicon ratios. Amplicon identities were first checked using diagnostic restriction enzyme digestions and then by direct sequencing of gel-purified amplicons using the PCR primers as sequencing primers.

Follicle isolation

Ovaries were recovered 20-22 days after grafting and transferred to culture medium containing 1 mg/ml collagenase type 1 (Sigma-Aldrich, Oakville, ON). Follicles were liberated following culture for 30-45 minutes at 37°C in an atmosphere of 5% CO_2 in air by repeatedly aspirating and expelling the medium with a 1 ml pipette. Care was taken to ensure that the follicles taken from control ovaries were comparable in size with those from mutant ovaries.

Dye microinjection

Follicles were washed through two dishes of culture medium and then transferred to coverslips in 200 µl drops of medium. They were incubated in a humidified chamber at 37°C in an atmosphere of 5% CO_2 in air for 24 hours to allow the follicles to attach to the glass. The coverslips were transferred to 35×10 mm Petri dishes containing 3 ml culture medium and assayed immediately or incubated for an additional 24 hours. A gap junction-permeant fluorescent dye (2′,7′-dichlorofluorescein, Molecular Probes, Eugene, OR) was backfilled via capillary action through a 1 mm thin-wall glass capillary (World Precision Instruments, Sarasota, FL) pulled to 1 µm. The concentration of 2′,7′-dichlorofluorescein was 7 mg/ml in solution containing 100 mM KCl and 20 mM KH_2PO_4, pH 7.4. One granulosa cell from each follicle was impaled and the dye allowed to diffuse into the cell for 1 minute. After 5 minutes, recipient granulosa cells were counted.

Immunocytochemistry

Except where otherwise noted, all steps were carried out at room temperature. Ovaries were fixed with either Bouin's solution (5 ml glacial acetic acid, 25 ml 40% formalin and 75 ml picric acid) for 2 hours, or 4% paraformaldehyde overnight at 4°C, embedded in paraffin, and sectioned at a thickness of 5 µm. Sections were deparaffinized in xylene and rehydrated in graduated ethanol solutions before blocking with 2% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 1 hour. Sections were then treated with either a monoclonal anti-GDF9 antibody (Kaivo-Oja et al., 2003) or a monoclonal anti-PCNA antibody (from the PC10 hybridoma) (Waseem and Lane, 1990) in 2% BSA for 1 hour at 1:100 and 1:200, respectively. The GDF9 antibody was a gift from Aaron Hsueh, Stanford University, Stanford, CA whereas the PCNA antibody was purchased from Sigma-Aldrich. Slides were washed with PBS and then incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (Caltag Laboratories, Burlingame, CA) diluted 1:2000 in 2% BSA for 1 hour. After further washing in PBS, sections were incubated with TrueBlue™ Peroxidase Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) for 15 minutes. Slides were rinsed in distilled water, dehydrated and mounted with Permount (Fisher Scientific, Nepean, ON). PCNA-positive granulosa cells were counted to determine the mean frequency of PCNA staining. The data were analyzed by one-way analysis of variance followed by Tukey's multiple comparison test.

For Cx43 immunostaining, ovary sections were treated as described previously (Roscoe et al., 2001) using an affinity purified, peptide-specific polyclonal antibody (Mitchell et al., 2003) raised against residues 360-382 of the C-terminal tail of Cx43. This antibody was provided by Stephen Lye, Samuel Lunenfeld Research Institute, Toronto, ON. The secondary antibody was fluorescein-conjugated goat anti-rabbit from ICN Biomedicals, Irvine, CA.

TUNEL assay

Ovaries fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 5 µm 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 (Promega, Madison, WI) according to the manufacturer's instructions. Nuclei were counterstained with 1 µg/ml propidium iodide (Sigma-Aldrich) and sections were mounted with anti-fade medium. To quantify the TUNEL staining in follicles, the total pixel

intensity for a given area of follicles was determined using Northern Eclipse software (Empix Imaging, Mississauga, ON). Owing to the accumulation of TUNEL label at the edge of some sections (which was variable between sections), follicles within 25 μm of the ovary surface were not selected for analysis. The data were analyzed by unpaired, two-tailed *t*-test.

