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A spatial model showing differences between juxtacrine and paracrine mutual oocyte-granulosa cells interactions

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The bidirectional communication between oocytes and granulosa cells are mediated by several factors *via* a local feedback loop(s). The current model was carried out to study the spatial mutual interaction of porcine denuded oocytes and granulosa cells either in direct contact (juxtacrine) or paracrine co-culture using transwell system. Transwell 0.4 µm polyester membrane inserts were used to permit oocytes-granulosa cells paracrine communication with a distance of 2 mm between them in co-culture. Oocytes were cultured with granulosa cells in a defined basic maturation medium for 44 h. In results, oocyte secreted factors (OSFs; GDF9 and BMP15) temporal expression showed progressive decrement by the end of culture in case of direct contact with granulosa cells while it was increased progressively in the paracrine co-culture groups. However, oocytes that were cultured in direct contact showed a significant increase in blastocyst development after parthenogenetic activation than the paracrine co-cultured ones (20% *vs.* 11.5%, respectively). By the end of culture, granulosa cell count in direct contact showed a significant decrease than the indirect co-culture group (1.2×10^5 cell/mL *vs.* 2.1×10^5 cell/mL, respectively). Steroids (P4 and E2) and steriodogenesis enzymes mRNA levels showed significant temporal alterations either after 22 h and 44 h of IVM in both juxtacrine and paracrine co-culture systems ($P \leq 0.05$). CX43 was much more highly expressed in the granulosa of the direct contact group than the indirect co-culture group. These results indicate the difference in mutual communication between oocytes and granulosa cells that were cocultured either in direct contact (juxtacrine) or with a short distance (paracrine) and propose a new paradigm to study different ovarian follicular cells interaction.

Keywords: Cell-cell communication, CX43, Ovarian follicular cells, Steroidogenesis

The organization and normal functioning of the ovary during follicle formation, through folliculogenesis to ovulation, is heavily dependent upon close bidirectional interactions between the germ cells and the surrounding somatic cells. Growing follicles support oocyte growth like granulosa cells and further, acquisition of meiotic and developmental competence[1]. They also regulate the progression of meiosis[2], and modulating the global matrix and steroid hormone production[3]. Gilchrist *et al*[4]. have reviewed this cellular transcriptional activity in the oocyte genome. In turn, the oocyte influences several aspects of granulosa cell development, including proliferation, differentiation, and extracellular interdependence as well as some paracrine or juxtacrine signaling factors are involved in the molecular and cellular processes that control their healthy development. The paracrine signals include factors from both sides; oocytes secreted factors (OSFs) and KIT ligands (KITL). Gap-junctional communication (GJC) is juxtacrine interaction between the germ cells and the surrounded granulosa cells through specialized structures named connexons[5,6]. Several models were used to study this interaction in vitro in order to clarify the interfollicular and luteal cellular interaction[7-11]. In the current study, we designed a model to compare the spatiotemporal bidirectional communication between oocytes and granulosa cells; oocytes were either directly attached (juxtacrine) or in distance (paracrine) with granulosa cells for 44 h during the period of oocytes *in vitro* maturation.

Materials and Methods

Ovaries and cumulus-oocyte complexes (*COCs*) *and granulose cells recovery*—Ovaries were obtained from gilts and sows at a local slaughterhouse and were transported to the laboratory within 4 h after

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collection in 0.9% NaCl at 25-30 °C. Follicular fluid including cumulus-oocyte complexes (COCs) were aspirated from antral follicles (3-6 mm in diameter) with an 18-gauge needle fixed to a 10 ml disposable syringe. Oocytes were separated from granulosa cells and washed three times with tissue culture medium (TCM)-199-HEPES (Invitrogen, Carlsbad, CA) and selected for *in vitro* maturation on the basis of visual assessment of morphological features, i.e., a compact multi-layered cumulus mass and a dark, evenly granulated cytoplasm. Oocytes were freed from cumulus cells by pipetting with 0.1% hyaluronidase in Dulbecco's PBS (Invitrogen) supplemented with 0.1% polyvinyl alcohol. Denuded oocytes were co-cultured with granulosa cells monolayers as mentioned below and subjected to one of the following: (i) total RNA extraction for RT-PCR and qPCR; or (ii) parthenogenetic activation for subsequent culture *in vitro*. For co-culture, cumulus granulosa cells, isolated after oocyte denuding by centrifugation (1500 rpm / 2 min) with viable cells (as determined by trypan blue dye exclusion) were initially seeded in tissue culture dishes at a density 1×10^6/mL and cultured at 37 °C under a water-saturated atmosphere of 95% air and 5% CO_2 in DMEM supplemented with Pen-Strep (Invitrogen) and 10% FBS for 48 h. Sub-culturing of confluent cells was done according to the experimental design.

