Selective assembly of connexin37 into heterocellular gap junctions at the oocyte/granulosa cell interface

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Summary

Studies of mice with targeted disruptions of specific connexin genes have revealed that at least two connexins, connexin37 (Cx37) and connexin43 (Cx43), play essential roles in ovarian follicle development. To explore the respective roles of these two connexins in gap-junctional communication between the developing murine oocyte and its surrounding cumulus granulosa cells, we used confocal immunofluorescence microscopy and oocyte preloading functional assays. Immunofluorescence microscopy located Cx37 within gap-junction plaques between granulosa cells and the oocyte, and Cx43 between surrounding granulosa cells. Preloading assays combining denuded oocytes and cultured granulosa cells expressing or lacking Cx37 or Cx43 revealed that Cx37 must be present in both cell types for the establishment of heterocellular gap-junctional coupling. Furthermore, immunofluorescence microscopy of cultured granulosa cells after incubation with denuded oocytes showed that the oocyte induces the formation of gap junctions containing Cx37 at the surface of granulosa cells. Continuous Cx37 expression in granulosa cells was confirmed using RT-PCR. Together, these results indicate that the growing murine oocyte is functionally coupled with granulosa cells by homotypic gap junctions composed of Cx37, and that the formation and/or stabilization of Cx37 junctions is selectively induced at the oocyte-granulosa interface by cell contact.

Key words: Gap junctions, Cx37, Cx43, Ovarian follicle, Oocyte, Cumulus granulosa cell, Connexin 43

Introduction

Gap junctions are clusters of intercellular channels that aggregate at sites of close cell-cell apposition, connecting the cytoplasm of adjacent cells and permitting the passage of inorganic ions, second messengers, and small metabolites (less than ~1000 daltons) between cells (Bruzzone et al., 1996). The primary unit of the gap-junction channel is the connexon, a hexameric structure of oligomerized connexin (Cx) subunits that forms a hemichannel in the plasma membrane. The end-to-end docking of two compatible hemichannels from neighbouring cells forms the intercellular channel (White and Paul, 1999). The connexins are members of a closely related family of integral membrane proteins with at least 19 members in the mouse genome (Willecke et al., 2002). Most cell types express multiple connexins, but whether different connexin isoforms can hetero-oligomerize into functional channels is dependent upon the compatibility of the connexins involved. This situation arises in vivo in granulosa cells of adult mice and rats where Cx43 and Cx45 are co-localized to the same gap-junctional plaques (Kidder and Mhawi, 2002; Okuma et al., 1996). It also occurs in cardiac myocytes that express Cx40 and Cx43, where the two connexins co-localize in gap-junction plaques (Gros et al., 1994). In 1995, Elfgang and others published an important series of experiments that investigated which connexins could form functional heterotypic channels in vitro (Elfgang et al., 1995). Gap-junctional-communication-deficient HeLa cells were transfected with seven murine connexin expression vectors, and these engineered cells were then cultured in pairs and assessed for dye coupling. Of specific interest to our study, Cx43 demonstrated the ability to form functional heterotypic channels with Cx37 as well as functional Cx43 homotypic channels. This in vitro model is particularly useful when determining the ability of different connexins to form functional gap-junction channels with one another, because although two connexins may co-localize in the same plaque, they could be forming distinct channels, due to their inability to form heterotypic channels.

Gap-junctional intercellular communication (GJIC) is a selective process mediated by the connexin isoform(s) that comprise each hemichannel within a gap junction. This communication selectivity and the implied physiological significance of such a system is demonstrated by disease states caused by specific connexin mutations and the phenotypes exhibited by connexin-knockout mice, suggesting that vital intercellular signalling is mediated by specific connexin isoforms (White and Paul, 1999).

The mammalian cumulus-oocyte complex (COC) represents a clear example of a multicellular unit that exhibits expression of multiple connexins and is reliant upon GJIC for proper development (Ackert et al., 2001; Juneja et al., 1999; Kidder and Mhawi, 2002; Simon et al., 1997). The COC is housed in the mature ovarian follicle. It consists of a developing oocyte

surrounded by stratified layers of cumulus granulosa cells (referred to here as granulosa cells) which are in direct contact with the oocyte through transzonal projections spanning the encapsulating zona pellucida (Albertini et al., 2001). Mural granulosa cells are found on the periphery of the follicle, next to the thecal cell layer. Gap junctions transfer amino acids and glucose metabolites to the growing oocyte, as well as compounds that regulate the meiotic maturation of the oocyte (Eppig, 2001). Gap junctions have been detected in rodent ovaries from the earliest stages of folliculogenesis (Juneja et al., 1999), and provide coupling throughout the follicle until gonadotropins interrupt GJIC and initiate ovulation (Granot and Dekel, 1998).

Multiple connexins are expressed within the developing ovarian follicle (reviewed by Kidder and Mhawi, 2002). Cx32, Cx43 and Cx45 have been localized between granulosa cells in mouse ovarian follicles, whereas Cx37 has been localized to the oocyte surface. Cx43 and Cx37 play critical roles in ovarian function as the absence of either connexin causes a loss of cell coupling and disruption of folliculogenesis (Ackert et al., 2001; Gittens et al., 2003; Simon et al., 1997). Gap junctions containing Cx43 were observed in pre-granulosa cells in mouse primordial follicles as early as postnatal day 1, and are believed to primarily mediate communication between granulosa cells (Juneja et al., 1999; Gittens et al., 2003). Cx37 is expressed within oocytes and at the interface between granulosa cells and the oocyte, but was not detected in the granulosa cell layers (Simon et al., 1997); in a later report, however, some Cx37 immunoreactivity was detected between granulosa cells in antral follicles (Wright et al., 2001). Cx37 may be the only connexin supplied by the oocyte for gap junction formation at the oocyte surface; this is supported by its presence in oocytes and the absence of oocyte-granulosa cell dye-coupling in Cx37-deficient follicles (Simon et al., 1997). However, the identity of the connexins supplied by granulosa cells for oocyte-granulosa cell gap junctions remains uncertain (Kidder and Mhawi, 2002).