Granulosa cell proliferation assays

Two types of experiment were performed to assess the ability of cultured granulosa cells to proliferate in response to GDF9. In preliminary experiments, wild-type follicles from CD1 strain ovaries were cultured in triplicate wells at a density of 10 follicles per well in 1 ml culture medium containing 0, 25, 50 or 100 ng/ml of recombinant mouse GDF9 (a gift from Martin M. Matzuk, Baylor College of Medicine, Houston, TX) (see Elvin et al., 1999a). The recombinant GDF9 was added to the medium as culture supernatant from transfected CHO cells; control cultures received comparable amounts of culture supernatant from CHO cells transfected with empty vector as described (Joyce et al., 2000). The cultures also contained either 200 μM carbenoxolone (CBX; Sigma-Aldrich), an inhibitor of gap junctional coupling or 200 μM glycyrrhizic acid (GZA; Sigma-Aldrich), an inactive analogue of carbenoxolone (Davidson and Baumgarten, 1988). The appropriate concentration of carbenoxolone for use with granulosa cells was determined in preliminary experiments in which cells were treated with a range of concentrations between 50 and 300 μM; 200 μM was the lowest concentration giving complete blockage of Lucifer Yellow dye transfer. Following 24 hours of culture with these compounds, fresh medium containing the drug and 1 μCi/ml [^{3}H]methyl thymidine (ICN Biomedicals, Montreal, QC) was added. Follicles were cultured for an additional 24 hours and the follicle diameters were measured using an ocular micrometer. They were then gently washed three times in phosphate-buffered saline (PBS) and extracted with 1 ml of 5% trichloroacetic acid (TCA) on ice for 20 minutes. The TCA-soluble fractions were transferred to scintillation vials to measure the intracellular unincorporated pool of [^{3}H]methyl thymidine. The wells were then washed twice with cold 100% ethanol and TCA-insoluble macromolecules were dissolved in 0.1 M NaOH/2% Na_2CO_3 overnight at room temperature. 360 μl of 1 M HCl was used to neutralize every 1 ml of 0.1 M NaOH/2% Na_2CO_3 solution, and 1 ml neutralized solution was counted to determine the intracellular incorporated pool of [^{3}H]methyl thymidine. The incorporation data were normalized to DNA content and expressed as proportion of total uptake of the label. The amount of DNA in each sample was quantified using ethidium bromide as previously described (Karsten and Wollenberger, 1977; Louis and Fritz, 1979).

Subsequent experiments examined granulosa cell proliferation in response to GDF9 using an assay based on PCNA staining and comparing wild-type with Cx43 null mutant follicles. The follicles were cultured as described above. They were treated with either conditioned medium containing recombinant GDF9 (6 or 50 ng/ml) or equivalent amounts of control medium. Following 48 hours of culture, follicles were washed with PBS, fixed in 80% methanol/20% acetone for 15 minutes at 4°C and stored in PBS at 4°C. PCNA was detected in granulosa cells as described above for paraffin sections. Photomicrographs were taken of individual follicles and PCNA-stained cells were counted blind by two independent observers. At least four follicles were counted for each genotype and treatment group. The two counts of each follicle were averaged and expressed as a ratio of the mean PCNA-positive cell count for the appropriate control group. Significance of differences between mean ratios was determined by two-way analysis of variance followed by Tukey's test.

Table 2. Effect of the *Gja1* null mutation on levels of mRNA encoding KITL, KIT and GDF9 in mouse ovaries

mRNA	No. ovaries	Amplicon ratio (mean±s.e.)		Significance
		Mutant	Wild type	
KITL	5	1.617±0.201	1.499±0.081	*P*=0.60
KIT	5	0.785±0.147	3.411±0.252	*P*<0.001
GDF9	5	0.992±0.038	1.322±0.118	*P*=0.04

Results

Gene expression and gap junctional coupling in mutant follicles

Semi-quantitative RT-PCR was used to explore the possibility that paracrine signaling pathways are disrupted in developing ovaries lacking Cx43 (Table 2). The results did not reveal any change in the level of mRNA encoding KITL, at least on a per ovary basis after normalizing to the level of mRNA encoding the housekeeping enzyme, APRT. However, as there are fewer oocytes in Cx43 null mutant ovaries to induce KITL-expressing cells from the ovarian stroma, KITL expression may actually be increased on a per cell basis. Thus, we can only conclude that *Kitl* gene expression is maintained without reduction in Cx43 null mutant ovaries. In contrast, KIT mRNA level per ovary was reduced by 77%. This reduction is similar to the ~80% reduction in oocyte number caused by the same mutation on the CD1 background (Juneja et al., 1999) indicating that the amount of KIT mRNA per oocyte was not appreciably reduced. Thus the arrest of folliculogenesis in ovaries lacking Cx43 is not likely to be due to an effect on KITL or KIT expression because the KITL/KIT signaling pathway does not depend on Cx43-mediated intercellular coupling. The same approach revealed that mRNA encoding GDF9 is more abundant in the mutant ovaries: GDF9 mRNA is reduced by 25% per ovary, but as the number of oocytes per ovary is reduced even further, the level of GDF9 mRNA per oocyte is actually increased. This indicates that gap junctional coupling via Cx43 channels influences *Gdf9* gene expression. Continued expression of GDF9 in oocytes of Cx43 null mutant ovaries was confirmed by immunocytochemistry (Fig. 1). Therefore, the reduction in follicle growth associated with the absence of Cx43 cannot be explained by loss of GDF9.

Immunostaining of GDF9 null mutant ovaries indicated that Cx43 expression is maintained in the absence of GDF9, and Cx43-containing gap junctions appear to be as abundant in the follicles of mutant ovaries as they are in heterozygous controls (Fig. 2). Strong immunoreactivity for Cx43 was detected in punctate structures between the granulosa cells of the arrested primary follicles; in contrast, no Cx43 immunostaining was detected in follicle sections from Cx43 null mutant females (not shown). Furthermore, dye injection experiments revealed that granulosa cells remain coupled in the absence of GDF9 (Fig. 3). For both heterozygous and homozygous GDF9 null mutant follicles, dye readily passed from the injected granulosa cell to contacting granulosa cells and thence to their neighbors, indicating extensive intercellular coupling.

