

Oocyte-directed depletion of connexin43 using the Cre-LoxP system leads to subfertility in female mice

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Abstract

Gap junctions, predominately comprising connexin43 (Cx43), mediate cell-to-cell communication within the ovarian follicle. However, the partaking of Cx43 in the formation of the gap junction channels, between the oocyte and the somatic cells, is controversial. We addressed this dispute by crossing females that carry a Cx43 coding region, flanked by loxP recognition sites, with males expressing the Cre recombinase under the control of *Zp3* promoter. Oocytes of the resultant *Zp3Cre;Gjal^{lox/lox}* mice did not express Cx43 and were referred to as *Cx43^{del/del}*. Unexpectedly, a decrease in Cx43 was observed in cumulus/granulosa cells of some follicles as well. Nevertheless, no histological abnormalities were detected in the ovaries of the *Zp3Cre;Gjal^{lox/lox}* mice. Furthermore, these mice ovulated normally and developed fully functional corpora lutea. Additionally, the ovarian *Cx43^{del/del}* oocytes were meiotically arrested and transferred Lucifer yellow to the surrounding cumulus cells. However, mating *Zp3Cre;Gjal^{lox/lox}* females with wild-type males resulted in a reduced rate of parturition and a substantial decrease in litter size. Further examination revealed that although preimplantation development of *Zp3Cre;Gjal^{lox/+}* embryos was normal, the blastocysts exhibited impaired implantation. Our data suggest that total ablation of Cx43 in the oocyte, combined with its decrease in the surrounding somatic cells, allows normal oogenesis and folliculogenesis, ovulation and early embryonic development but severely impairs the implantation capacity of the resulting blastocysts.

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Introduction

The ovarian follicle consists of two major cellular compartments: the somatic cumulus/granulosa cells and the female gamete. Gap junction-mediated cell-to-cell communication between these two compartments allows the ovarian follicle to function as one physiological unit. Specifically, it has been demonstrated that oocyte growth and development are dependent on the transmission of nutrients from the follicle cells via gap junctions (reviewed in Buccione et al., 1990). Moreover, it

has been shown that the meiotic status of the oocyte is subjected to regulation by its communication with the somatic follicle cells (Dekel and Piontkewitz, 1991; Sela-Abramovich et al., 2006). Other studies suggested that oocytes do not only receive but also provide regulatory signals that control folliculogenesis (Canipari et al., 1995; Li and Mather, 1997; Vanderhyden et al., 1993). Some of these messages could possibly be transmitted via gap junctions.

Gap junctions are transmembrane channels; each gap junction comprises two hemi-channels, referred to as connexons, contributed by opposing membranes of adjacent cells. Every connexon consists of six connexin (Cx) subunits organized around a central pore. The hydrophilic pore of the channel allows the transcellular flow of ions and small molecules of up to 1.8 kDa, such as cyclic nucleotides (cAMP and cGMP), inositol

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phosphates and calcium ions. The Cx family of proteins in the mouse includes at least 20 members, the homologues of which are present in other vertebrate species. Connexins exhibit a tissue-specific, developmentally regulated expression (Granot and Dekel, 1998; Rozental et al., 2000; Shibata et al., 2001).

Several Cxs have been detected in ovarian follicles of different species, among which the indispensability of Cx37 (encoded by the gene *Gja4*) and Cx43 (encoded by the gene *Gjal*) has been clearly demonstrated. In Cx37-deficient mice, both the ovarian follicles and oocytes were arrested at an early stage of their development, pointing towards the major role of this protein in establishing bidirectional communication (Simon et al., 1997). Mice that lack Cx43 die soon after birth as a result of cardiac malfunction (Reaume et al., 1995), precluding determination of its function in ovarian development. In order to circumvent postnatal lethality, analysis of the role of Cx43 in folliculogenesis utilized ovaries removed from prenatal Cx43 knockout mice that were allowed to further develop either in vitro, in organ culture, or in vivo, under the kidney capsule of wild-type female mice. Under both experimental conditions, folliculogenesis in Cx43-deficient ovaries did not proceed beyond the primary follicle stage, and the oocyte growth was retarded (Ackert et al., 2001; Juneja et al., 1999). These studies indicated that Cx43 plays a major role in germ cell development and ovarian folliculogenesis.

Identification, localization and regulation of Cx43 have been extensively investigated in the somatic compartment of the ovarian follicle. The data generated by these studies strongly support the idea that this Cx predominantly participates in the network of cell-to-cell communication established in the cumulus/granulosa cells. However, the information regarding the identity of the specific Cx, contributed by the oocyte to form the gap junctions with its neighboring somatic cells, is somewhat controversial. Using several complementary techniques, we detected the presence of the Cx43 mRNA and its protein product in rat zona pellucida-free oocytes at different stages of their development (Granot et al., 2002). Our findings confirmed earlier studies demonstrating expression of the Cx43 mRNA and protein in mouse and cattle oocytes, respectively (Kehler et al., 2004; Sutovsky et al., 1993; Valdimarsson et al., 1993). However, these reports were not supported by other studies that failed to detect the presence of Cx43 protein in mouse oocytes (Simon et al., 1997; Veitch et al., 2004). If indeed Cx43 is expressed by the oocyte, it may take part in the formation of the gap junction channels between the female gamete and the somatic cells of the ovarian follicle. In that case the oocyte Cx43 could play a role in regulating normal ovarian development and function. A definitive evidence to support this possibility could be provided by oocyte-specific ablation of the *Gjal* gene.

In order to investigate the role of the oocyte Cx43, we used the Cre-LoxP system for targeting the deletion of Cx43 specifically at the oocyte. For this purpose, a mouse line carrying a *Gjal* coding region, flanked by LoxP recognition sites for the Cre recombinase (Theis et al., 2001), was crossed with mice in which Cre expression is controlled by regulatory sequences from the zona pellucida 3 (*Zp3*) gene; This gene is

expressed exclusively in the oocyte (de Vries et al., 2000). Here we discovered that the excision of the *Gjal* gene was not restricted to the oocyte but, to some extent, also took place in the follicle cells. Nevertheless, the complete deletion of *Gjal* gene in the oocyte, combined with its reduced expression in the follicle cells, did not affect oogenesis, folliculogenesis and ovulation. The ovulated oocytes were successfully fertilized and the early embryos developed normally. The subfertility exhibited by these mice apparently represents the poor implantation capacity of the resulting blastocysts.

Materials and methods

Animals

C57BL/6-Tg(*Zp3-cre*)3Mrt/J transgenic male mice expressing the Cre recombinase, under the control of *Zp3* promoter (de Vries et al., 2000), were purchased from Jackson Laboratories. Cx43^{lox/lox} animals (Theis et al., 2001) were kindly provided by Klaus Willecke, University of Bonn, Germany. The experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academy of Science, Bethesda, MD).

Genotype analysis

Mouse tail genomic DNA was extracted, using DirectPCR kit (Viagen) according to the manufacturer's protocol and genotype by PCR. Cx43^{lox/lox} mice were genotyped by PCR analysis, using primers UMP (5'-TCATGCCCGGCA-CAAGTGAGAC-3') and UMPR (5'-TCACCCCAAGCTGACTCAACCG-3'). PCR reaction for the Cx43^{lox/lox} transgene was carried out for 35 cycles (initial denaturation at 94 °C for 3 min, then 35 cycles at 92 °C for 1 min, 65 °C for 1.5 min, 72 °C for 2 min and a final incubation at 72 °C for 10 min). The PCR conditions for genotyping the Cre transgene in *Zp3-Cre* mice were described previously (Lan et al., 2004).

Meiotic status assessment

Mammalian oocytes enter meiosis during embryonic life, proceed to the diplotene stage of the first prophase and are arrested at around birth. Meiotically arrested oocytes are characterized by diffused chromosomes, surrounded by an intact nuclear membrane termed "germinal vesicle" (GV). Resumption of meiosis, also known as oocyte maturation, is triggered in vivo by the estrus surge of LH. This process involves chromatin condensation, dissolution of the nuclear membrane (germinal vesicle breakdown, GVB) and formation of the first metaphase (MI) spindle. The first meiotic division is completed by segregation of the homologous chromosomes and the emission of the first polar body (PBI). Immediately thereafter, the oocytes progress to the second meiotic division where they are once again arrested at the second metaphase (MII). Morphological examination for either the presence of GV or its absence (GVB) and the presence of PBI were employed for the assessment of the meiotic status of the oocytes.