Denuded oocytes in vitro maturation (*IVM*)—Granulosa cells initial count was 1×10^5 cell/mL based on trypan blue dye exclusion. The oocytes were divided into two groups of 70 oocytes each; the direct contact (juxtacrine, JC) with granulosa cells and transwell coculture (parcrine, PC) using 0.4 µm polyester membrane inserts (Corning Inc., Pittston, PA) to permit oocytes-granulosa cells communication with distance of 2 mm between them (Fig. 1) in basic maturation medium, TCM-199 supplemented with 1 µg/ml follicle stimulating hormone (FSH) (Antrin, Teikoku, Japan), 10 ng/ml epidermal growth factor (EGF), 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 µg/ml insulin, 1% (v/v) Pen-Strep (Invitrogen), at 39 °C in a humidified atmosphere of 5% CO_2 for 44 h.

Parthenogenetic activation and in vitro culture (*IVC*)—Denuded oocytes were kept in a four-well cell culture plate (SPL Lifesciences Co. Ltd., Kyung Gi-Do, Korea) containing mannitol (0.25 M) for 1.5 min to allow oocytes to settle down. The oocytes were then transferred into a chamber between two electrodes connected to a BTX electro cell manipulator 2001 (BTX, Inc., San Diego, CA) and activated by a single pulse of 1.5 kV/cm for 100 µs[12]. Electro-activated oocytes were then washed in TCM and transferred into 500 µl of porcine zygote medium-3 (PZM-3) supplemented with 4 mM 6-dimethylaminopurine (6-DMAP) and cultured for 4 h to produce diploid zygotes in an atmosphere of 39°C, 5% CO_2, 5% O_2 and 90% N_2. Presumptive diploid zygotes were washed, transferred into 30 µl microdrops of PZM-3[13] covered with mineral oil (Sigma-Aldrich Corp., St. Louis, MO) and cultured in an atmosphere of 39 °C, 5% CO_2, 5% O_2 and 90% N_2. On day-2, embryos were evaluated for cleavage to the 2-cell stage or beyond. Blastocyst formation was assessed on day-7.

Total RNA extraction, reverse transcription and real-time PCR—A total of 50 oocytes were used in each batch for total RNA extraction. Three pools of oocytes were used for analysis and each pool was repeated 3 times. Total RNAs were extracted from detached cumulus granulosa cells and from denuded oocytes using the easy-spin™(DNA-free) Total RNA Extraction Kit (iNtRON Biotechnology, Inc., KyungGi-Do, Korea) according to the manufacturer's instructions. In brief, denuded oocytes were washed twice with PBS and then treated with diethyl pyrocarbonate (DEPC)-treated water and kept at −80 °C or used fresh. Total RNAs were then eluted from all samples following the manufacturer's protocol (iNtRon Biotechnology Inc.). Reverse transcription was carried out at 50 °C for 50 min. Individual RT reaction was performed using random hexamer and superscript ™ III reverse transcriptase (Invitrogen) in a 20 µL reaction. 1-2 µL cDNA was subjected to reverse transcription-polymerase chain reaction (RT-PCR) using Maxime PCR PreMix kit-*i*-starTaq (Intron Biotech., Seoul, Republic of Korea). The PCR amplification was carried out for one cycle of denaturation at 95 °C for 5 min and subsequent

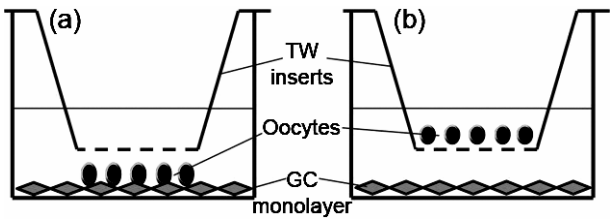

Fig.1—Experimental design. Denuded oocytes were divided into two groups of 70 oocytes each; the direct touch (juxtacrine, JC) with granulosa cells (GC) and indirect coculture (paracrine, PC) in transwell (TW) 0.4 µm polyester membrane inserts with distance 2 mm.