Cell cycle progression in mutant follicles

We previously demonstrated that the ovaries of Cx43 null mutant mice maintained on the C57BL/6 genetic background lack multilaminar follicles. Granulosa cells, having formed a single cuboidal layer around each oocyte, do not thereafter

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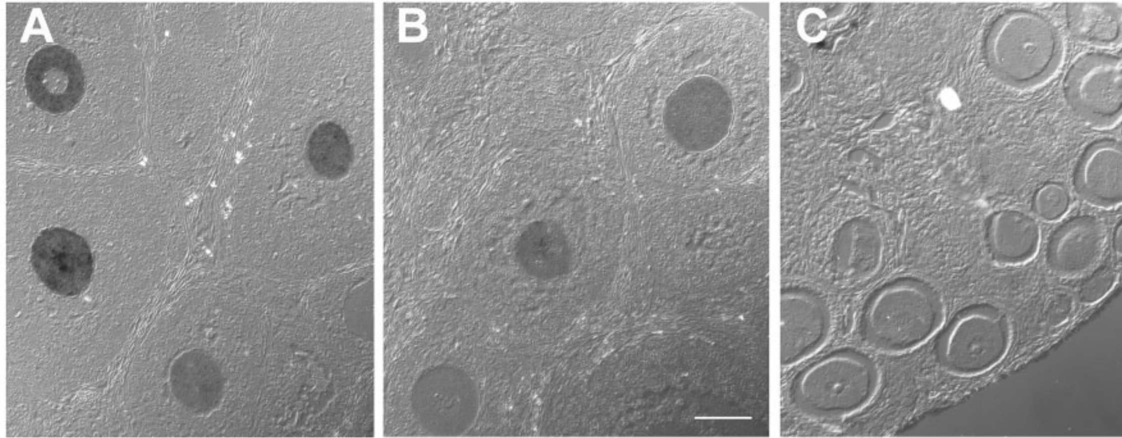

Fig. 1. GDF9 expression is maintained in oocytes of Cx43 null mutant follicles. Wild-type (A), *Gja1*⁻/*Gja1*⁻ (B) and *Gdf9*⁻/*Gdf9*⁻ (C) ovary sections were immunostained for GDF9. Bar, 50 µm.

increase in number (Ackert et al., 2001). To determine if the granulosa cells of these mutant follicles can proliferate, we tested for the expression of proliferating cell nuclear antigen (PCNA) in sections of wild-type and mutant ovaries (Fig. 4). PCNA, an accessory protein of DNA polymerase δ, is most abundant during S phase (Waseem and Lane, 1990). PCNA was highly expressed by the granulosa cells of wild-type ovaries throughout the three week period of follicle development that we observed (Fig. 4A,B). Primary follicles demonstrated a complete ring of PCNA-positive granulosa cells, but staining was not detected in the squamous granulosa cells of quiescent primordial follicles, or in stromal cells. PCNA was also detected in the nuclei of oocytes of primary through to antral follicles, which is consistent with the findings of others (Oktay et al., 1995). Mutant granulosa cells also demonstrated evidence of PCNA staining, but the frequency was low in most follicles (Fig. 4C). To evaluate further the level of granulosa cell proliferation persisting in the absence of Cx43, Cx43 null mutant ovaries were stained for PCNA in parallel with GDF9 null mutant ovaries, which do not develop follicles beyond the

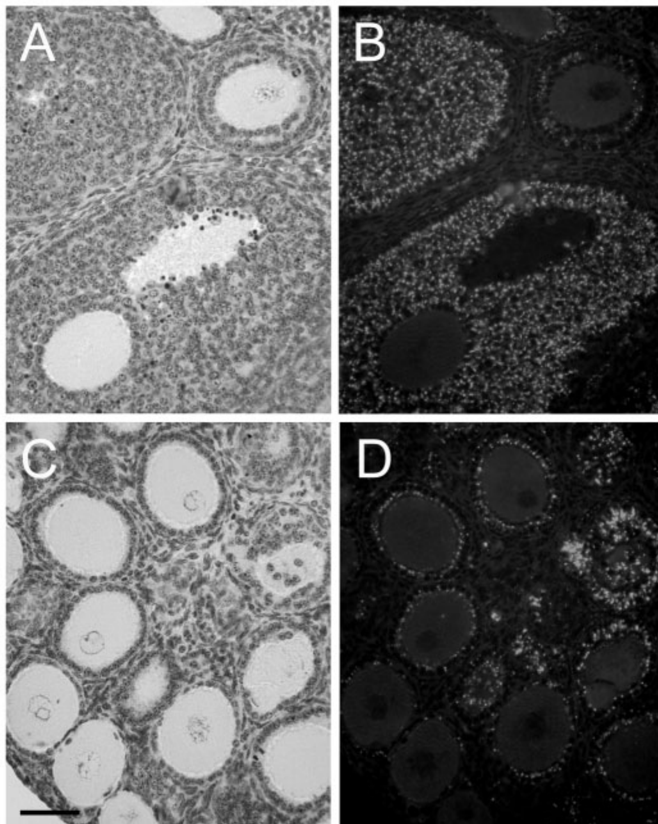

Fig. 2. Cx43 expression is maintained in GDF9 null mutant follicles. *Gdf9*⁺/*Gdf9*⁻ (A,B) and *Gdf9*⁻/*Gdf9*⁻ (C,D) ovary sections were immunostained for Cx43. Punctate immunoreactivity characteristic of gap junctions is evident between the granulosa cells of both control (B) and null mutant (D) follicles. Bar, 50 µm.