Oocyte recovery

Sexually immature 23-day-old female mice were injected with 5IU of pregnant mare's serum gonadotropin (PMSG Chrono-gest Intervet, The Netherlands) for the recruitment of follicles to develop to the antral stage. Euthanasia was performed by cervical dislocation 48 h later and the ovaries were removed. Follicles were punctured and the cumulus–oocyte complexes (COC) recovered and placed into acidic L-15 medium (pH 6.0) to remove the cumulus cells. The cumulus-free oocytes were monitored microscopically, using differential interference contrast (DIC) optics for reinitiation of meiosis, while employing the morphological parameters described previously. Ovulation was obtained by injecting 5IU of human chorionic gonadotropin (hCG, Chrono-gest Intervet, The Netherlands) 48 h after PMSG administration. Euthanasia was

Table 1
List of primers and number of cycles used for PCR analysis

Gene name	Primer forward	Primer backward	No. of cycles
Cx43	CTTCCAGTATCATTGTTGGAAAAG	TGGATTGTTCTTCATCTC	20
Cx37	CTTCGTGACACACCCACCC	TACAAATGGGGGAGGTCTGG	26
Cre	ATGCTTCTGTCCGTTTGCC	CGCTCGACCAGTTTAGTTACC	35
β -actin	CCCATTGAACATGGCATTGTTAC	TTGATGTCACGCACGATTTC	20

performed by cervical dislocation 24 h later and the number of oocytes present in the oviduct was counted.

Zona-free oocytes were isolated as described previously in rat oocytes (Granot et al., 2002).

LacZ staining

Staining was performed, as described previously (Jorgez et al., 2004). Briefly, ovaries were fixed in 0.2% glutaraldehyde (Sigma) for 4 h on ice with shaking. Tissues were bisected after the first hour. The ovaries were washed three times for 15 min each in wash buffer (2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% nonident-P40 in PBS). Staining was carried out in 0.5 mg/ml X-gal, 5 mM potassium ferrocyanid and 5 mM potassium ferricyanide in wash buffer at room temperature for 4 h, with shaking and protection from light. Samples were then washed three times for 10 min each in PBS. Stained ovaries were paraffin embedded and sections of 7 μ m were made. LacZ stained sections were visualized using an E600 microscope (Nikon).

Follicle culture

Ovaries were recovered from PMSG-primed female mice and the intact ovarian follicles were isolated and incubated in L-15 tissue culture medium containing 5% fetal bovine serum (FBS, both purchased from Biological Industries, Israel) in 25 ml flasks gassed with 50% O₂ and 50% N₂ (Sela-Abramovich et al., 2006). Incubations were carried out at 37 °C in an oscillating water bath in the presence or absence of 1 μ g/ml of ovine LH (o-LH, NIH LH S-24). At the end of the incubation period the follicles were incised and the COCs were recovered. The oocytes were monitored microscopically, using DIC optics for reinitiation of meiosis, as described previously.

Reverse transcription analysis

Total RNA was extracted from 120 oocytes, using tri-reagent (Sigma). At the end of the procedure, the RNA pellet was dissolved in 9 μ l DDW. Reverse transcription (RT) was performed by mixing 8 μ l RNA with 4 μ l of MMLV-RT 5 \times buffer (Promega), 10 mM deoxynucleotide triphosphates (dNTP), 1 μ l of oligo (dT)_{12–18} (Promega), 40 units of RNasin a RNases inhibitor (Promega), 200 units of Moloney murine leukemia virus reverse transcriptase (M-MLV Reverse transcriptase, Promega) and 3 μ l of DDW. This mixture was incubated at 37 °C for 2 h.

Ovaries were isolated and total RNA was extracted, using tri-reagent (Sigma). Reverse transcription (RT) reaction was performed by dissolving 7.5 μ g RNA in 11 μ l DDW. The rest of the procedure was performed, as described above.

PCR reaction

The above cDNAs were used for PCR amplification with primer sets for Cx37, Cx43, Cre and β -actin (Table 1) in a 25 μ l reaction volume with 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100 (Promega, Madison, WI), 2.5 mM MgCl₂, 400 μ M each d-NTP and 0.625 units of Taq DNA Polymerase (Promega, Madison, WI). PCR was performed for the indicated number of cycles (Table 1, initial denaturation at 94 °C for 3 min, then 20–35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final incubation at 72 °C for 7 min). The number of cycles used ensured that the reaction could be quantified within the log phase of the amplification reaction.

The reaction mix (24 μ l) was run on a 1.5% agarose gel stained with ethidium bromide and quantified using UV imaging (Gel Doc 1000, Bio-Rad, Hercules, CA) and Molecular Analyst software (Bio-Rad, Hercules, CA). Experimental replication of each time point was performed in triplicate for all three sets.

Culture of granulosa cells and genomic DNA analysis

Granulosa cells were recovered from the ovaries of the abovementioned female mice. The cells were plated onto serum-coated wells (equivalents of two ovaries per six wells) in 24-multiwell plates (16 mm; Nunc, Copenhagen, Denmark) containing 0.5 ml of L15 tissue culture medium. The plates were incubated in a humidified incubator at 37 °C for 3 days. The cells were harvested and genomic DNA was extracted, using MasterPure complete DNA and RNA purification kit (Epicentre). PCR analysis was performed, using Cx43 deletion specific primers (forward: 5'-GGCATAACAGACCCCTTGGACTCC-3', reverse: 5'-TGCGGGCCTCTTCGCTATTACG-3'). The reaction mix (24 μ l) was run on a 1.5% agarose gel stained with ethidium bromide and quantified using UV imaging (Gel Doc 1000, Bio-Rad, Hercules, CA).

Western blot analysis

Proteins were extracted from follicles isolated from ovaries 48 h after PMSG injection, and Laemmli buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β -mercaptoethanol) was added. The samples were then boiled for 5 min and samples of 40 μ g protein was electrophoretically separated on a 12% acrylamide gel followed by their transfer to a nitrocellulose membrane. After blocking with 5% skimmed milk, the membranes were incubated with anti-CX43 antibody (BD Bioscience) overnight at 4 °C followed by incubation with the secondary antibodies for 1 h at room temperature. The immunoreactive bands were detected by ECL (Amersham). Anti- β -tubulin antibody (Sigma) was used as a marker for equal protein amount loading on the gel. The intensity of the signal was quantified by computerized densitometry (quantity one).

Histology

Ovaries obtained from the abovementioned female mice were fixed in paraformaldehyde (BDH chemicals) for 24 h and paraffin embedded. Sections of

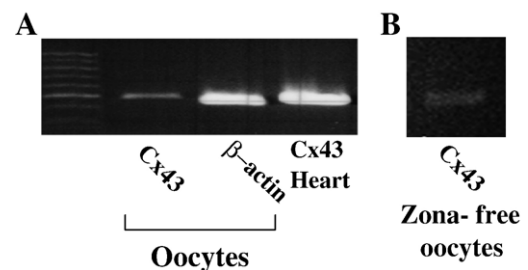


Fig. 1. Cx43 expression in the oocyte. (A) Cx43 mRNA. RNA was extracted from wild-type animals. RT-PCR analysis was performed using primers for Cx43 and β -actin. One representative out of at least three independent experiments with similar results is presented. (B) RNA was extracted from zona-free oocytes. RT-PCR analysis was performed using primers for Cx43.

5 μm were mounted and the slides were either stained by hematoxylin and eosin (H&E) or processed for immunofluorescence analysis. Assessment of the distribution of follicles among the different developmental stages was performed, as described previously (Israely et al., 2003).

Immunofluorescence

Cx37 and Cx43 immunofluorescence was performed on deparaffinized sections washed in tap water and PBS. Blocking of non-specific binding sites was obtained by incubating the sections for 30 min in 3% fetal calf serum in PBS. Sections were then incubated overnight at 4 °C with either anti-Cx37 (1:300, Alpha Diagnostic international) or anti-Cx43 antibody (1:300, transduction

laboratories), washed with PBS and immunoreacted with the Alexa 488-conjugated secondary antibody for 1 h at room temperature. The sections were washed three times with PBS and visualized, using fluorescence microscope (Nikon). All images were taken under identical conditions.