The present study was designed to investigate the relative contributions of Cx43 and Cx37 to folliculogenesis in the mouse and also to determine whether granulosa cells express both Cx37 and Cx43. If both connexins are expressed, are they differentially assembled into distinct gap-junction complexes depending upon whether the adjacent contacting cell is an oocyte or granulosa cell? The ability of a cell to assemble connexins selectively into specific gap-junction complexes would indicate that gap-junction formation is not solely dependent on the expression of compatible connexins, but also on other factors contributed by the contacting cell.

Materials and Methods

Cell lines and culture conditions

All media, sera and culture reagents were obtained from InVitrogen (Burlington, ON), Becton Dickinson (St Laurent, QC) or Sigma (Oakville, ON). LipofectAMINE PLUS was obtained from InVitrogen. NRK cells (NRK 52-E, ATCC, Manasses, VA) endogenously expressing Cx43 were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Cells were plated on 60-mm tissue culture dishes with or without glass coverslips and maintained at 37°C in an environment of 95% air and 5% carbon dioxide. Cells were sub-cultured by treating with 0.25% trypsin and 2 mM EDTA for 5 minutes and were replated at a 5-10 fold dilution.

Antibodies

Rabbit anti-Cx37 polyclonal antibodies were raised against a peptide sequence representing amino acid residues 306-323 of the carboxy-terminal tail of the mouse Cx37 sequence (Genemed Synthesis, San Francisco, CA). Both the polyclonal (CT-360) and monoclonal (P4G9 E3, Fred Hutchison Cancer Research Center Hybridoma Facility, Seattle, WA) anti-Cx43 antibodies were raised against a peptide sequence that represents amino acid residues 360-382 of rat Cx43. The CT-360 antibody was purified against the peptide as previously described (Laird and Revel, 1990). Rabbit polyclonal anti-giantin was purchased from BioCan Scientific (Mississauga, ON). Anti-rabbit or anti-mouse secondary antibodies (conjugated to the fluorescent dyes Texas Red or FITC) were purchased from Jackson ImmunoResearch (West Grove, PA).

Engineering of the Cx37-GFP chimeric cDNA expression vector

In order to test the efficacy and specificity of the new anti-Cx37 antibody, we engineered cells to express Cx37-GFP. Cx37 cDNA was PCR-amplified using oligonucleotides TTTCTCGAGATGGGCGA-CTGG to create an *Xho*I site and TGTCGGATCCTGCACATACTG to create a *Bam*HI site at the 5′ and 3′ ends of Cx37, respectively, as well as removing the stop codon. PWO DNA polymerase (Boehringer, Indianapolis, IN) was used to ensure fidelity of the PCR reaction. PCR products and the vector pEGFP-N1 (Clontech, Palo Alto, CA) were digested with *Xho*I and *Bam*HI. GFP was fused in frame to the carboxyl terminus of Cx37 with the addition of a 7-amino acid polylinker (GTCCTAGGTGGCCAGCGGTGG). After ligation, competent JM109 *Escherichia coli* were transformed with the plasmid, and selected positive colonies were identified. Finally, the cDNA encoding the chimeric protein was verified by the Applied Biosystems (Foster City, CA) dye terminator cycle sequencing method. Cx37-GFP cDNA was additionally cloned into an AP2 replication-defective retroviral vector (Galipeau et al., 1999) at the *Eco*47III-*Not*I restriction digest site.

Retroviral infection of NRK cells with cDNA encoding Cx37-GFP

The retroviral AP2 construct encoding Cx37-GFP was transfected into 293GPG packaging cells using the LipofectAMINE PLUS procedure (InVitrogen, Burlington, ON). Forty-eight hours after transfection, 293GPG culture medium was replaced with fresh DMEM. Culture medium containing a high-titre of virus was collected after 24 hours, filtered through a 0.45 μm syringe filter, and used to deliver transgenes into target cells (Mao et al., 2000; Qin et al., 2001; Qin et al., 2002). NRK cells grown to 20-40% confluency were infected in viral culture medium twice over 48 hours at 37°C. After infection, the medium was replaced with DMEM and cells were subcultured repeatedly as necessary. Infection efficiency was determined 48 hours after infection by visualizing live or fixed cells for Cx37-GFP expression under a fluorescence microscope.

Preparation of COC primary cultures

Wild-type CD-1 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). *Gja4*$^{-/-}$ (C57BL/6J strain lacking Cx37) and *Gja1*$^{-/-}$ (CD-1 strain lacking Cx43) mice were obtained by mating mice heterozygous for the mutant allele. These were bred and maintained in the John P. Robarts Research Institute Barrier Facility on the campus of the University of Western Ontario. The *Gja4* mutant line was generously provided by Dr David Paul, Harvard Medical School, Boston, MA (Simon et al., 1997). To obtain oocytes and granulosa cells from mice lacking Cx43 (which die at birth), ovaries from late-gestation fetuses were grafted into adult, ovariectomized female hosts as described previously (Ackert et al., 2001; Gittens et

al., 2003). Females (either graft hosts or normally cycling) were anaesthetized with CO_2 and killed by cervical dislocation. Ovaries were removed and placed in culture in Waymouth MB 752/1 medium supplemented with 10% fetal bovine serum, 0.025 M HEPES (N-2-hydroxyethylpiperazine-N′2-ethane sulfonic acid) buffer, 100 units/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml Fungizone™ (from InVitrogen or Sigma). Fat and connective tissue surrounding the ovary was removed using fine 30-gauge needles. Ovaries were then transferred to a second dish of culture medium, where follicles were released from the ovary by slashing with fine needles. Type-1 collagenase (1 mg/ml of culture medium, Sigma) was added to the medium at this stage to increase ovarian tissue separation, facilitate follicle release, and remove surrounding theca cells (Eppig et al., 1992). Repeated pipetting of remaining ovarian tissue in culture medium also enhanced follicle removal. Follicles were then collected from the second dish, and placed into a new dish containing the above culture medium without collagenase. COCs were selected based on size and morphology (with the oocyte surrounded by layers of granulosa cells having an undefined outer border). Selected COCs were then washed with culture medium and transferred to glass cover slips with approximately 150 μl of culture medium and incubated for either 1 or 2 days, before fixation with a solution of 80% methanol/20% acetone. In some experiments, cultured COCs were treated with 10 μg/ml of brefeldin A (BFA; Cedarlane Labs, Hornby, ON) for 5 hours. For granulosa cell cultures, twice-washed COCs were treated with 0.05% trypsin and 2 mM EDTA for 30 minutes, centrifuged at 9000 ***g*** for 2 minutes and resuspended in culture medium.