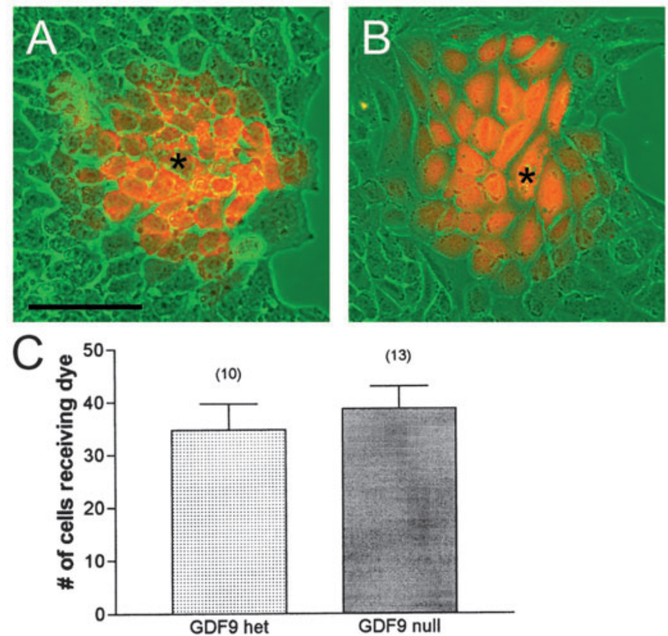

Fig. 3. Intercellular coupling is maintained in GDF9 null mutant follicles. Dichlorofluorescein was injected into a single granulosa cell (asterisk) of a *Gdf9*⁺/*Gdf9*⁻ (A) and a *Gdf9*⁻/*Gdf9*⁻ follicle (B). The extent of intercellular dye spread did not differ significantly between the two genotypes (C). These images are representative of the ten control and 13 mutant follicles that were tested. Bar, 50 µm.

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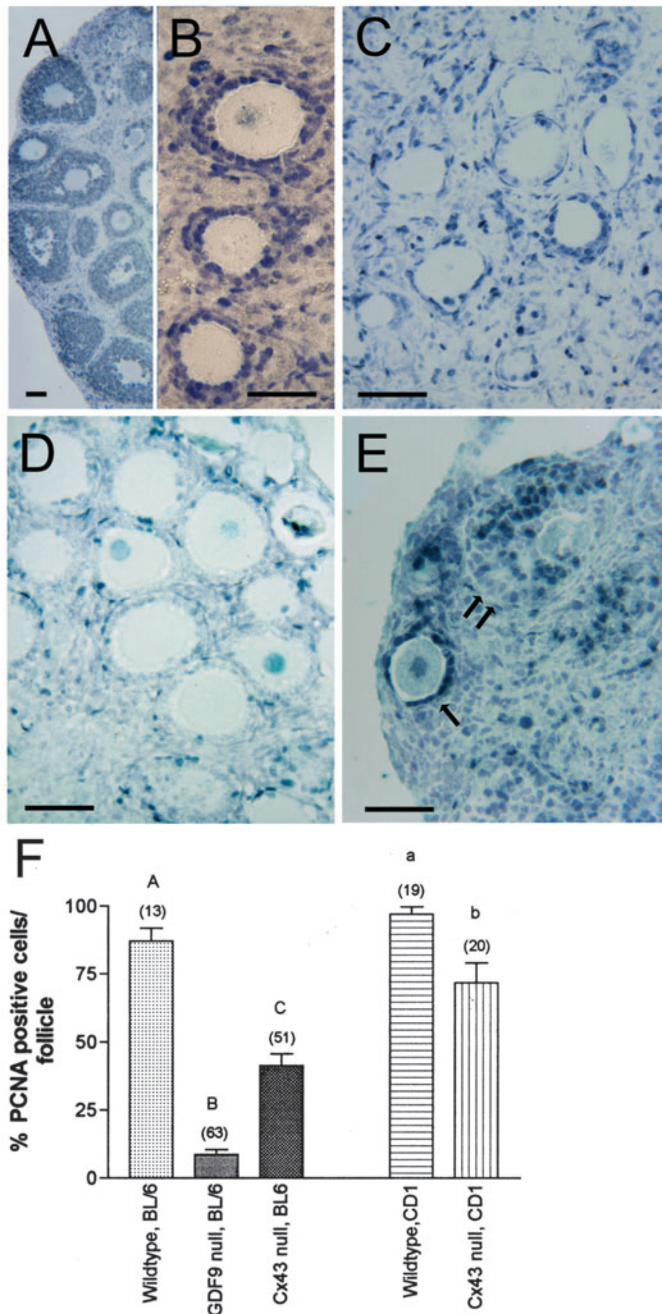