Dye transfer

Dye injections into oocytes surrounded by their cumulus cells were performed, as described previously (Veitch et al., 2004). Briefly, COCs isolated from ovaries of PMSG-primed female mice were microinjected with 5% Lucifer yellow (Molecular Probes, Eugene, OR) in ddH₂O for 1 min. Injections resulted in dye filling of the oocyte and in the indicated cases further spread of the dye to

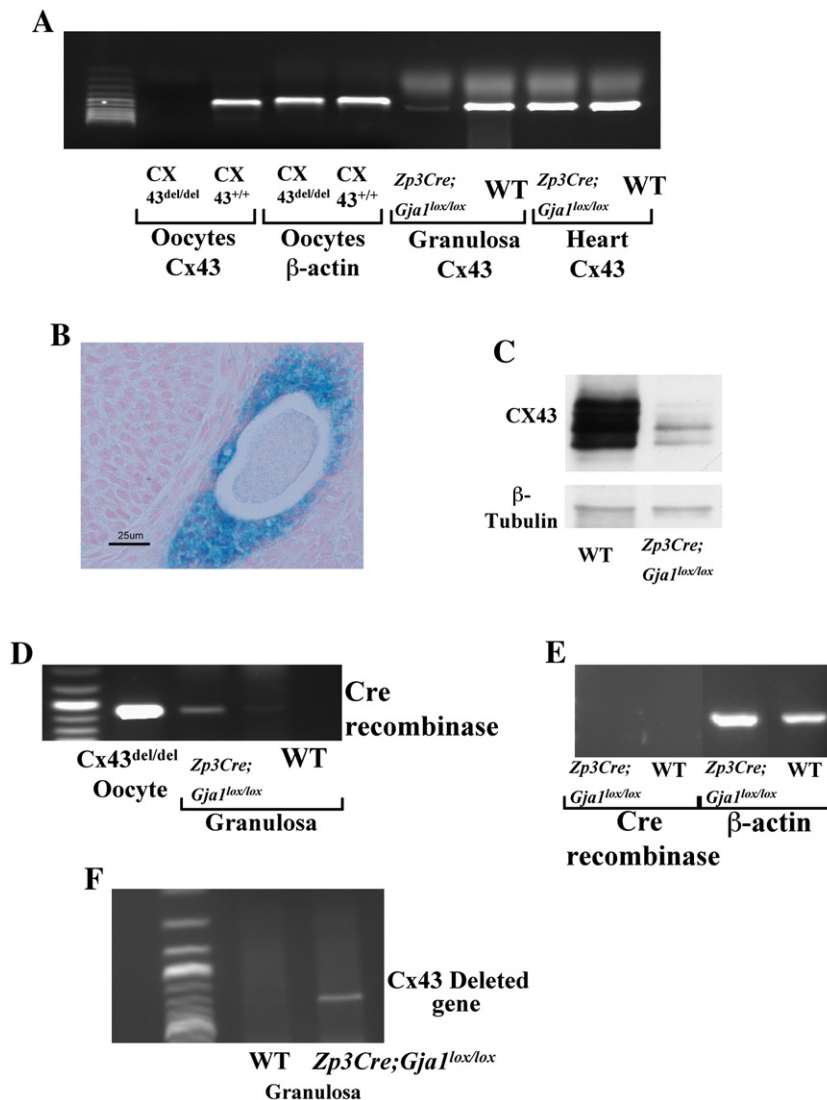


Fig. 2. Depletion of Cx43 in the ovary using the Cre-LoxP system. (A) Ablation of Cx43 in the ovary. RNA was extracted from oocytes, granulosa cells and heart of either *Zp3Cre;Gja1^{lox/lox}* or WT animals. RT-PCR analysis was performed using primers for Cx43 and β -actin. One representative out of at least three independent experiments with similar results is presented. (B) LacZ reporter gene expression after Cre excision. PMSG-primed *Zp3Cre;Gja1^{lox/lox}* ovaries were sectioned and stained for LacZ expression as described in Materials and methods. Note staining in somatic follicle cells. Scale bar—25 μm . (C) Western blot analysis of Cx43 protein expression in WT and *Zp3Cre;Gja1^{lox/lox}* intact ovarian follicles. One representative out of two independent experiments, in which a total of 42 large antral follicles recovered from 6 individual mice for each group with similar results, is presented. (D, E) Expression of the enzyme Cre recombinase in granulosa cells (D) and in embryo implantation site of E5.5 pregnant females (E) of *Zp3Cre;Gja1^{lox/lox}* animals. RNA was extracted from granulosa cells and RT-PCR analysis was performed using primers for Cre recombinase. One representative experiment out of a total of three independent experiments with similar results is presented. (F) Deletion of the *Gja1* gene in the genome of granulosa cells of *Zp3Cre;Gja1^{lox/lox}* animals. Genomic DNA was extracted from a primary culture of granulosa cells. RT-PCR was performed using specific primers for the deletion site of *Gja1* in the genome. One representative experiment out of a total of three independent experiments with similar results is presented.

the cumulus cells. The experiments were recorded by a confocal microscope (Zeiss). The fact that the spread of dye observed reflected transfer through gap junctions was confirmed by the concomitant injection of rhodamine dextran, which cannot pass the gap junction channels due to its high molecular weight.

Fertility and parturition evaluation

To assess their fertility, 2 month old *Zp3Cre;Gja1^{lox/lox}* females were mated with either C57bl/6 wild-type (WT) or *Cx43^{lox/lox}Cre^{-/-}* males. Each male was housed with 5 females in a cage for 2 months. Each experiment included four WT or *Zp3Cre^{-/-};Gja1^{lox/lox}* males. The number of pups of each female was counted and recorded.

Preimplantation embryo collection

To monitor preimplantation development in vivo, embryos were washed from the oviduct and/or the uterus at E1.5, E2.5 or E3.5 of pregnancy (vaginal plug=E0.5, indicating mating had occurred the night before) using M2 medium (Sigma-Aldrich Corp. St. Louis, MO, USA) and their developmental stage was evaluated, using standard light microscopy.

Evaluation of embryo implantation

On E4.5, mice were intravenously injected with Evans blue (Sigma); 1% w/v in saline, 100 μ l (Greep Ro and Geiger, 1973). Evans blue binds serum proteins and accumulates around the implanting embryos (Plaks et al., 2006). Mice were then sacrificed 10 min after injection and implantation sites were counted. A mouse was considered pregnant upon the appearance of at least one implantation site.

Determination of serum progesterone

Serum progesterone (P4) was determined using time-resolved fluorescence immunoassay as follows: progesterone was extracted from the serum using petroleum ether which was subsequently evaporated. The extracts were reconstituted with assay buffer (0.05 M Tris-HCl, 0.5% BSA, 0.02 M NaCl, 0.1% Tween-20, pH 7.7). ELISA plates were coated with rabbit anti-mouse immunoglobulins (Dako, Gostrup, Denmark) and blocked (2% BSA). Progesterone standards (1–320 pg in 50 μ l/well) or samples (50 μ l/well) were diluted in assay buffer and incubated for 30 min at room temperature with 50 μ l highly specific anti-P4 monoclonal antibodies (final dilution 1:100,000). The anti-P4 antibodies (clone 1E11, de Boever et al., 1994) were generously provided by Dr. Fortune Kohen (The Weizmann Institute of Science, Rehovot, Israel). Europium-P4 (50 μ l/well, PerkinElmer Life and Analytical Sciences Inc. (Wallac), Wellesley, MA) was then added for an additional 1 h of incubation. After washing (0.02 M NaCl, 0.05% Tween-20), enhancement solution (200 μ l/well) was added and incubated for 10 min and the fluorescence was then measured using the Victor2 1420 multilabel counter (PerkinElmer Life and Analytical Sciences Inc. (Wallac), Wellesley, MA). Each sample was analyzed in triplicates.

Statistical analysis

Each experiment was carried out at least three times, with at least 3 to 4 mice at each time point. Data points are presented as mean \pm SE. Statistical significance was done using Student's 2 tailed unpaired *t*-test (Microsoft Excel).

Results

Confirmation and characterization of the *Cx43* targeted deletion

The controversial information regarding the expression of *Cx43* by mouse oocytes led us to revisit this issue. Using RT-PCR, we demonstrated that mouse oocytes do express the

Cx43 mRNA (Fig. 1A). Furthermore, in order to exclude the possibility that the transcript detected in the oocytes may represent *Cx43* mRNA from the cumulus cell processes in the zona pellucida, RT-PCR was performed on zona-free oocytes. We demonstrated that zona-free oocytes also express *Cx43* mRNA (Fig. 1B).

In order to generate a mouse line that bears *Cx43* deficient oocytes, female mice carrying a *Gja1* coding region, flanked by loxP (Theis et al., 2001), were crossed with males in which Cre recombinase expression is controlled by *Zp3* regulatory sequences. Genotyping of the resultant offspring revealed the expected Mendelian distribution between WT, heterozygous *Cx43^{lox/+}* and homozygous *Cx43^{lox/lox}* (1:2:1 respectively) individuals. RT-PCR analysis demonstrated that the oocytes of the *Zp3Cre;Gja1^{lox/lox}* mice indeed failed to express *Cx43* (Fig. 2A). These oocytes are referred to as *Cx43^{del/del}* throughout this report. Unexpectedly, a substantial decline in the *Cx43* mRNA levels in the ovarian somatic cells, in addition to the