Immunofluorescence

Cells grown on coverslips were rinsed with PBS (pH 7.4) and fixed with 80% methanol/ 20% acetone for 10 minutes at 4°C. Fixed cells were immunolabelled as previously described (Laird et al., 1995). Briefly, coverslips were washed 2-3 times with PBS and incubated for 1 hour in 2% bovine serum albumin (BSA) in PBS to block non-specific binding. They were further washed 2-3 times in PBS and incubated with primary antibody diluted in 2% BSA/PBS for 1 hour. After incubation with the primary antibody, coverslips were washed 3 times over 15 minutes in PBS, and then incubated for 1 hour with anti-rabbit or anti-mouse secondary antibodies conjugated to the fluorescent dyes Texas Red or FITC. Coverslips were washed as before with PBS, rinsed with distilled water and mounted on microscope slides using Airvol (Air Products and Chemicals, Allentown, PA). For double-labelling experiments, cells were labelled with two primary antibodies using consecutive rounds of immunofluorescent labelling as described above. Fixed cells were labelled in triplicate in at least 10 trials (unless specified otherwise), with each trial including at least two positive and negative control repetitions to ensure antibody efficacy.

For peptide competition studies, the anti-Cx37 antibody was pre-incubated for 90 minutes at room temperature with the corresponding peptide (5 μg/ml), and immunofluorescent labelling was performed with the pre-treated antibody as described above.

RT-PCR

RT-PCR was performed with RNA isolated from cultured granulosa cells and whole lung homogenates from groups of wild-type and Cx37-deficient mice (6-10 weeks old). Total RNA was collected using the TRIzol® method (InVitrogen). RT-PCR was carried out according to instructions supplied with the One-Step RT-PCR Kit (Qiagen, Mississauga, ON). Briefly, 1 μg of total RNA was mixed with kit contents to a total of 25 μl with RT at 50°C (30 minutes) and PCR activation at 95°C (15 minutes). Cycling parameters were as follows: denaturation 94°C (1 minute), annealing 60°C (1 minute), extension 72°C (1 minute) for 35 cycles, with a final extension of 10 minutes. The primers used for PCR were: Cx37 sense 5′-ACGGTCGTCCC-CTCTACAT-3′; Cx37 antisense 5′-GGTAGATCAGGGTGGGTGTG-3′.

Oocyte microinjection

Follicles were isolated and cultured for 0, 1, or 2 days on 12-mm-diameter coverslips in Petri dishes as above. Oocytes were impaled with a microinjection needle containing 5% Lucifer yellow (Molecular Probes, Eugene, OR) in ddH_2O for 20 minutes. Injections resulting in rapid filling of the oocyte within 1 minute and showing no evidence of granulosa cell injection were recorded. To be certain that the spread of dye observed reflected transfer from the oocyte to the granulosa cells via gap junctions, we also examined follicles from Cx37-deficient mice which lack gap-junctional coupling between the oocyte and the granulosa cells (Simon et al., 1997).

Dye transfer by preloading

COCs were carefully dissected from early antral follicles of adult female mice as described above. They were washed through two dishes of the same medium and placed in 1 ml fresh medium and incubated at 37°C in an atmosphere of 5% CO_2 in air for 2 days to allow granulosa cells to form a monolayer. Oocytes of similar size were harvested from early antral follicles of wild-type and Cx37-deficient follicles as described above and immersed in acid Tyrode's solution (Nagy et al., 2003) for a few seconds to remove the zonae pellucidae. The denuded (ZP-free) oocytes were washed several times in culture medium and preloaded with calcein AM (a membrane-permeable molecule that gives rise to the membrane-impermeable green fluorescent dye, calcein, once inside the cell) and DiI (a lipophilic, fluorescent dye that stains membranes red) as described previously (Goldberg et al., 1995). Preloaded oocytes were transferred onto the unlabelled granulosa cell monolayers. The monolayers received only one preloaded oocyte per dish. Dishes were then incubated for 4 hours to allow sufficient time for the oocytes to settle and establish gap junctions with the granulosa cells. Calcein transfer from preloaded oocyte to unlabelled granulosa cells was determined by fluorescence microscopy and taken as an indication of intercellular coupling. At least 20 trials were conducted for each combination.

Oocyte-granulosa cell co-culture

Wild-type granulosa cell monolayers or granulosa cells at the edge of a cultured COC were immunofluorescently labelled with both Cx37 and Cx43 antibodies after they had been in contact with denuded, unlabelled wild-type or Cx37-deficient oocytes for 4 hours. The positions of the attached oocytes were carefully marked prior to fixation and immunlabelling with anti-connexin antibodies. The oocytes became detached from the monolayers during this process. The presence or absence of Cx37 gap-junction plaque-like structures in granulosa cells that had been in contact with wild-type or Cx37-deficient oocytes, was scored by three independent, blinded observers.

Imaging

Immunofluorescently labelled cells grown and fixed on mounted coverslips were analyzed on a Zeiss (Thornwood, NY) LSM 410 inverted confocal microscope with a 63× oil (1.4 numerical aperture) objective. Fluorescent signals were imaged by excitation with either a 488 nm (FITC or GFP) or 568 nm (Texas red) laser line from a Krypton/Argon laser and collected on a photomultiplier after passage through appropriate filter sets (Laird et al., 1995). Microinjected and preloaded cells were imaged on a Zeiss inverted microscope equipped with a Sensicam CCD camera. Digital images were then prepared using Adobe Photoshop 5.5 and CorelDraw 9 software.