Fig. 4. Granulosa cell proliferation is reduced in Cx43 null mutant follicles as revealed by PCNA staining. (A,B) In wild-type ovaries of the CD1 strain, virtually every granulosa cell is positive for PCNA. (C) In *Gja1⁻/Gja1⁻* ovaries of the C57BL/6 strain, many granulosa cells of the arrested primary follicles lacked PCNA immunoreactivity. (D) *Gdf9⁻/Gdf9⁻* follicles had very little PCNA immunoreactivity. (E) In *Gja1⁻/Gja1⁻* ovaries of the CD1 strain, some primary follicles (single arrow) stained intensely for PCNA whereas many granulosa cells of secondary follicles (double arrows) stained weakly or were negative for PCNA. (F) Quantitative differences in the frequency of PCNA staining between mutant lines are evident . Bars represent the % of PCNA-positive cells in all follicles and error bars indicate the s.e. Different letters above the bars indicate significant differences between mutant lines within the same genetic background ($P<0.01$ in all cases). The number of follicles is indicated in parentheses above each bar. Bar, 50 µm.

unilaminar stage because the granulosa cells fail to proliferate (Elvin et al., 1999b). The incidence of PCNA-positive granulosa cells was greater in Cx43 null mutant than in GDF9 null mutant follicles (compare Fig. 4C and D). These data are presented graphically in Fig. 4F. As has been observed (Elvin et al., 1999b), less than 10% of the granulosa cells in sections of GDF9 null mutant ovaries stained positively for PCNA,

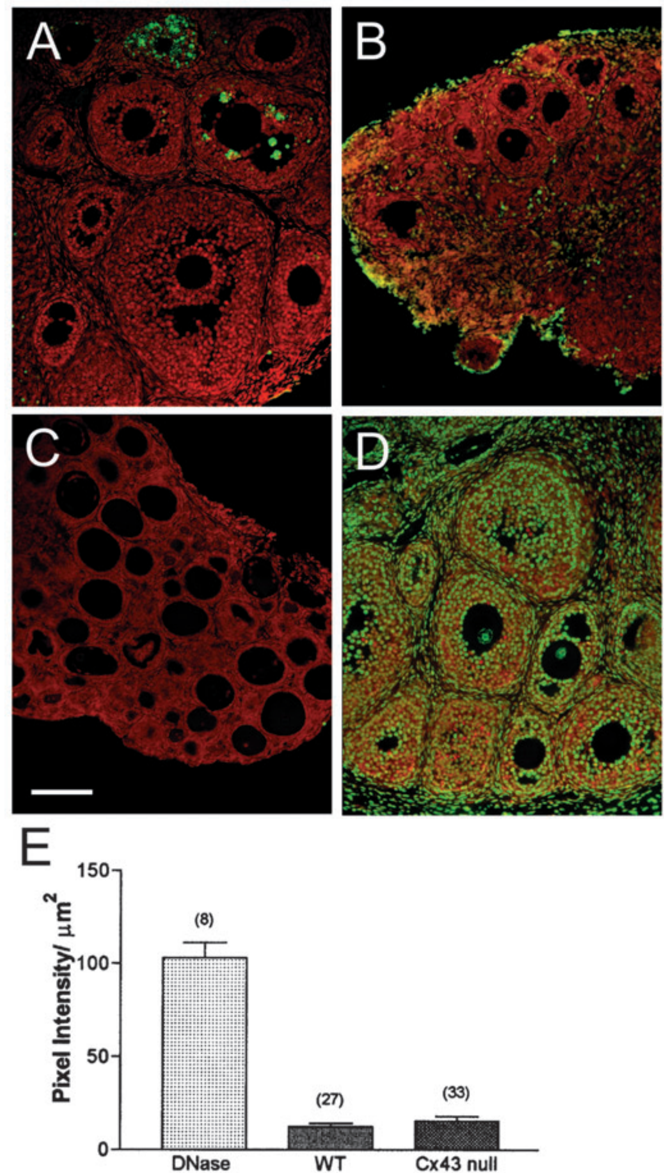

Fig. 5. Granulosa cell apoptosis is not increased in Cx43 null mutant follicles as indicated by the TUNEL assay. (A) In wild-type ovaries, apoptosis can be readily detected in some follicles, indicative of atresia. (B) Very few apoptotic cells were observed within *Gja1⁻/Gja1⁻* follicles, although apoptotic cells were observed in the stroma. (C) GDF9 null mutant ovaries did not display evidence of any apoptotic granulosa or stromal cells. (D) A positive control section, treated with DNase before TUNEL staining. (E) A quantitative comparison of TUNEL staining in wild-type and Cx43 null mutant follicles failed to reveal a significant difference ($P>0.3$). The bars indicate the total pixel intensity normalized to follicle area, the error bars indicate s.e. and the number of follicles surveyed is indicated in parentheses above each bar. Bar, 100 µm.

whereas 41% were positive in Cx43 null mutant granulosa cells of the C57BL/6 background. We also examined ovaries of CD1 strain mice lacking Cx43, in which some follicles progress to early antral stages (Ackert et al., 2001). We found strong PCNA staining in granulosa cells of some primary follicles, but reduced frequency (72%) and intensity of staining among the granulosa cells, especially those of multilaminar secondary and antral follicles (Fig. 4E,F). Thus, the lack of gap junctional communication between granulosa cells of mutant follicles impairs cell cycle progression, contributing to eventual arrest of follicle development in the C57BL/6 strain. The reduction is much less severe in the CD1 strain, allowing folliculogenesis to proceed to more advanced stages.