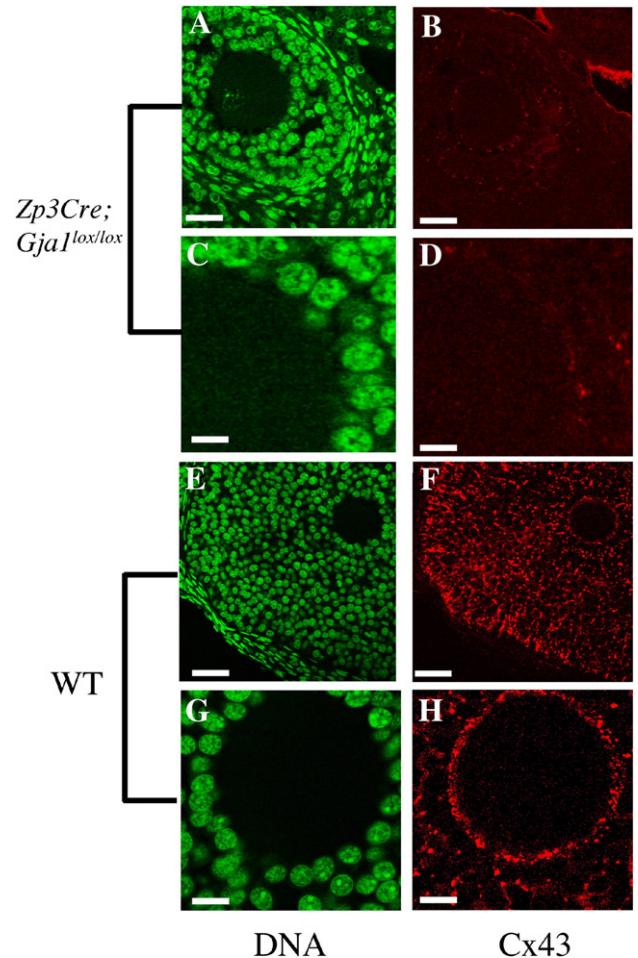


Fig. 3. *Cx43* protein in the ovaries of *Zp3Cre;Gja1^{lox/lox}* animals. Ovarian sections of PMSG-primed *Zp3Cre;Gja1^{lox/lox}* mice were stained for DNA (A) or incubated with anti-*Cx43* antibody (B). Scale bars A, B—30 μ m. A closer view on a representative cumulus–oocyte complex of *Zp3Cre;Gja1^{lox/lox}* animals (C, D). Scale bars C, D—9 μ m. Ovarian sections of WT of PMSG-primed mice were stained for DNA (E) or incubated with anti-*CX43* antibodies (F). Scale bars E, F—50 μ m. A closer view on a representative WT cumulus–oocyte complex (G, H). Scale bars G, H—15 μ m.

complete deletion of Cx43 in the oocytes, was also observed (Fig. 2A). Heart Cx43 mRNA of the *Zp3Cre;Gjal^{lox/lox}* mice did not differ from that of WT animals (Fig. 2A).

A cryptic lacZ reporter gene, expressed under the control of the endogenous Cx43 regulatory elements, was engineered in the previously mentioned *Cx43^{lox/lox}* mice (Theis et al., 2001). Accordingly, Cre mediated excision of the “floxed” Cx43 gene in the *Cx43^{del/del}* oocytes was demonstrated by their positive blue staining (Fig. 2B). However, careful examination of the X-gal treated ovarian sections revealed some staining in the cumulus/granulosa cells of a few of the follicles and confirmed our RT-PCR results (Fig. 2B). The reduction in CX43 expression in both the oocyte and the granulosa cells was further examined in WT and *Zp3Cre;Gjal^{lox/lox}* isolated intact ovarian follicles. Western blot analysis demonstrated a lower level of CX43 protein expression in *Zp3Cre;Gjal^{lox/lox}* follicles as compared to WT (Fig. 2C). Quantitation of the Western blot signal obtained from the analysis of 42 individual large antral follicles,

recovered from each group, *Zp3Cre;Gjal^{lox/lox}* and WT mice revealed an $88 \pm 1.4\%$ reduction.

Our results that Cx43 mRNA is partially depleted in the ovarian somatic cells are in accordance with complementary findings that Cre mRNA was expressed in the granulosa cells of *Zp3Cre;Gjal^{lox/lox}* mice, but not in that of the WT animals (Fig. 2D). However, Cre was not expressed in E5.5 embryo implantation site of *Zp3Cre;Gjal^{lox/lox}* females that were mated with WT males (Fig. 2E). Moreover, PCR analysis of genomic DNA using specific primers for the *Gjal* allele detected deletion of the *Gjal* gene in the granulosa cells of the *Zp3Cre;Gjal^{lox/lox}* mice (Fig. 2F). These results were also supported by immunofluorescence staining of ovarian sections, using anti-Cx43 antibody. As expected, expression of Cx43 could not be detected in the *Cx43^{del/del}* oocytes. However, a relatively lower availability of the protein was also observed in the somatic compartment of the corresponding ovaries (Figs. 3A–D), as compared to that of wild types (Figs. 3E–H).

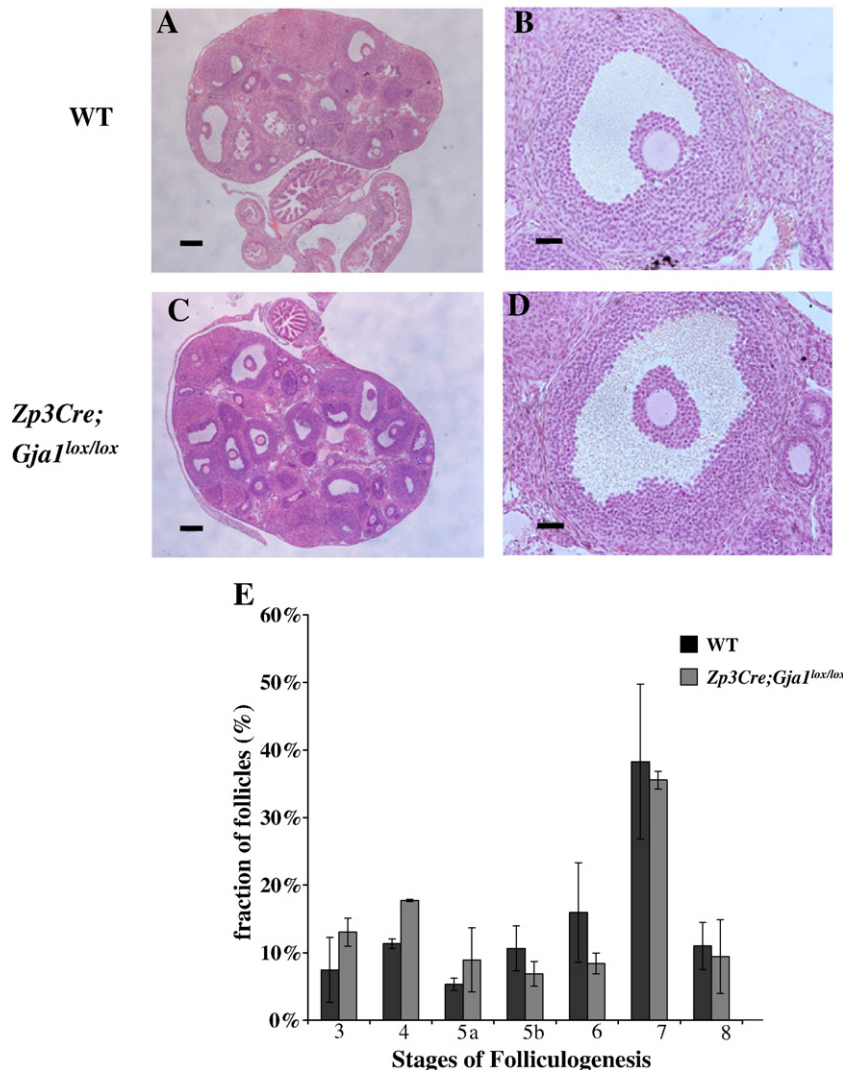


Fig. 4. Histological analysis of ovaries of PMSG-primed mice. Wild-type (A, B) and *Zp3Cre;Gjal^{lox/lox}* (C, D) animals were injected with PMSG. The ovaries were removed 48 h later, fixed and sectioned. Scale bar in panels A and C—200 μ m. Scale bar in panels B and D—50 μ m. (E) Quantitative analysis of the follicles at different stages of folliculogenesis. Ovaries from 4 WT and 4 *Zp3Cre;Gjal^{lox/lox}* mice were examined as described in Materials and methods.

Histological analysis of the ovaries and assessment of the meiotic status of the *Cx43^{del/del}* oocytes

In view of the previously demonstrated indispensability of *Cx43* for normal folliculogenesis and oogenesis (Ackert et al., 2001; Juneja et al., 1999), it was quite surprising that histological analysis of the ovaries of our *Zp3Cre;Gja1^{lox/lox}* mice revealed no abnormalities (Figs. 4A–D). Furthermore, the follicles did not show any obvious growth restriction; as a consequence, the distribution of follicles among the different developmental stages and the fraction of Graafian follicles observed in PMSG-primed *Zp3Cre;Gja1^{lox/lox}* and the WT mice were very similar ($35.6\% \pm 1.3\%$ and $38\% \pm 11\%$ respectively, Fig. 4E).