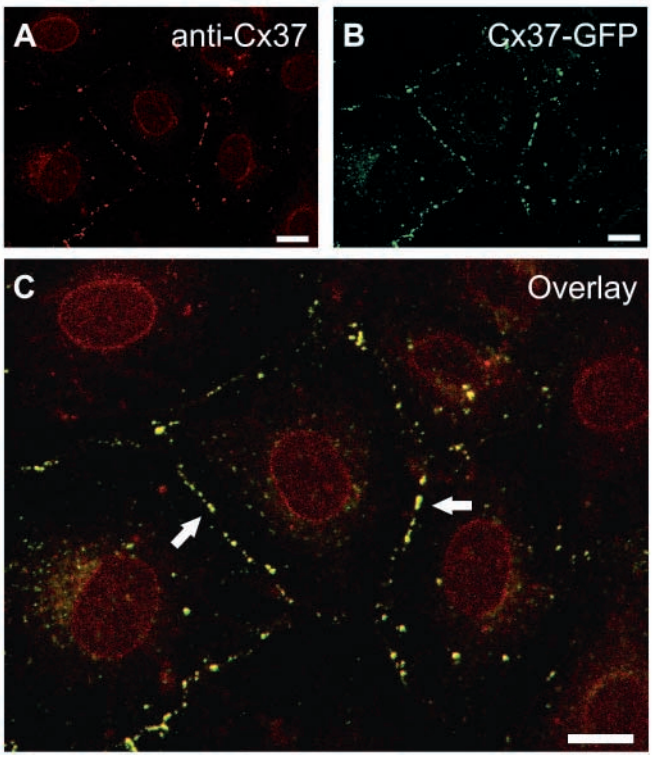

Fig. 1. A new anti-Cx37 antibody is specific for Cx37 gap-junction plaques. NRK cells were infected with a retroviral vector encoding Cx37 tagged with GFP (B, green) and immunolabelled with anti-Cx37 antibodies (A, red). Co-localization is indicated by yellow color and arrows (C). Bars=10 μm.

Results

Characterization of a new anti-Cx37 antibody

In order to determine the efficacy and specificity of our new anti-Cx37 antibody, it was first used to immunofluorescently label NRK cells engineered to express Cx37-GFP. Anti-Cx37 antibody binding co-localized with the Cx37-GFP fluorescent signal in all cases (Fig. 1), with low-level immunostaining of the nuclear envelope. These results suggest that Cx37-GFP was retained as an intact fusion protein when expressed in NRK cells and the new anti-Cx37 antibody was specific and reliable. As a negative control, wild-type NRK cells expressing Cx43 but not Cx37 exhibited no immunoreactivity with the anti-Cx37 antibody (results not shown).

Connexin localization in cultured murine COCs and ovarian follicle sections

After characterization in engineered cell lines, the new Cx37 antibody was used to localize Cx37 in fixed murine COCs. Confocal analysis revealed that Cx37 was localized to large, punctate gap-junction-like plaques at the border between the oocyte and granulosa cells in cultured COCs (Fig. 2A) and ovarian follicle sections (Fig. 2B). These results support previous findings using fixed ovary sections where Cx37 was localized to the interface between granulosa cells and the oocyte (Simon et al., 1997). As negative controls, the anti-Cx37 antibody was competitively inhibited with the immunizing peptide prior to immunofluorescent labelling of

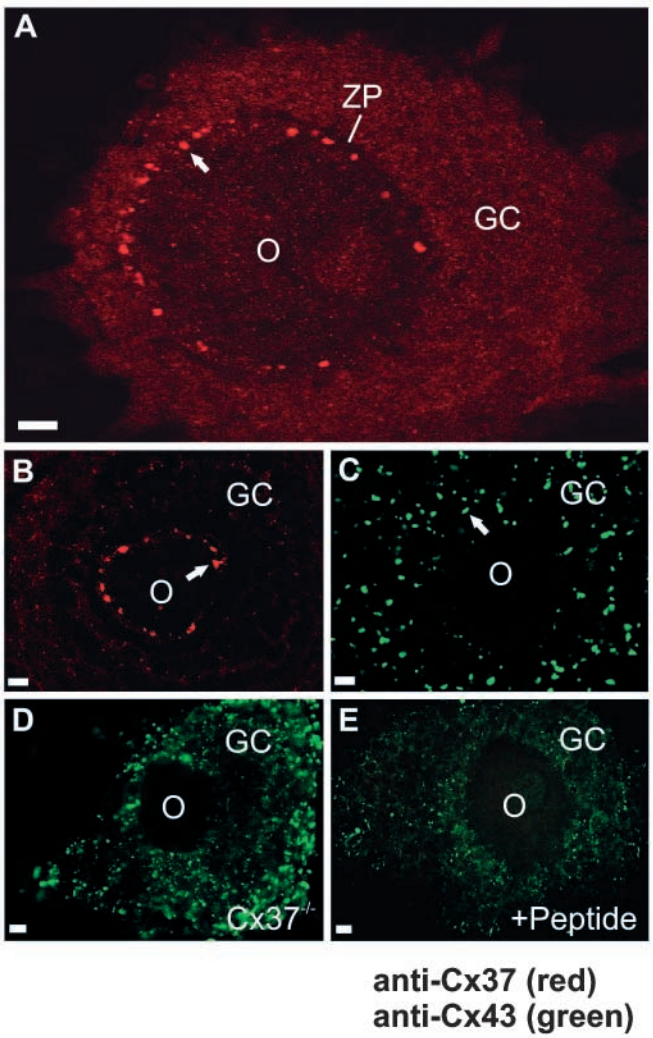

Fig. 2. Connexin localization in the cumulus-oocyte complex (COC) as detected by confocal immunofluorescence microscopy. In single-labelling experiments, Cx37 (anti-Cx37, red) was localized to gap-junction plaques (arrow) on the surface of the oocyte (O), beneath the zona pellucida (ZP) in both cultured COCs (A) and follicle sections (B). In other single-labelling studies, Cx43 (anti-Cx43, green) was localized to plaques (arrow) between contacting granulosa cells (GC) in follicle sections (C). In double-labelling experiments, no Cx37 immunostaining was detected when Cx37-deficient COCs were immunolabelled (D, note lack of red signal) or when the anti-Cx37 antiserum was competed with the immunizing peptide in cultured COCs (E, note lack of red signal). Cx43 was localized to plaques between granulosa cells in D and E. Bars=10 μm.