Apoptosis in mutant follicles

As a reduced proportion of mutant granulosa cells express PCNA with increasing follicle development, we wondered whether the rate of apoptotic cell death might be elevated. To evaluate this, we used the TUNEL assay to fluorescently label fragmented DNA of apoptotic cells. Cx43 null mutant ovaries from both C57BL/6 and CD1 genetic backgrounds were examined in parallel with ovaries from mice lacking GDF9. As shown in Fig. 5, apoptotic granulosa cells were prominent in atretic follicles of wild-type ovaries; other than that, the incidence of apoptosis observed in Cx43 null mutant follicles did not differ from that of the wild type. No apoptotic cells were observed in mutant follicles arrested at the unilaminar stage on either genetic background (Fig. 5B), although apoptotic cells were observed in the stroma of mutant CD1 ovaries. GDF9 null mutant ovaries did not display evidence of any apoptotic granulosa cells or stromal cells (Fig. 5C). These data are presented graphically in Fig. 5E. Thus, the failure of the granulosa cell population of mutant follicles to increase in number cannot be attributed to an increase in apoptotic cell death.

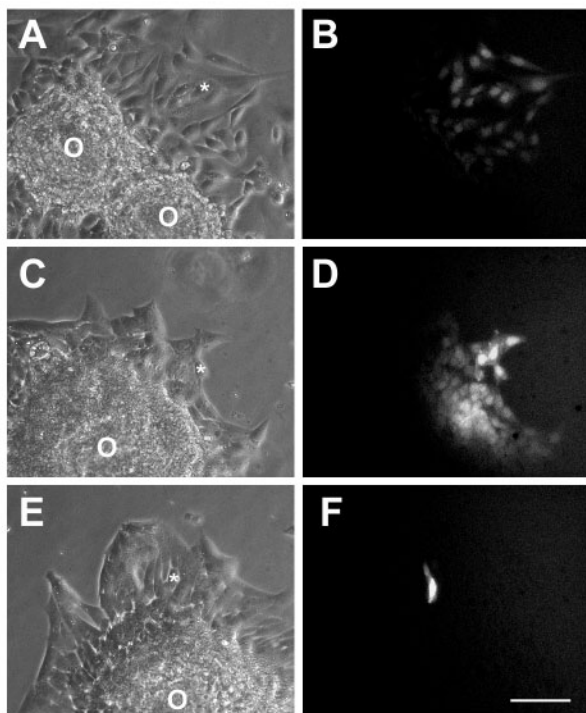

Fig. 6. Carbenoxolone completely blocks dye coupling in cultured granulosa cells whereas its inactive analog does not. Follicles were cultured for 24 hours without drug (A,B), with 200 μM glycyrrhizic acid (C,D) or with 200 μM carbenoxolone (E,F), then tested for dye coupling using Lucifer Yellow. The asterisk in A,C and E indicates the injected cell. O, oocyte. Bar, 50 μm.

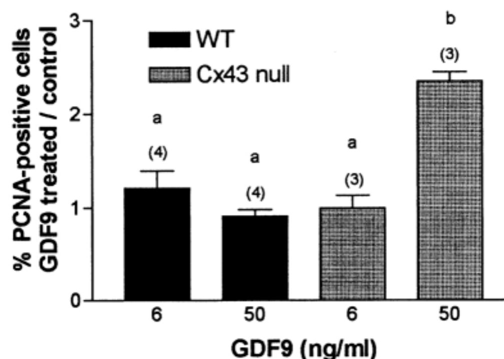

Fig. 7. Exogenous GDF9 can override the impairment of proliferation in Cx43 null mutant granulosa cells (CD1 strain) that lack gap junctional coupling. The frequency of PCNA staining for each treatment group is normalized to the respective control. The error bars indicate s.e.; different letters above the bars indicate significant differences (P<0.01). The number of follicles examined is indicated in parentheses above each bar.

Effect of exogenous GDF9 on proliferation of granulosa cells lacking gap junctional coupling

The failure of follicles deficient in either Cx43 or GDF9 to develop beyond the primary (unilaminar) follicle stage on the C57BL/6 background suggests that there may be a relationship between gap junctional coupling and paracrine signaling during folliculogenesis. The lack of granulosa cell proliferation in mice lacking GDF9 (Elvin et al., 1999b) and the demonstration that GDF9 increases granulosa cell proliferation and growth of preantral follicles (Hayashi et al., 1999; Vitt et al., 2000) caused us to hypothesize that the lack of gap junctional coupling between granulosa cells was interfering with the ability of those cells to respond to GDF9. To test this hypothesis, we first isolated preantral follicles from wild-type CD1 mice and cultured them in the presence of radiolabeled thymidine and carbenoxolone, a gap junction channel blocker, to mimic the coupling-deficient state of granulosa cells lacking Cx43. As a control, follicles from the same mice were treated with the same concentration of glycyrrhizic acid, an inactive analogue of carbenoxolone. The incorporation of labeled thymidine, normalized to DNA content, was expressed relative to total cellular uptake. Carbenoxolone (200 μM) completely abolished dye coupling, with none of the four follicles tested showing dye transfer (Fig. 6E,F); it is possible that the cells remained electrically coupled. In contrast, glycyrrhizic acid had no effect: each of the three follicles tested was strongly dye coupled (Fig. 6C,D). Dye coupling blockade reduced the growth of follicles: at the end of the treatment period, the mean area of carbenoxolone-treated follicles was 0.154±0.027 mm^2 compared to 0.598±0.125 mm^2 for glycyrrhizic acid-treated control follicles (significantly different according to unpaired,