cycles with denaturation at 95 °C, annealing for 30 s, extension at 72 °C for 45 s and final extension at 72 °C for 5 min. Then 10 µl of PCR products were fractionated on 1% agarose gel (Intron Biotech.) and stained with RedSafe ™ (Intron Biotech.). Based on the optimization curves testing different numbers of PCR cycles to select exponential amplification conditions, 35 and 30 PCR cycles were chosen for semiquantitative analysis of all candidate genes and *GAPDH*, respectively. In all assays, cDNA template negative and reactions without reverse transcription resulted in negative amplification. Real-Time PCR (qPCR) was done according to the Takara Bio Inc. guidelines. Total 22 µL PCR reaction was made by adding 2 µL cDNA, 1 µL forward primer, 1 µL reverse primer, 8 µL SYBR Premix Ex Taq, 0.4 µL ROX Reference (Takara Bio Inc. Shiga, Japan) and 9.6 µL of Nuclease-free water (Ambion Inc., Austin, TX). The reaction was done using 7300 Real Time PCR System (Applied Biosystems, Forest City, CA) according to the company instructions. The thermal profile for real-time RT-PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 40 s. RT-qPCR was used for comparison of relative gene expression patterns in oocytes (*GDF9* and *BMP15*asoocytes maturation markers[14]) and in granulosa (β-HSD, CYP19A as steriodogenesis markers and CX43) against *GAPDH* expression. Primer sequences, annealing temperatures and approximate size of the amplified fragments are listed in Table 1.

Hormonal assay—Steroids (E2 and P4) were measured in the maturation medium after 22 h and 44 h through commercial kits. Serum progesterone concentrations were measured by radioimmunoassay (RIA) using a Coat-a-Count Progesterone Kit (Siemens Medical Solutions Diagnostics, Flanders, NJ). The kit contains rabbit anti-progesterone antibody and has a minimum detection limit of 0.02 ng/ml. The intra- and inter assay precision (coefficient of variation, CV %) of the samples ranged from 2.7-8.8 and 3.9-9.7, respectively. Estradiol concentrations were measured by electrochemiluminescence immunoassay using Estradiol II kit (RocheDiagnostics Corp., Indianapolis, IN). The kit contains rabbit anti-estradiol antibody and has a minimum detection limit of 5.0 pg/ml. The intra- and inter assay precision (CV %) of the samples ranged from 2.3-6.2 and 6.2 -13.0, respectively.

Immunofluorescence detection of CX43 in granulosa cells—Granulosa cells were fixed in 4% paraformaldehyde (w/v) in phosphate buffered saline (PBS), pH 7.4 for 30 min at room temperature. After washing in PBS, cells were permeabilized by incubation in PBS containing 0.1% Triton-X100 (volume/volume; v/v) and then were blocked by 1% goat serum (v/v; Vector Laboratories, Burlingame, CA) for 1 h at room temperature then overnight at 4 °C. Primary antibody directed against CX43; mouse monoclonal anti-connexin 43/GJA1 (Abcam, Cambridge, MA) with dilution 1:100 was prepared in 1% goat serum; cells were incubated in primary antibody solution for 2 h at room temperature, washed in PBS, then incubated in secondary antibody (Cy™3-conjugated goat anti-mouse IgG) (Jackson ImmunoResearch lab. Inc. PA) diluted 1:200 in PBS for 1 h at room temperature before washing in PBS. Granulosa cells nuclei were counterstained with Vectashield mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI) (Vector Lab. Inc., CA).

Statistical analysis—Statistical analysis was done using GraphPad Prism 4.02 (Graphpad Software Inc, San Diego, CA). The data were analyzed by one way-ANOVA followed by Tukey's test. Significance was determined when the *P* value was less than 0.05.

Results

Effect of direct contact between oocyte and granulosa cells on oocyte gene expression and

Table 1—List of primers that were used in real-time PCR

Gene	Forward (5′—3′)	Reverse (5′—3′)	Size (bp)	Annealing Temperature (°C)	Accession No.
CX43	GGTGGACTGTTTCCTCTCTCG	GGAGCAGCCATTGAAATAAGC	232	60	NM_001244212.1
HSD3B1	GCCAGCGTGCCGGTCTTCAT	GAATGGGCTCCCCTCCCCGT	249	60	NM_001004049.1
CYP19A	CATGCGAAAAGCCTTAGAGGA	GCTGGAAGTACCTGTAAGGA	167	60	NM_214429.1
GDF9	CAGTCAGCTGAAGTGGGACA	TGGATGATGTTCTGCACCAT	135	60	NM_001001909.1
BMP15	CCTCCATCCTTTCCAAGTCA	GTGTAGTACCCGAGGGCAGA	112	60	NM_001005155.1
GAPDH	ACACTCACTCTTCTACCTTTG	CAAATTCATTGTCGTACCAG	90	60	NM_001206359.1