COCs (Fig. 2E), and no labelling was evident when COCs were obtained from Cx37-null mice (Fig. 2D). Together, these studies suggest that the anti-Cx37 antibody is specific and effective for detecting Cx37 in mammalian cells.

Double-immunofluorescent labelling of cultured COCs revealed extensive Cx43 localization in punctate, gap-junction-like plaques between contacting granulosa cells (Fig. 3), consistent with Cx43 localization in ovarian follicle sections (Fig. 2C). As above, Cx37 was localized to large, punctate gap-junction-like plaques at the border between the oocyte and

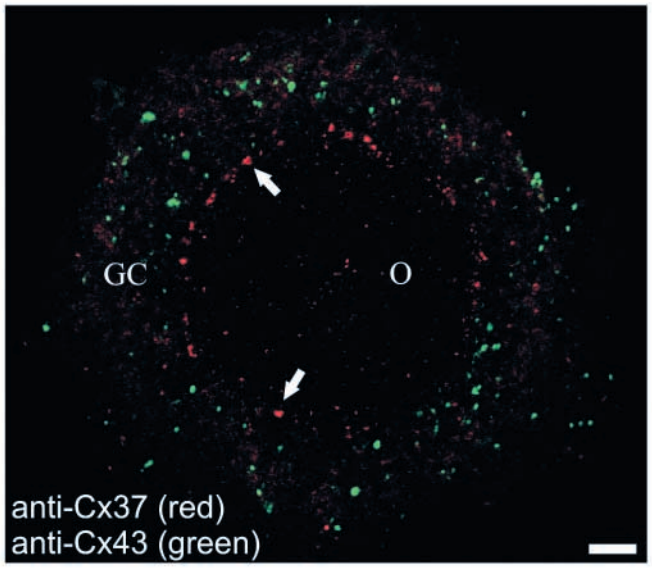

Fig. 3. Double-immunofluorescent labelling of cultured COCs revealed that Cx37 is localized to gap-junction plaques at the oocyte surface whereas Cx43 is localized to plaques between adjacent granulosa cells. Arrows indicate punctate localization of Cx37 (anti-Cx37, red) at the border between the oocyte (O) and granulosa cells (GC). Populations of intracellular Cx37 are visible within the oocyte and at candidate gap-junction plaques between adjacent GC. Cx43 (anti-Cx43, green) is localized to plaques between granulosa cells and is rarely found at the oocyte surface. Bar=10 μm.

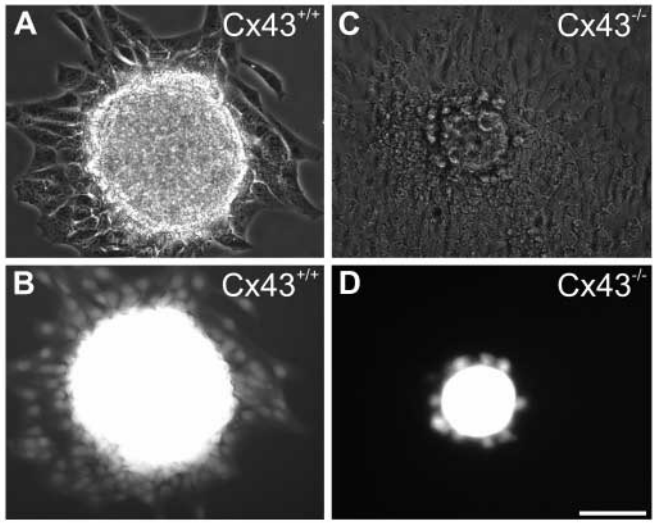

Fig. 4. Oocyte-granulosa cell coupling is maintained in the absence of Cx43. Wild type (A,B) and Cx43-deficient (C,D) follicles were isolated, cultured, and oocytes were microinjected with the gap-junction-permeable dye, Lucifer yellow. Dye transferred throughout the wild-type follicle after oocyte injection (B), whereas dye spread to only the first layer of granulosa cells directly in contact with the oocyte in Cx43-deficient follicles (D). Bar=50 μm.

granulosa cells. Importantly, there was no evidence of Cx37 co-localization with Cx43. Populations of intracellular Cx37 within the oocyte may represent connexins undergoing transport and/or degradation. Interestingly, Cx37 immunostaining was occasionally seen within the granulosa cell layers but this labelling was random and not consistently found between adjacent cells.

Functional analysis of Cx43 and Cx37 in cultured COCs

Gap-junctional intercellular communication between the oocyte and the granulosa cells of wild-type and Cx43-null follicles was assessed by examining the transfer of the microinjected gap-junction-permeable dye, Lucifer yellow, from the oocyte to surrounding cells. The granulosa cells intimately associated with the oocyte of wild-type follicles demonstrated strong fluorescence, indicating that dye had spread from the oocyte to the granulosa cells (Fig. 4A,B). Dye was also passed to distant granulosa cells of the monolayer in 13 out of 13 trials, regardless of follicle culture period (0, 1 or 2 days). The lack of Cx43-containing channels between the oocyte and the granulosa cells of Cx43-deficient follicles did not prevent dye transfer to the first layer of granulosa cells (dye transferred to the first layer of granulosa cells in 8 out of 14 trials, regardless of follicle culture period). However, dye did not spread to further cell layers (Fig. 4C,D). This result is in accord with our previous study demonstrating that Cx43 is required for granulosa cell intercommunication via gap junctions (Gittens et al., 2003), and implies further that Cx43 is not required for oocyte-granulosa cell coupling.

Previous experiments were unable to identify which members of the connexin family are contributed by the granulosa cells and the oocyte, or the composition of gap junctions between these two cell types. Therefore, to determine the specific connexin content of gap junctions between the oocyte and surrounding granulosa cells, denuded oocytes from wild-type or Cx37-deficient mice were preloaded with a gap-junction-permeable fluorescent dye (calcein) and seeded onto wild-type or Cx37-deficient granulosa cells. Extensive dye transfer was detected in 18 out of 24 trials (75%) when wild-type preloaded oocytes were seeded on monolayers of wild-type granulosa cells (Fig. 5A,D). Thus denuded oocyte/granulosa cell co-cultures can re-establish heterocellular coupling, reproducing the intercellular gap-junctional communication that exists in vivo.