one-tailed *t*-test, $P<0.05$). Correspondingly, carbenoxolone caused a significant decrease in thymidine incorporation such that the ratio of incorporation to uptake in carbenoxolone-treated cells was 0.584±0.046 compared to 0.864±0.039 for control cells ($P<0.05$). This effect could be overcome by the addition of 50 ng/ml recombinant GDF9, which restored the incorporation ratio of thymidine into carbenoxolone-treated cells to 0.872±0.039, not significantly different from control cells. This experiment was then repeated using follicles from Cx43 null mutant mice, which lack both dye and electrical coupling (Gittens et al., 2003), with proliferation being monitored by PCNA staining. Once again, 50 ng/ml of exogenous GDF9 restored the proliferation of coupling-deficient (in this case mutant) granulosa cells. Null mutant cells treated with GDF9 underwent an almost twofold increase in PCNA staining frequency (from 0.22±0.05 to 0.38±0.02, $P<0.05$) to achieve a proliferation rate not significantly different from wild-type cells, which were not significantly affected by GDF9. These results are presented graphically in Fig. 7, where PCNA staining frequency for each treatment group is normalized to the respective control. Thus, the impaired proliferation of granulosa cells lacking gap junctional coupling may be due at least in part to reduced responsiveness of the population as a whole to endogenous GDF9, a deficit that can be overcome by administration of GDF9 exogenously. The reduction in PCNA staining frequency in vitro because of the loss of Cx43 was more severe than in the experiment of Fig. 4, where the frequency of PCNA-positive granulosa cells in CD1 ovaries in vivo was reduced by only 23% (0.93±0.03 in wild-type ovaries compared to 0.72±0.07 in the mutant ovaries). This comparison suggests that the proliferation rate is lower and the effect of Cx43 deficiency is more severe in vitro.

Discussion

Our experiments were inspired by the observation that certain inactivating mutations in the genes encoding Cx43, KITL and GDF9 cause follicle development to arrest in the primary stage. This similarity led us to hypothesize that paracrine and gap junctional signaling pathways within the developing follicle interact. The results demonstrated that loss of gap junctional coupling among the granulosa cells does not compromise expression of the genes encoding KITL, its receptor or GDF9, nor is gap junctional coupling abolished by the loss of GDF9. On the other hand, loss of coupling among granulosa cells altered their response to exogenous GDF9 in vitro: mutant cells responded by increasing their proliferation whereas wild-type cells did not. Thus, the main finding of our study is that there is an interaction between paracrine signaling and gap junctional communication in developing follicles. Apparently, the increased expression of GDF9 in oocytes lacking Cx43 as revealed by RT-PCR is not sufficient to overcome the reduction in granulosa cell proliferation.

GDF9 is a member of the TGFβ superfamily of paracrine signaling factors that, within the rodent ovary, is exclusively produced by oocytes (reviewed by Vitt and Hsueh, 2002). Its roles in folliculogenesis have been studied in GDF9 null mutant mice and in cultured granulosa cells treated with the recombinant protein. In GDF9 null mutant ovaries, oocytes develop abnormally, eventually degenerating (Dong et al., 1996; Carabatsos et al., 1998). Oocyte growth is accelerated, coincident with enhanced expression of KITL by the granulosa cells (Elvin et al., 1999b). The mutant follicles arrest their growth in the primary stage, demonstrating that GDF9 is an essential mitogen for granulosa cells. These findings are consistent with other data demonstrating that oocytes stimulate granulosa cell proliferation in vitro (Vanderhyden et al., 1992). Correspondingly, recombinant GDF9 has been shown to stimulate granulosa cell proliferation along with inducing the expression of several genes and suppressing the expression of others, including *Kitl* (Elvin et al., 1999a; Joyce et al., 2000; Vitt et al., 2000; Varani et al., 2002). Based on these findings, it has been proposed that the functions of GDF9 include maintaining the proliferation of granulosa cells during follicular growth and establishing the distinctive properties of cumulus and mural granulosa cells based on their distance from the oocyte (Vitt and Hsueh, 2002).