It is from around birth that all oocytes in mammalian ovaries are arrested at the diploten of the first meiotic prophase. Fully grown oocytes exit meiotic arrest upon their release from the ovarian follicle. This observation raised the idea that a meiosis inhibitor of a follicle origin is transferred to the oocyte through gap junctions (reviewed by Sherizly et al., 1988). In that case, impaired junctional communication should reverse the state of meiotic arrest. Morphological analysis of oocytes performed immediately upon their release from the ovarian follicles revealed that, similar to the *Cx43^{+/+}*, the *Cx43^{del/del}* oocytes were meiotically arrested, as indicated by the presence of an intact GV (Fig. 5A).

The ability of the oocyte lacking *Cx43* to resume meiosis in response to LH was further examined in vitro, in isolated intact ovarian follicles exposed to this gonadotrophin. Confirming the abovementioned findings, in the absence of LH, almost all oocytes were meiotically arrested. Upon the addition of LH to the culture medium, $85\% \pm 9\%$ of the *Cx43^{del/del}* oocytes resumed meiosis. This response was not significantly different from that of the *Cx43^{+/+}* oocytes ($80\% \pm 13\%$, Fig. 5B).

Dye transfer analysis in cumulus–oocyte complexes

Our findings that *Zp3Cre;Gja1^{lox/lox}* females exhibit a normal ovarian phenotype, populated by meiotically arrested oocytes, raised some intriguing questions with regard to cell-to-cell communication between the *Cx43^{del/del}* oocytes and the surrounding cumulus cells. We found that Lucifer yellow, a small molecular weight marker that flows through gap junctions, microinjected into *Cx43^{del/del}* oocytes, was clearly transferred to the cumulus cells (Fig. 6B). No transfer of a high molecular weight marker was observed (Figs. 6C, F). The expression of *Cx37* mRNA (Fig. 6G) and localization of its corresponding protein in the oolema of the *Cx43^{del/del}* oocytes were not affected (Figs. 6H–K). This may suggest that *Cx37* contributes to the gap junctional communication in *Cx43* depleted mouse oocytes.

Examination of ovulation in *ZP3Cre-Cx43^{lox}* mice

For induction of ovulation, *Zp3Cre;Gja1^{lox/lox}* and WT female mice were primed with PMSG followed by hCG administration, as described in the Materials and methods section. A full ovulatory response was obtained in all animals examined (8/8 WT

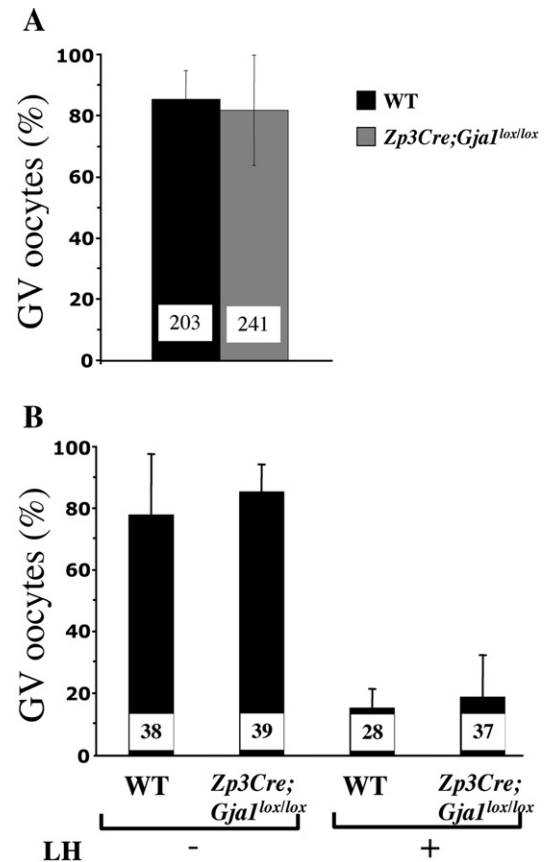


Fig. 5. Assessment of the meiotic status of the *Cx43^{del/del}* oocytes. (A) Oocytes in the ovaries are meiotically arrested. Oocytes were released from ovaries of PMSG-primed either WT or *Zp3Cre;Gja1^{lox/lox}* mice. The oocytes were monitored immediately for their meiotic status as indicated by the presence or the absence of GV. Number of oocytes is indicated at bottom of columns. (B) LH-induced resumption of meiosis. Follicles were isolated from ovaries of PMSG-primed either WT or *Zp3Cre;Gja1^{lox/lox}* mice. The follicles were incubated in the presence or absence of LH (1 μ g/ml) for 5 h. At the end of incubation, the oocytes were released and monitored for their meiotic status indicated by the presence or the absence of GV. Total number of follicles/oocytes examined in each group is indicated.

mice and 10/10 *Zp3Cre;Gja1^{lox/lox}* animals). The rate of ovulation in WT was similar to that of *Zp3Cre;Gja1^{lox/lox}* animals (Fig. 7A). The ovulated oocytes of both groups completed the first meiotic division, as indicated by the presence of PBI. The cumulus cells, encapsulating both *Cx43^{+/+}* and *Cx43^{del/del}* oocytes, were expanded. In accordance, no histological differences were found between the ovulating ovaries of WT and *Zp3Cre;Gja1^{lox/lox}* female mice (Figs. 7B–D, E–G, respectively). Specifically, corpora lutea were observed in ovarian sections of both types of animals (Figs. 7B, C, E, F) and ovulated oocytes were detected in their oviducts (Figs. 7D and G).

Fertility evaluation of *Zp3Cre;Gja1^{lox/lox}* mice

Despite the apparent normal folliculogenesis and oogenesis, *Zp3Cre;Gja1^{lox/lox}* females exhibited a significant reduction ($p < 0.05$) in the rate of parturition (Table 2) and a significant ($p < 0.05$) decrease in litter size. Specifically, upon mating of

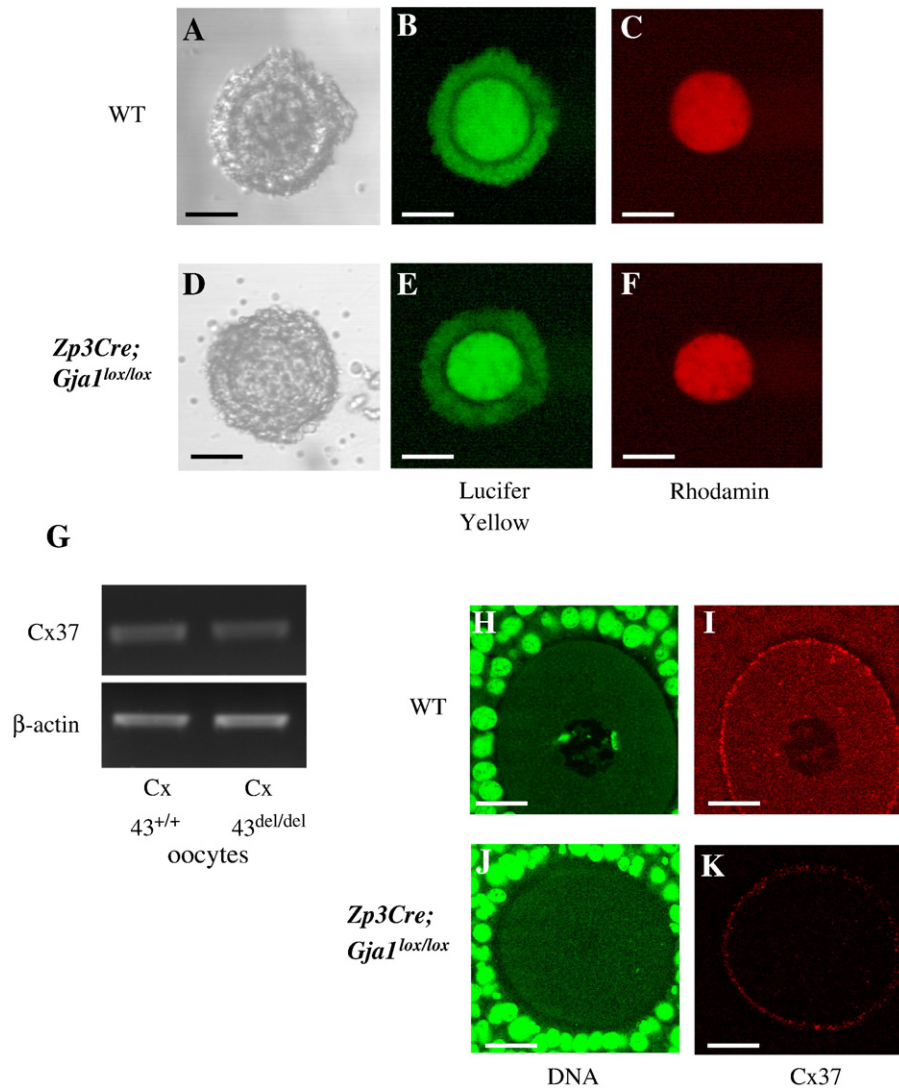


Fig. 6. Dye transfer in the cumulus–oocyte complex. WT (A) and *Cx43^{del/del}* (D) oocytes surrounded by their cumulus were isolated and injected with Lucifer yellow (B, E). Rhodamine, a high molecular weight marker that is not transferred via gap junctions, served as a negative control (C, F). Scale bars A to F—50 μ m. (G) Expression of Cx37 mRNA in the ovaries of ZPCre-Cx43^{lox} mice. RNA was extracted from oocytes of *Zp3Cre; Gja1^{lox/lox}* and WT animals and RT-PCR analysis was performed using primers for Cx37 and β -actin described in Table 1. One representative experiment out of three independent experiments with similar results is presented. (H–K) Localization of the Cx37 protein in the ovary. Ovarian sections of PMSG-primed either WT or *Zp3Cre; Gja1^{lox/lox}* mice were stained for DNA (H and J) or incubated with anti-CX37 antibody (I and K). Scale bars H to K—20 μ m.

these females with *Zp3Cre^{-/-}; Gja1^{lox/lox}* males, an almost 40% reduction in litter size was observed as compared to WT females (Table 2). These males are considered as WT since the *Gja1* gene is expressed normally in the absence of Cre recombinase. As an additional control, *Zp3Cre; Gja1^{lox/lox}* females were mated with C57bl WT males (Table 2). The reduction in litter size in that case was 60%. The surviving pups of the *Zp3Cre; Gja1^{lox/lox}* females developed normally; their weight was similar to that of pups born to WT animals and no histological abnormalities were detected in the different tissues examined.