Wild-type, preloaded oocytes were then seeded onto granulosa cell monolayers from mutant mice lacking Cx37 (Fig. 5B,E), to determine if Cx37 expression in granulosa cells is essential to permit heterocellular coupling with the oocyte. No dye transfer was detected in any of the trials (n=31). In addition, preloaded Cx37-deficient oocytes were seeded onto wild-type granulosa cells (Fig. 5C,F), to determine if Cx37 must be present in oocytes to establish heterocellular coupling. Again, no dye transfer was detected (n=28). There was no apparent difference in the stability of wild-type and mutant cell attachments, indicating that cell adhesion was not a limiting factor in the ability of the mutant cells to form gap junctions. Additionally, wild-type preloaded oocytes were seeded onto granulosa cell cultures deficient in Cx43, to confirm results from oocyte dye injections indicating that Cx43 is not required for granulosa-oocyte coupling. Dye transfer to at least a few cells in direct contact with the oocyte was detected in 20 out of 27 trials (74%, results not shown).

Identification of Cx37 in granulosa cells

The finding that Cx37 must be present in both oocytes and

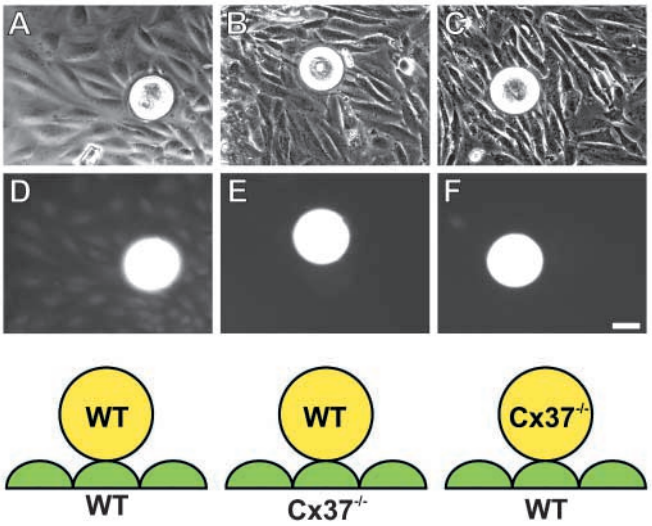

Fig. 5. Cx37 is essential for coupling between the oocyte and granulosa cells. Wild-type oocytes (preloaded with calcein) transfer dye extensively to wild type granulosa cells (A,D) whereas no dye transfer occurs when Cx37 is missing from either the granulosa cells (B,E) or the oocyte (C,F). Bar=25 μm.

granulosa cells for heterocellular coupling to be established implies that this connexin must be expressed by granulosa cells, even though our immunofluorescence experiments could not reliably detect Cx37 in cells which were not directly adjacent to the oocyte. To test the possibility that Cx37 assembly into gap junctions is restricted to those granulosa cells in direct contact with the oocyte, wild-type granulosa cells were immunofluorescently labelled with both Cx37 and Cx43 antibodies after they had been in contact with denuded, unlabelled, wild-type oocytes for 3-5 hours. Although Cx43 was localized to numerous sites of granulosa cell contact (Fig. 6A-C), Cx37 localization was restricted to sites where oocytes had attached (n=10; Fig. 6B, arrows). It is improbable that this localized Cx37 immunostaining was caused by a portion of the oocyte membrane remaining attached to the granulosa cells, as oocyte integrity was maintained during fixation, indicating that the oocytes had not been damaged. No significant Cx37 localization was detected in the granulosa cells that had not been in contact with denuded oocytes. In addition, denuded, unlabelled Cx37-deficient oocytes were seeded onto wild-type granulosa cells for 4 hours followed by immunofluorescent labelling as above. No significant Cx37 localization was detected in granulosa cells in contact with Cx37-deficient oocytes (n=10, Fig. 6C).

RT-PCR was performed to determine if Cx37 mRNA could be detected in cultured granulosa cells. Total RNA was collected from granulosa cells cultured for 24 hours, as well as from wild-type and Cx37-null mouse lung homogenates. Specific primers amplified Cx37 amplicons of the expected size in both the granulosa cell and wild-type lung samples (Fig. 6D). No specific amplicons were amplified from Cx37-null mouse lung cDNA or when no cDNA template was used.

To determine whether Cx37 and Cx43 gap junctions in COCs undergo rapid turnover and reassembly as is described for some cultured cells (Laird et al., 1995; Musil and Goodenough, 1991), protein transport was blocked with BFA