developmental competence—First, we examined the developmental competence of the oocytes that were matured either in paracrine or juxtacrine pattern with granulosa cells (Table 2). Oocytes that were cultured in juxtacrine pattern with granulosa cells significantly increased the blastocyts yield more than the paracrine one after parthenogenetic activation (20% *vs*. 11.85%, respectively). In order to detect the molecular changes between the group that cause this significant difference, we analyzed the temporal expression of oocytes maturation markers, GDF9 and BMP15 (Fig. 2). Both genes were progressively increased alongside the maturation period in paracrine group while they tended to decline significantly after 22 h from maturation in juxtacrine group that reflected a major change during the oocytes maturation period between the two experimental groups.

Effect of direct contact of oocyte on granulosa cells number, gap junction and steroidogenesis—Culturing of oocytes in direct contact (juxtacrine) with granulosa cells significantly decreased the 44 h granulosa cell number when compared to the paracrine group, 1.2 ± 0.19 $\times 10^5$/mL vs. 2.1 ± 0.12 $\times 10^5$/mL, respectively (Table 2), initial number at 0 h was 1 $X10^5$/mL. Moreover, presence of oocytes in direct contact with granulosa cells for 44 h directly affected the gap junction protein CX43 expression. As it is shown in Fig. 3, CX43 in JC group was much more highly expressed than that of PC group either on protein (immunofluorescence, Fig. 3A-B) or mRNA (real-time PCR, Fig. 3C) levels. On the other hand, the level of steroids, P4 and E2 showed temporal differences between the experimental groups; both groups showed no significant difference in the first 22 h of culture however they showed significant difference after 44 h of IVM when they compared to the sham group, i.e. granulosa cells only without oocytes. After 22 h, P4 in PC was 0.93 ± 0.74 ng/mL and in JC it was 2.67 ± 0.91 ng/mL. Likewise, E2 was 32.6 ± 4.3 ng/mL *vs*. 25.3 ± 3.4 ng/mL of the same groups, respectively. While after 44 h, P4 in PC was significantly lower than JC group compared to the sham group (39.2 ± 4.6 ng/mL *vs*. 13.9 ± 3.3 ng/mL, respectively). E2 showed significant increase in PC group (79.2 ± 3.6 ng/mL) than that of JC (35.5 ± 3.1 ng/mL) and sham group (29.1 ± 2.1 ng/mL) (Fig. 4). Similarly, steroidogenesis enzymes mRNA levels showed similar patterns either after 22 h or 44 h (Table 3) of IVM. Additionally, the temporal expressions of *β-HSD* and *CYP19A* were compared to the expression of an arbitrary group which is the granulosa cells without oocytes cultured for the same

Table 2—Effect of co-culturing the denuded oocytes, either juxtacrine (JC) or paracrine (PC) to granulosa cells, on oocyte developmental competence, granulosa cell number. The values are presented as (mean±SE).

		Direct touch (JC)	Indirect coculture (PC)
Oocyte developmental competence	Number	200	194
	Blastocyst (%)	40 (20%)	23 (11.85%)**
Granulosa cell number* ($X10^5$/mL)		1.2±0.19	2.1±0.12**

*initial number was 1 $X10^5$/mL. **Values are significant when $P < 0.05$.

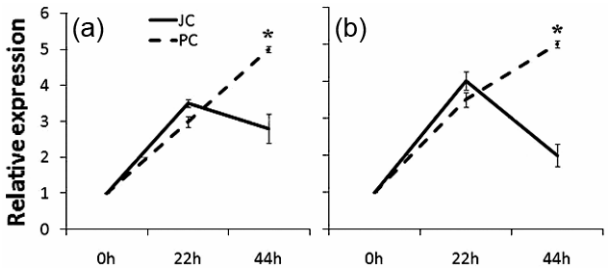

Fig. 2—Temporal relative gene expression of oocytes secreted factors *GDF9* and *BMP15* in the two experimental groups (JC, juxtacrine and PC, paracrine) by real-time PCR. The expression in 0 h was used as an arbitrary unit. *P <0.05.