Preimplantation embryo collection

To monitor early embryonic development in vivo, embryos were washed from the oviduct and/or the uterus at E1.5, E2.5 or

E3.5 of pregnancy (embryos cannot be washed from the uterus at E4.5 since they undergo implantation at that time). There was no significant difference in the mean number of embryos at each developmental stage in *Zp3Cre; Gja1^{lox/lox}* as compared to WT females (Fig. 8).

Assessment of implantation rate

Embryo implantation was assessed in vivo on E4.5 as described in Materials and methods. The mean number of implantation sites detected in *Zp3Cre; Gja1^{lox/lox}* females (3.7 ± 0.7) after mating with *Cx43^{lox/lox}/Cre^{-/-}* males was significantly reduced (49%) compared with detected in WT females (7.5 ± 1.1 , $p=0.009$) mated with the same males (Fig. 9).

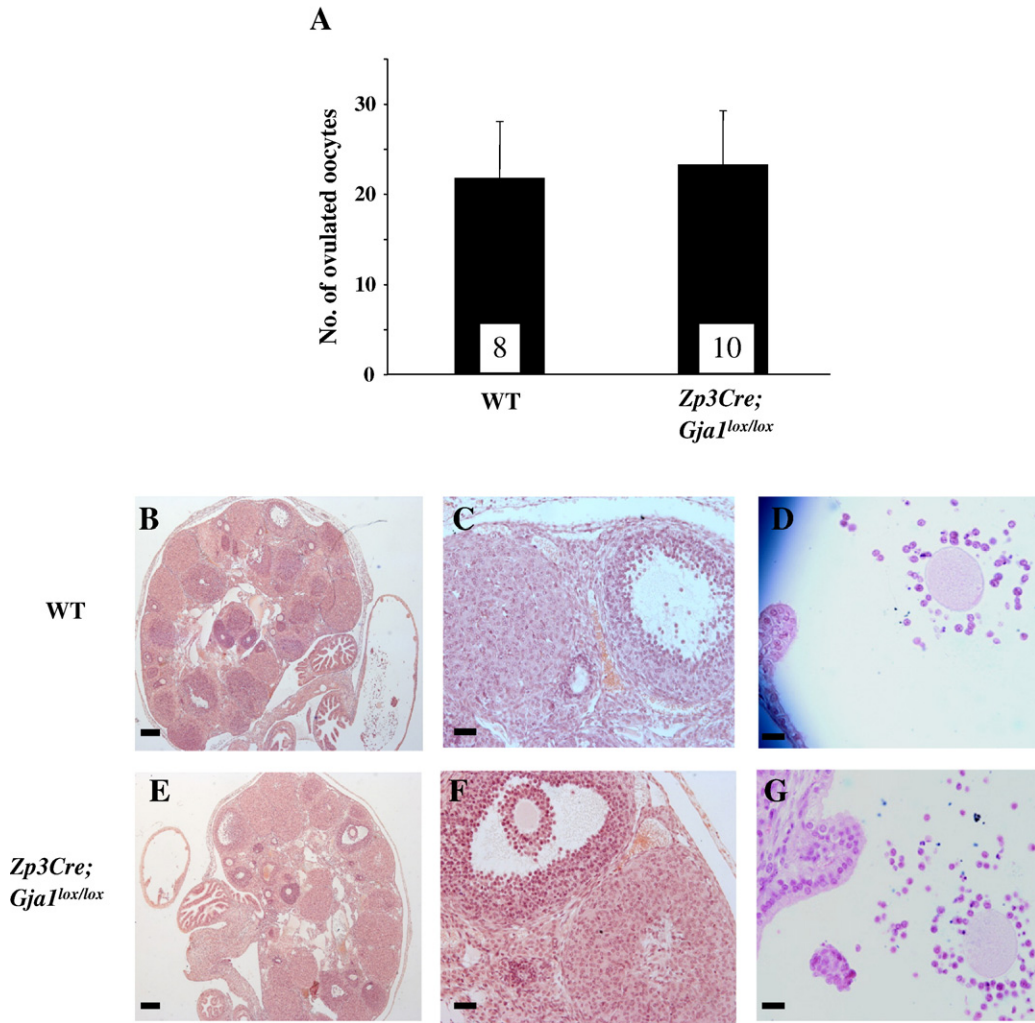


Fig. 7. Ovulatory response and histological analysis of ovaries of PMSG/hCG-treated mice. (A) either WT or *Zp3Cre;Gja1^{lox/lox}* animals were primed with PMSG and administrated by hCG 48 h later. Oviductal oocytes were counted 24 h after hCG injection. Number of animal is indicated at bottom of columns. (B–G) The ovaries were removed 24 h after hCG administration, fixed and sectioned. Cx43^{+/+} (D) and Cx43^{del/del} (G) ovulated oocytes were observed in the fallopian tubes. Scale bars B and E—200 μ m. Scale bars C and F—50 μ m. Scale bars D and G—25 μ m.

Serum progesterone in pregnant Zp3Cre;Gja1^{lox/lox} mice

Implantation failure in the *Zp3Cre;Gja1^{lox/lox}* females could possibly be attributed to the reduced expression of Cx43 in the granulosa cells that in turn may lead to corpus luteum insufficiency. To examine this possibility serum progesterone

Table 2
Fertility evaluation of *Zp3Cre;Gja1^{lox/lox}* females

Females	Male	No. of females	No. of pregnant females	Total no. of pups	Average litter per female
WT	<i>Zp3Cre^{-/-}; Gja1^{lox/lox}</i> (=WT)	20	19 (95%)	136	7.15
<i>Zp3Cre; Gja1^{lox/lox}</i>	<i>Zp3Cre^{-/-}; Gja1^{lox/lox}</i> (=WT)	20	10 (50%)	41	4.1
WT	C57/bl	19	18 (95%)	151	8.4
<i>Zp3Cre; Gja1^{lox/lox}</i>	C57/bl	19	14 (73%)	46	3.3

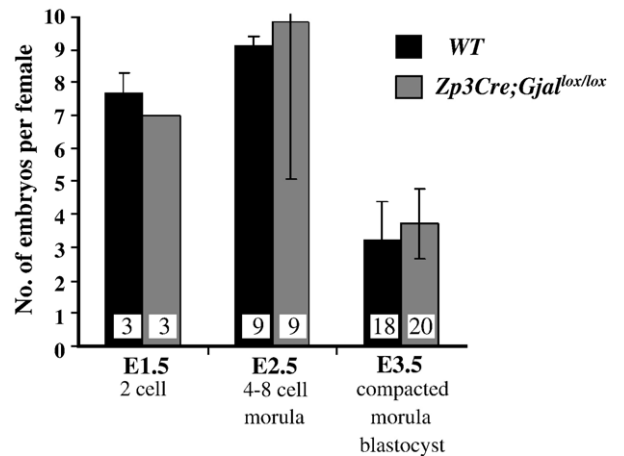


Fig. 8. Preimplantation embryonal development. Preimplantation embryos were washed from the oviduct and/or the uterus of WT and *Zp3Cre;Gja1^{lox/lox}* females at E1.5, E2.5 or E3.5 of pregnancy (vaginal plug=E0.5) and their developmental stage was evaluated. The total number of females examined at each day of pregnancy is noted in the white box.