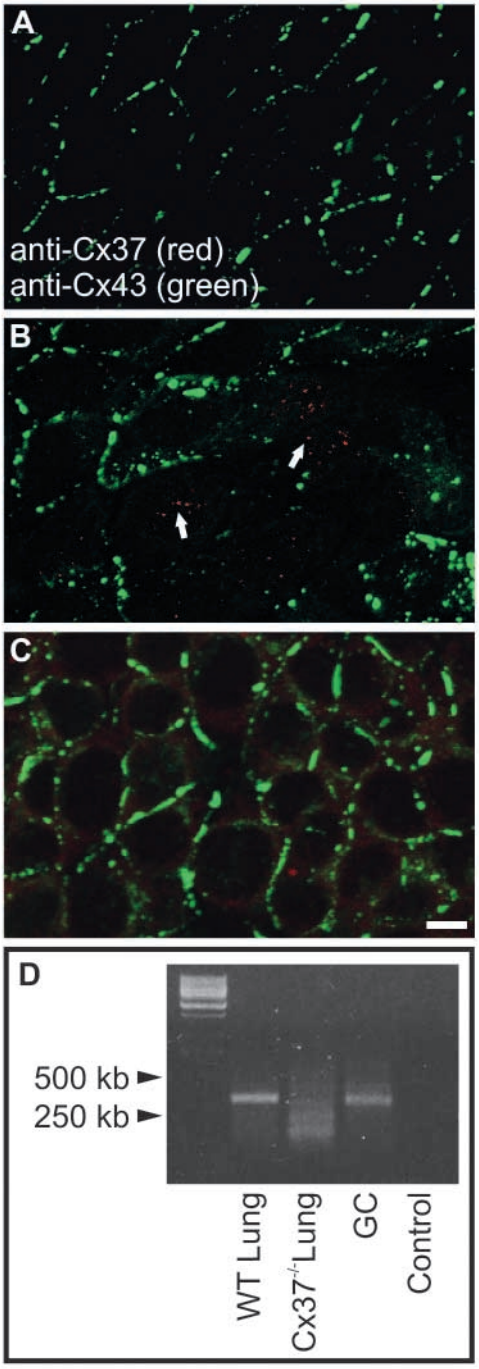

Fig. 6. Cx37 is recruited to sites where seeded Cx37-positive oocytes contact granulosa cells. Wild-type (B) or Cx37-deficient (C) denuded oocytes were seeded onto cultured granulosa cell monolayers for 4 hours, fixed, and immunofluorescently labelled for Cx37 (anti-Cx37, red) and Cx43 (anti-Cx43, green). The oocytes became detached from the granulosa cells during fixation. Wild-type oocytes recruited Cx37 (arrows) to sites of oocyte-granulosa cell contact (B), whereas granulosa cells contacting Cx37-deficient oocytes (C) or not in contact with oocytes (control, A) exhibited little or no localized Cx37. RT-PCR using Cx37 specific primers was performed with RNA isolated from wild-type mouse lung, lung from Cx37-deficient mice, and cultured mouse granulosa cells (D). Amplicons of the predicted size were found in wild-type lung and granulosa cells, but were absent from lungs obtained from Cx37-deficient mice and when RNA templates were absent (negative control). Bar=10 μm.

for 5 hours, a sufficient time to observe a major reduction in gap-junction plaques at cell-cell interfaces. Treatment with BFA successfully disrupted the Golgi apparatus (Fig. 7C; red) as shown by immunolabelling for the resident Golgi protein giantin, but the localization and distribution pattern for Cx37 (Fig. 7B,D; red) and Cx43 (Fig. 7A,C; green) was not detectably altered. This suggests that the assembled Cx43 and Cx37 gap junctions are not subject to rapid turnover as described in some cell lines.

Discussion

In this study we present convincing evidence that Cx37 homotypic gap junctions provide an essential avenue for communication between the developing mouse oocyte and the granulosa cells immediately surrounding it. In addition, our results indicate that Cx37 is also expressed in granulosa cells more distant from the oocyte, but not stabilized for assembly into gap-junction plaques. Finally, these studies highlight the finding that cell-cell contacts, in addition to connexin compatibility, regulate the connexins that will assemble into stable gap junctions between adjacent cells.

The main objective of this study was to develop a primary culture model of the COC that could be used to examine the localization and functional importance of Cx37 and Cx43, which ultimately would elucidate their respective roles in the developing follicle. Therefore, we developed a new anti-Cx37 antibody that was shown to be effective and specific for localizing Cx37 in cell lines engineered to express Cx37-GFP, in sectioned ovaries, and also in an in vitro COC culture model. Furthermore, our COC culture model was shown to retain the spatial distribution of connexins found in vivo and the intercellular coupling, supporting its use as an in vitro model of granulosa-oocyte coupling. A modified preloading approach for the in vitro system, where the granulosa cells and the oocyte are isolated independently, was chosen to examine the differential roles of Cx37 and Cx43 in the COC. This model was useful because the connexin complement of each cell type could be manipulated, unlike studies using cultured follicles or COCs from connexin-deficient mice, where both cell types are missing the same connexin.

Using both new and previously characterized antibodies, we localized connexins in our cultured COC model to two distinct regions: gap-junction plaques found between all granulosa cells (Cx43-positive) and those found at the interface between the oocyte and surrounding granulosa cells (Cx37-positive). This is consistent with other results (Simon et al., 1997) obtained from frozen sections of mouse ovaries where diffuse Cx37 immunostaining was also detected in granulosa cells but there was no convincing evidence of gap-junction plaques in this area. These results are in contrast with other published data (Wright et al., 2001) reporting immunolabelling of Cx37 in gap junctions between granulosa cells in antral follicles, but no Cx37 immunolabelling at the oocyte surface. The discrepancy between these results is attributed to differences between strains of mice or to the different specificity of the antibody used in the studies (Wright et al., 2001). Our double-immunofluorescent labelling of COC cultures revealed that Cx37 and Cx43 are not present within the same plaques, consistent with each having a unique physiological role, an idea that is strongly supported by the different ovarian

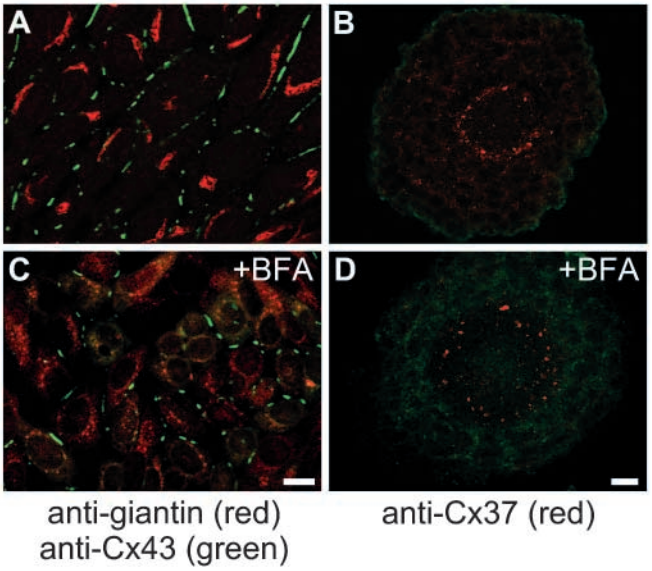

Fig. 7. Distribution/localization of Cx37 is not affected by BFA treatment. Cultured COCs were treated with 10 μg/ml of BFA for 5 hours to block connexin transport and gap-junction plaque regeneration. Immunostaining for anti-giantin (A, red) revealed that the Golgi apparatus was effectively disrupted upon BFA treatment (C, red). However, Cx43 gap-junction plaques between adjacent granulosa cells (A,C; anti-Cx43, green) and Cx37 at oocyte-granulosa cell interfaces (B,D; anti-Cx37, red) were not markedly altered by BFA treatment. To enhance cellular architecture, COCs were double-labelled with a non-specific antibody that provided background immunofluorescence (B,D, green). Bars=10 μm (A,C); 25 μm (B,D).

phenotypes demonstrated by Cx37- and Cx43-deficient mice (Simon et al., 1997; Ackert et al., 2001).