The results reported here showing that recombinant GDF9 stimulates the proliferation of cultured granulosa cells from preantral follicles, reinforce earlier work (Vitt et al., 2000a) in which a similar effect was seen in granulosa cells from antral follicles. However, the stimulation recorded in our experiments was smaller in magnitude and limited to cells with impaired gap junctional coupling. The differential response of Cx43 null mutant and wild-type granulosa cells in our experiments probably reflects the fact that the oocytes remained in the cultures as an endogenous source of GDF9. In work to be published elsewhere, it was demonstrated that *Gdf9* gene expression increases in follicles cultured for three days, leveling off thereafter (F. H. Thomas, J.-F. Ethier, S. Shimasaki and B.C.V., submitted). Hence, even without exogenous GDF9, the granulosa cells in our experiments were being stimulated to proliferate. We interpreted our results as indicating a reduction in responsiveness of Cx43 null mutant granulosa cells to oocyte-derived GDF9, a deficit that could be overcome by increasing the supply of GDF9 exogenously. This conclusion is supported by several observations. First, the mutant cells in our experiments were clearly handicapped: their proliferation rate in vitro in the absence of any treatment was reduced despite the presence of the oocyte. Furthermore, it is clear that the proliferation of mutant granulosa cells is also reduced in vivo. This situation did not result from disruption of GDF9 production by mutant oocytes as GDF9 mRNA is not diminished in the mutant, nor is there any obvious reduction in GDF9 itself. An alternate interpretation would be that, despite the continued expression of GDF9 by Cx43-deficient oocytes, its processing or secretion might be impaired such that the granulosa cells were not being stimulated. This would imply that paracrine signaling is impaired by a defect in the oocytes, not the granulosa cells. In work to be published elsewhere, we have ruled out this possibility by demonstrating that oocytes lacking Cx43 can direct normal follicle development when combined with wild-type granulosa cells (J.E.I.G. and G.M.K., in preparation).

Our conclusion that the functions of GDF9 depend on gap junctional coupling within the responding granulosa cell population is consistent with what is known about the roles of gap junctions in other cells that respond to extracellular signals. In vitro studies, as well as analysis of organ pathophysiology in connexin transgenic and knockout mice have revealed a role for gap junctional coupling in glucose-stimulated insulin secretion from pancreatic β cells (Charollais et al., 2000),

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ACTH-stimulated corticosteroid production by adrenocortical cells (Munari-Silem and Rousset, 1996; Shah and Murray, 2001), carbachol-stimulated fluid secretion from lacrimal glands (Walcott et al., 2001) and glucose release from hepatocytes in response to sympathetic nerve stimulation, noradrenalin or glucagon (Nelles et al., 1996; Stümpel et al., 1998). In these cases, gap junctional coupling is understood to mediate intercellular propagation of second messengers generated by the extracellular signal. This concept has been most thoroughly developed in the case of hepatocytes lacking Cx32. Loss of this connexin causes a severe reduction in the number of gap junctions, although a small number of Cx26 gap junctions remain (Nelles et al., 1996; Valiunas et al., 1999). Consequently, coordination of calcium wave propagation between hepatocytes is compromised, probably because of reduced intercellular diffusion of inositol trisphosphate (Tordjmann et al., 1997; Niessen and Willecke, 2000). As in our study, the response of the coupling-deficient cells could be increased to the level of wild-type cells by increasing the concentration of the external signal (in that case noradrenalin or glucagon) to supraphysiological levels (Stümpel et al., 1998). By analogy, we hypothesize that impaired granulosa cell proliferation in Cx43 null mutant follicles reflects the failure of one or more signaling molecules, produced in response to GDF9, to propagate adequately throughout the population. Such a gap junction-mediated signal might be required to ensure that granulosa cells more distant from the oocyte and encountering less GDF9 maintain a rate of proliferation equal to the cells that are adjacent to the oocyte, where the concentration of GDF9 would be highest. According to this interpretation, exogenous GDF9 eliminates the need for a signal propagated through gap junctions by acting equally on all granulosa cells in the population. Rather than affecting proliferation directly, loss of gap junctional coupling might reduce the amount of a secreted autocrine factor released by granulosa cells in response to GDF9. In either situation, proliferation in response to GDF9 would be compromised.

As a member of the TGFβ superfamily, GDF9 would be expected to signal through type I and type II serine/threonine kinase receptors on granulosa cells to phosphorylate members of the SMAD family of transcriptional regulatory proteins, which would then enter the cell nuclei and alter gene expression (reviewed by Shi and Massagué, 2003). Recently, bone morphogenetic protein (BMP) receptor type II and the type I receptor, activin receptor-like kinase 5, were identified as mediating the actions of GDF9 in granulosa cells, acting upstream of SMAD2 and SMAD3 (Vitt et al., 2002; Mazerbourg et al., 2004). It is not known what gap junction-permeable second messengers are generated in cells responding to GDF9, but the molecules known to pass readily through Cx43 gap junctions include adenosine phosphates, glucose, glutamate, glutathione and inositol trisphosphate (Goldberg et al., 1998; Goldberg et al., 1999; Niessen et al., 2000). It remains to be determined whether the concentrations of these or any other small molecules are modulated in granulosa cells by GDF9 and whether obstruction of their cell-to-cell diffusion accounts for the failure of coupling-deficient granulosa cells to proliferate normally.

Our results demonstrate that gap junctional coupling is an important adjunct to paracrine signaling during ovarian follicle development; one can assume it is involved similarly in other organs. In the ovary, it may be essential for maximizing GDF9 responsiveness within growing follicles. Disruption of granulosa cell coupling not only impairs follicle growth, but also impacts negatively on the quality of the enclosed oocytes. Given the fact that GDF9 is expressed in human oocytes and its underexpression may be associated with aberrant folliculogenesis (Filho et al., 2002), it will be important to determine whether Cx43-mediated gap junctional coupling, acting downstream of GDF9, might be a determinant of female fertility.

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