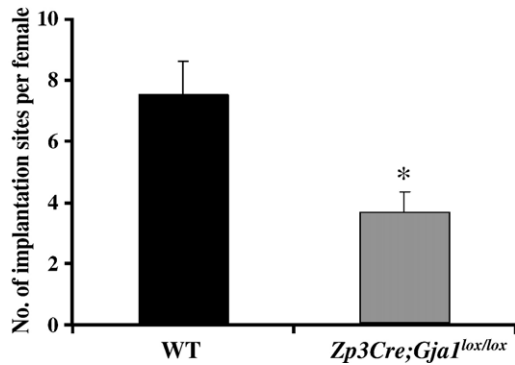


Fig. 9. The rate of implantation. Implantation was analyzed on E4.5 in WT and *Zp3Cre;Gja1^{lox/lox}* pregnant dams ($n=10$ in each group) after i.v. injection of Evans blue (EB). Pregnancy was determined upon revealing at least one implantation site after EB injection. (*) indicates significant difference ($p<0.05$).

(P4) concentrations were determined in pregnant females. Determinations were performed on E5.5 at which serum progesterone reaches its maximal levels (Girsh and Dekel, 2002). No significant difference in serum progesterone was found in *Zp3Cre;Gja1^{lox/lox}* (61.9 ± 12) and WT (75.6 ± 8.5 , $p=0.4$) females (Fig. 10). This result is further supported by the ovarian histology of PMSG/hCG-treated *Zp3Cre;Gja1^{lox/lox}* ovaries that exhibited normal corpora lutea (Figs. 7E, F).

Discussion

Our study aimed at identification of the role of the oocyte Cx43 in ovarian development. For this purpose, we employed the Cre-loxP conditional knockout strategy that exclusively deletes the *Gja1* gene from the oocyte. Unexpectedly, partial depletion of Cx43 mRNA and the subsequent lower levels of the corresponding protein were observed in the cumulus/granulosa cells of some follicles as well. Somewhat surprisingly, we found that the complete elimination of Cx43 in the oocyte, combined with its reduced expression in the granulosa cells, did not affect oogenesis and folliculogenesis, allowing a normal ovulatory response. However, the rate of implantation was severely impaired. Specifically, the ovaries of the *Zp3Cre;Gja1^{lox/lox}* mice were populated by follicles that represent the entire range of folliculogenesis, with the large antral follicles exhibiting a normal response to PMSG. Moreover, the oocytes that reside in these follicles were meiotically arrested and exhibited fully established gap junctional communications with their surrounding cumulus cells. Furthermore, follicle-enclosed Cx43^{del/del} oocytes responded to LH and resumed meiosis. In addition, the ovulation rate in the *Zp3Cre;Gja1^{lox/lox}* was similar to that of WT and fully functional corpora lutea were developed. Moreover, early embryonic development was also not impaired. However, even though an abnormal ovarian phenotype was not identified and normal preimplantation embryonic development was monitored as well, a substantial reduction in both parturition rate and litter size was observed in these animals. Further analysis revealed that the difference in fertility of those *Zp3Cre;Gja1^{lox/lox}* females as compared to WT can be attributed to the impaired implantation of the *Zp3Cre;Gja1^{lox/+}* embryos.

Oocyte expression of Cre recombinase has been previously reported in females, produced by either TNAP-Cre or *Zp3*-Cre lines (Kehler et al., 2004; Lewandoski et al., 1997). However, in spite of the common knowledge that either of these two genes is specifically expressed in the oocyte, both these papers described some ectopic Cre excision. An oocyte specific activity of Cre recombinase has been reported by de Vries et al. (2000) that used a slightly different construct for the establishment of the *ZP3*-Cre transgenic founder. This transgenic line was later employed by Lan et al. (2004), showing the exclusive expression of the *ZP3Cre* in oocytes. Using these same *Zp3Cre* mouse lines (purchased from Jackson Laboratories) for the establishment of the *Zp3Cre;Gja1^{lox/lox}* animal colony, we expected to reproduce these findings. However, to our disappointment, we found that, in addition to its expression in the oocyte, the Cre recombinase was also detected in the somatic cells of some ovarian follicles. This peripheral expression of Cre recombinase resulted in a partial deletion of Cx43 in the granulosa cells. Nevertheless, ablation of Cx43 in the oocyte, combined with partial deletion of this gene in the granulosa cells, did not result in an abnormal ovarian phenotype. These findings seem to disagree with those reported for the systemic Cx43 knockout mice.

As mentioned previously, mice that lack the Cx43 die soon after birth. To explore the effect of Cx43 ablation, ovaries were removed prenatally from the Cx43 knockout mice and allowed to further develop either in vitro, in organ culture, or in vivo under the kidney capsule of WT mice. In both cases, folliculogenesis in Cx43-deficient ovaries did not proceed beyond the primary follicle stage, and oocyte growth was retarded. Furthermore, oocytes recovered from these grafts failed to undergo meiotic maturation and could not be fertilized (Ackert et al., 2001; Juneja et al., 1999).

This apparent controversy could possibly represent the different time points during development at which the deletion of Cx43 took place. Unlike the oocytes of the systemic knockout mice that never expressed Cx43, the deletion of Cx43 in our animal model did not occur before the 3rd postnatal day, which is the age at which the *Zp3* gene is initially expressed (Epifano et al., 1995). In addition, it has been shown that Cre recombinase activity can be delayed by at least 2 days after the expression of

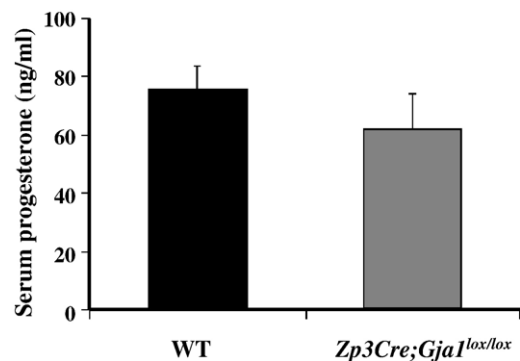


Fig. 10. Serum progesterone concentrations. Luteal function was assessed by the analysis of progesterone (P4) levels in serum of E5.5 pregnant WT ($n=4$) and *Zp3Cre;Gja1^{lox/lox}* ($n=5$) pregnant females as described in Materials and methods.

the targeting gene (Kehler et al., 2004). Indeed, positive LacZ staining was observed in the ovaries of *Zp3Cre*-R26R females at the age of 5 days and older (Lan et al., 2004). Therefore, the oocytes and the ovarian cells in our model do express Cx43 throughout prenatal life, as well as during the first 3–5 postnatal days—an age at which folliculogenesis proceeds to the primary stage (Epifano et al., 1995). Taking this information into account, our study suggests that follicles that reached the primary stage are no longer dependent on Cx43 for their development. Furthermore, we herein demonstrate that the oocyte Cx43 is not involved in the negative control of meiosis. Since Cx43^{del/del} oocytes do exhibit gap junctional communication with the surrounding cumulus cells, we suggest that Cx37 is apparently responsible for executing this function. This role of Cx37 has also been suggested by the use of chimeric follicles containing Cx37-deficient oocytes and wild-type granulosa cells (Veitch et al., 2004). In addition it has been shown recently in mouse follicles that the presence of other connexins such as Cx45 and Cx32 can compensate for the loss of CX43 and possibly establish gap junctional communication between the oocyte and the somatic cells (Tong et al., 2006).

A recent study showed that treating granulosa cells with a specific inhibitor of Cx43 prevented progesterone secretion (Ke, 2005). Along this line, subfertility of the *Zp3Cre;Gjal^{lox/lox}* females may possibly be attributed to the reduced expression of Cx43 in the granulosa cells. In this case, the relatively low Cx43 expression in the granulosa cells of *Zp3Cre;Gjal^{lox/lox}* ovaries may be manifested by corpus luteum insufficiency. However, this assumption is not supported by histological analysis of *Zp3Cre;Gjal^{lox/lox}* ovaries that seems to develop normal corpora lutea after hCG treatment. Moreover, serum progesterone determination in of *Zp3Cre;Gjal^{lox/lox}* females at day E5.5 which is 1 day post implantation, revealed no significant difference between WT and *Zp3Cre;Gjal^{lox/lox}* females. These findings exclude the possibility that the reduced implantation represents corpus luteum insufficiency that results from low levels of Cx43 in the granulosa cells. Subfertility due to an ectopic expression of Cre enzyme in E5.5 embryo implantation site of pregnant females was also excluded by RT-PCR analysis.

Alternatively, the sub-fertility in the *Zp3Cre;Gjal^{lox/lox}* females may represent an impaired quality of the Cx43^{del/del} oocytes. Indeed, early oocyte growth and maturation of Cx43^{del/del} oocytes seem to be appropriate. However, other processes within the oocyte that are essential for acquisition of developmental competence, and cannot be identified by the commonly established markers, may require the expression of Cx43. These processes, collectively defined as cytoplasmic maturation (reviewed in Eppig, 1996), may be essential for the developmental success before and after activation of the zygotic genome.