Previous studies using Lucifer yellow dye transfer indicated that Cx37 and Cx43 can form functional heterotypic gap junctions in vivo (Elfgang et al., 1995). We chose to assess functional cell coupling using Lucifer yellow and calcein, another widely used gap-junction-permeable dye. Calcein passed readily between wild-type, denuded oocytes and adjacent wild-type granulosa cells and thence to other granulosa cells, again indicating permeability on the part of both Cx37 and Cx43 channels. When Cx37 was missing from either cell type, however, calcein transfer did not occur. Although a more sensitive assessment by electrical coupling might have indicated residual conductance not detectable by calcein transfer, the most reasonable conclusion is that the normal level of coupling between oocytes and granulosa cells requires this connexin to be present in both cell types, probably forming homotypic channels.

Whereas another study (Elfgang et al., 1995) revealed that it is possible for Cx37 and Cx43 to form functional heterotypic junctions, our evidence suggests that this is not how these connexins are utilized in the mouse COC. This finding has wider implications, as it indicates that compatible connexins (Cx43 and Cx37) may form functional gap-junction channels in cell systems where they are overexpressed in vitro. However, factors beyond the connexins themselves must be involved in defining when compatible connexins will indeed make a heterotypic gap-junction channel in vivo. The fact that both

Cx43 and Cx37 plaques were relatively insensitive to BFA treatment suggests that the plaques that do form are not subject to rapid turnover at the cell surface as reported for some cell types (Laird et al., 1995; Musil and Goodenough, 1991).

The results presented here clearly demonstrate that Cx37 is the predominant (if not the only) connexin responsible for bi-directional gap-junctional communication between the oocyte and surrounding granulosa cells. It is not only necessary for oocyte-granulosa cell coupling and folliculogenesis (Simon et al., 1997), but it is likely to be sufficient as well, since Cx43 is not required. These two pieces of evidence imply that Cx37 and Cx43 are used in the mouse ovarian follicle to create separate 'communication compartments' based on the fact that gap-junction channels composed of different connexins have unique permeability properties (Goldberg et al., 2002; Nicholson et al., 2000). The fact that the connexin composition of gap junctions differs spatially within the follicle implies that molecules could be segregated within either the oocyte or adjacent granulosa cells or the whole granulosa cell population based on differential gap- junction permeability. In this way, an oocyte-derived factor that can traverse Cx37-homotypic junctions may only affect those granulosa cells in direct contact with the oocyte. This could represent a possible mechanism for the parallel development of both the oocyte and granulosa cells, as specific molecules could be separated based on their ability to traverse specific gap-junction types. The identification of these factors will provide invaluable insight into the physiological roles that gap junctions serve in folliculogenesis.

Immunostaining of granulosa cells with anti-Cx37 antibodies did not produce a clear localization pattern, probably due to the fact that few, if any, Cx37 plaques are formed between granulosa cells. Two lines of evidence suggest that granulosa cells do indeed express Cx37 beyond the cell layer adjacent to the oocytes. First, RT-PCR results using specific Cx37 primers indicated that the *Gja4* gene is transcribed in granulosa cells, so it is possible that if the Cx37 protein is being synthesized, it is only stabilized at sites of contact with the oocyte. Secondly, immunofluorescent labelling of granulosa cells that had been cultured with denuded oocytes revealed Cx37 plaque formation where the oocytes had been in contact with the granulosa cells. The plaques did not appear to be localized at boundaries between granulosa cells, implying that they had formed only between granulosa cells and the seeded oocytes. This finding suggests that not only do granulosa cells have the ability to express Cx37, but also that the presence of a contacting oocyte can induce its assembly into gap junctions with the oocyte. Furthermore, no significant Cx37 immunostaining was detected in granulosa cells that had been in contact with Cx37-deficient oocytes for the same culture period. This evidence implies that it is not just the presence of an oocyte that is required to induce assembly of Cx37 plaques in granulosa cells, but more specifically, the oocyte must contain Cx37. The mechanism of this induction remains unknown. The lack of a reliable antibody to detect Cx37 on western blots limited our ability to study Cx37 in granulosa cells further.

Is there an oocyte-derived factor or a specific cell-cell contact event that mediates Cx37 recruitment and/or stabilization? If so, that could explain why Cx37 is not localized in gap-junction plaques between granulosa cells, as this signal may be acting only on those granulosa cells in direct contact with the oocyte. Alternatively, the presence of hemichannels containing Cx37 at the oocyte surface may be the reason that Cx37 is able to stabilize at the surface of granulosa cells contacting the oocyte, and not between other granulosa cells. This explanation is supported by the fact that wild-type oocytes induced Cx37 plaques in granulosa cells, whereas Cx37-deficient oocytes did not. In this scenario, the presence of Cx37 at the oocyte surface is sufficient for the stabilization of Cx37 from the granulosa cells. If this is the case, why is Cx43 not recruited in the same way, when in vitro evidence (Elfgang et al., 1995) suggests that Cx43 and Cx37 can form functional heterotypic junctions? These questions regarding the means by which Cx37 in granulosa cells is selectively recruited to gap junctions with the oocyte are both interesting and important, and require further study.

In summary, the development of an in vitro granulosa cell-oocyte complex primary culture model in which oocytes and granulosa cells can be recombined has provided us with a unique system to examine connexin expression and function in the ovarian follicle. Analysis of intercellular coupling in this system indicated that gap-junctional coupling between oocytes and granulosa cells is via homotypic Cx37 gap junctions. Finally, factors beyond the compatibility of connexin family members to form a heterotypic gap-junction channel are necessary to regulate the temporal and spatial assembly of specific gap-junction channels.

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