The mechanism by which Cx43 may influence the developmental competence of the oocyte is not yet understood. However, some recent reports of the involvement of Cx43 in channel-independent cellular functions strongly support this idea (reviewed in Giepmans, 2004). Accordingly, it has been demonstrated that Cx43 might regulate neural crest cell migration via N-cadherin (Xu et al., 2001). Another study of

colocalized Cx43 with tubulin showed that microtubules in liver epithelial cells extend to, and terminate at the plasma membrane only at regions of Cx43 cell–cell contact, suggesting a role for Cx43 in microtubules stabilization (Giepmans et al., 2001). However, even if one of the abovementioned malfunctions had occurred as a consequence of Cx43 deletion, it did not interfere, according to our results, with the normal ovulatory response.

In summary, we generated an animal model that totally lacks Cx43 in the oocyte and expresses reduced levels of this protein in the granulosa cells. Analyzing this model, we suggest here, for the first time, that Cx43 plays a dispensable role in the advanced stages of folliculogenesis and/or oogenesis. Moreover, we clearly demonstrate that the expression of Cx43 in the ovary is essential for the acquisition of normal embryonic development by the oocyte.

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References

- Ackert, C.L., Gittens, J.E., O'Brien, M.J., Eppig, J.J., Kidder, G.M., 2001. Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. *Dev. Biol.* 233, 258–270.
- Buccione, R., Schroeder, A.C., Eppig, J.J., 1990. Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol. Reprod.* 43, 543–547.
- Canipari, R., Epifano, O., Siracusa, G., Salustri, A., 1995. Mouse oocytes inhibit plasminogen activator production by ovarian cumulus and granulosa cells. *Dev. Biol.* 167, 371–378.
- de Boever, J.G., Kohen, F., Bosmans, E., 1994. Binding of homologous and heterologous isoluminal- and enzyme-labelled progesterone conjugated to monoclonal antibodies. *Anal. Chim. Acta* 290, 239–245.
- de Vries, W.N., Binns, L.T., Fancher, K.S., Dean, J., Moore, R., Kemler, R., Knowles, B.B., 2000. Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes. *Genesis* 26, 110–112.
- Dekel, N., Piontkewitz, Y., 1991. Induction of maturation of rat oocytes by interruption of communication in the cumulus–oocyte complex. *Bull. Assoc. Anat. (Nancy)* 75, 51–54.
- Epifano, O., Liang, L.F., Familari, M., Moos Jr., M.C., Dean, J., 1995. Coordinate expression of the three zona pellucida genes during mouse oogenesis. *Development* 121, 1947–1956.
- Eppig, J.J., 1996. Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. *Reprod. Fertil. Dev.* 8, 485–489.
- Giepmans, B.N., 2004. Gap junctions and connexin-interacting proteins. *Cardiovasc. Res.* 62, 233–245.
- Giepmans, B.N., Verlaan, I., Hengeveld, T., Janssen, H., Calafat, J., Falk, M.M., Moolenaar, W.H., 2001. Gap junction protein connexin-43 interacts directly with microtubules. *Curr. Biol.* 11, 1364–1368.
- Girsh, E., Dekel, N., 2002. Involvement of endothelin-1 and its receptors in PGF2alpha-induced luteolysis in the rat. *Mol. Reprod. Dev.* 63, 71–78.
- Granot, I., Dekel, N., 1998. Cell-to-cell communication in the ovarian follicle: developmental and hormonal regulation of the expression of connexin43. *Hum. Reprod.* 13 (Suppl. 4), 85–97.
- Granot, I., Bechor, E., Barash, A., Dekel, N., 2002. Connexin43 in rat oocytes:

- developmental modulation of its phosphorylation. *Biol. Reprod.* 66, 568–573.
- Greep Ro, Astwood, A.E., Geiger, S.R., 1973. *Handbook of Physiology*. Washington.
- Israely, T., Dafni, H., Granot, D., Nevo, N., Tsafirri, A., Neeman, M., 2003. Vascular remodeling and angiogenesis in ectopic ovarian transplants: a crucial role of pericytes and vascular smooth muscle cells in maintenance of ovarian grafts. *Biol. Reprod.* 68, 2055–2064.
- Jorgez, C.J., Klysiak, M., Jamin, S.P., Behringer, R.R., Matzuk, M.M., 2004. Granulosa cell-specific inactivation of follistatin causes female fertility defects. *Mol. Endocrinol.* 18, 953–967.
- Juneja, S.C., Barr, K.J., Enders, G.C., Kidder, G.M., 1999. Defects in the germ line and gonads of mice lacking connexin43. *Biol. Reprod.* 60, 1263–1270.
- Ke, F.C., Fang, S.H., Lee, M.T., Sheu, S.Y., Lai, S.Y., Chen, Y.G., Huang, F.L., Wang, P.S., Stocco, D.M., Hwang, J.J., 2005. Lindane, a gap junction blocker, suppresses FSH and transforming growth factor β 1-induced connexin43 gap junction formation and steroidogenesis in rat granulosa cells. *J. Endocrinol.* 184, 555–566.
- Kehler, J., Tolkunova, E., Koschorz, B., Pesce, M., Gentile, L., Boiani, M., Lomeli, H., Nagy, A., McLaughlin, K.J., Scholer, H.R., Tomilin, A., 2004. Oct4 is required for primordial germ cell survival. *EMBO Rep.* 5, 1078–1083.
- Lan, Z.J., Xu, X., Cooney, A.J., 2004. Differential oocyte-specific expression of Cre recombinase activity in GDF-9-iCre, Zp3cre, and Msx2Cre transgenic mice. *Biol. Reprod.* 71, 1469–1474.
- Lewandoski, M., Wassarman, K.M., Martin, G.R., 1997. Zp3-cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. *Curr. Biol.* 7, 148–151.
- Li, R., Mather, J.P., 1997. Lindane, an inhibitor of gap junction formation, abolishes oocyte directed follicle organizing activity in vitro. *Endocrinology* 138, 4477–4480.
- Plaks, V., Kalchenko, V., Dekel, N., Neeman, M., 2006. MRI analysis of angiogenesis during mouse embryo implantation. *Magn. Reson. Med.* 55, 1013–1022.
- Reaume, A.G., de Sousa, P.A., Kulkarni, S., Langille, B.L., Zhu, D., Davies, T.C., Juneja, S.C., Kidder, G.M., Rossant, J., 1995. Cardiac malformation in neonatal mice lacking connexin43. *Science* 267, 1831–1834.
- Rozental, R., Srinivas, M., Gokhan, S., Urban, M., Dermietzel, R., Kessler, J.A., Spray, D.C., Mehler, M.F., 2000. Temporal expression of neuronal connexins during hippocampal ontogeny. *Brain Res. Brain Res. Rev.* 32, 57–71.
- Sela-Abramovich, S., Edry, I., Galiani, D., Nevo, N., Dekel, N., 2006. Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation. *Endocrinology* 147, 2280–2286.
- Sherizly, I., Galiani, D., Dekel, N., 1988. Regulation of oocyte maturation: communication in the rat cumulus–oocyte complex. *Hum. Reprod.* 3, 761–766.
- Shibata, Y., Kumai, M., Nishii, K., Nakamura, K., 2001. Diversity and molecular anatomy of gap junctions. *Med. Electron. Microsc.* 34, 153–159.
- Simon, A.M., Goodenough, D.A., Li, E., Paul, D.L., 1997. Female infertility in mice lacking connexin 37. *Nature* 385, 525–529.
- Sutovsky, P., Flechon, J.E., Flechon, B., Motlik, J., Peynot, N., Chesne, P., Heyman, Y., 1993. Dynamic changes of gap junctions and cytoskeleton during in vitro culture of cattle oocyte cumulus complexes. *Biol. Reprod.* 49, 1277–1287.
- Theis, M., de Wit, C., Schlaeger, T.M., Eckardt, D., Kruger, O., Doring, B., Risau, W., Deutsch, U., Pohl, U., Willecke, K., 2001. Endothelium-specific replacement of the connexin43 coding region by a lacZ reporter gene. *Genesis* 29, 1–13.
- Tong, D., Gittens, G.E., Kidder, G.M., Bai, D., 2006. 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, 290–297.
- Valdimarsson, G., De Sousa, P.A., Kidder, G.M., 1993. Coexpression of gap junction proteins in the cumulus–oocyte complex. *Mol. Reprod. Dev.* 36, 7–15.
- Vanderhyden, B.C., Cohen, J.N., Morley, P., 1993. Mouse oocytes regulate granulosa cell steroidogenesis. *Endocrinology* 133, 423–426.
- Veitch, G.I., Gittens, J.E., Shao, Q., Laird, D.W., Kidder, G.M., 2004. Selective assembly of connexin37 into heterocellular gap junctions at the oocyte/granulosa cell interface. *J. Cell. Sci.* 117, 2699–2707.
- Xu, X., Li, W.E., Huang, G.Y., Meyer, R., Chen, T., Luo, Y., Thomas, M.P., Radice, G.L., Lo, C.W., 2001. Modulation of mouse neural crest cell motility by N-cadherin and connexin 43 gap junctions. *J. Cell Biol.* 154, 217–230.