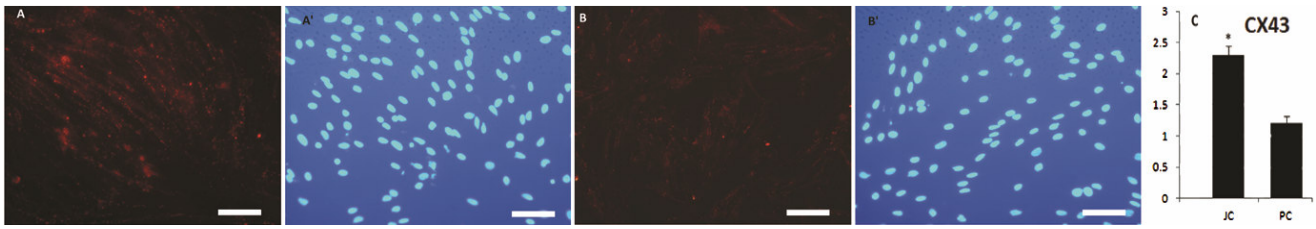

Fig. 3—Connexin 43 (CX43) expression in granulosa cells of the experimental groups. Localization of CX43 in granulosa cells (A, juxtacrine group; B, paracrine group; A′ and B′ images shows nuclear staining with DAPI of the same groups, respectively), scale bar = 20 µm; C, real-time PCR relative expression of *CX43* gene in the experimental groups (JC, juxtacrine and PC, paracrine). Data are the strength of gene expression of control group (Granulosa cells cultured without oocytes) after normalizing to *GAPDH* expression. *P <0.05.

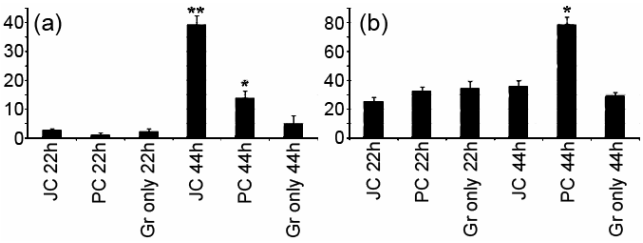

Fig. 4—Steroid hormones (P4, progesterone and E2, estradiol) level (ng/ml) in the two experimental groups (JC, juxtacrine and PC, paracrine) and control groups (Gr. Granulosa cells cultured without oocytes) after 22 h and 44 h culture. **P* <0.05, ***P* <0.01.

period. After 44 h *β-HSD* in JC group was 2-fold of PC group while *CYP19A* was about 2-fold of JC one.

Discussion

Several models were used to reveal the interaction between different ovarian cells *in vitro*, including 3D models[9-11]. In the current study, a new approach was used to study the interplay between the porcine denuded oocytes and granulosa cells in spatial and temporal manner. To our knowledge, this the first report showing the paracrine interaction between the oocyte and granulosa cells using the transwell technique. The oocytes were kept in co-culture with granulosa cells either directly attached to it (juxtacrine, JC) or with a 2 mm distance (paracrine, PC) during the *in vitro* maturation time. The paracrine communication in JC group cannot be excluded. However, the presence of oocytes in direct touch with granulosa cells refers to a stimulus which does not exist in PC group. This model can be used for further studying the interaction between different ovarian cell kinds *in vitro*. Oocytes that were cultured JC to granulosa cells showed significant increase in their developmental competence after parthenogenetic activation reflected by an increase in blastocyst yield (Table 2). While oocyte quality is reflected by the competence to develop to a blastocyst[15], culture conditions determine blastocyst quality, possibly including changes in gene expression[16,17]. Interestingly, the temporal gene expression of OSFs; GDF9 and BMP15, as oocytes maturation markers[14], showed different patterns between the two groups; a progressive increase in expression along with oocyte maturation was noticed in PC group however it showed a significant decrease in JC group (Fig. 2). The cumulative increase in temporal expression of both BMP15 and GDF9 might be involved in disturbing the meiosis and competence of the oocytes[18,19]. Our result does not agree with Li *et al.*[20] who have found that porcine GDF9 decreased with the oocyte maturation in cumulus-oocyte complex however BMP15 showed an increase with oocyte maturation. The difference might be due to the using of denuded oocytes instead of cumulus-oocyte complexes which reflects the regulatory loop of granulosa on oocytes function/gene expression[21].

Table 3—Relative quantification (RQ) of steriodogenesis enzymes genes expression in granulosa cells of juxtacrine and paracrine groups. RQ was the relative expression of specific gene to GAPDH and justified to its relative expression to graunlosa cells (Gr) as arbitatory units for 22 h and 44 h as experimentally designed.

The values are presented as (mean±SE).

Gene	Group	Arbitatory group	Fold change of relative expression
β-HSD/ GAPDH	JC 22 h	Gr 22 h	1.53±0.15*
	PC 22 h		0.82±0.11
	JC 44 h	Gr 44 h	12.1±0.85*
	PC 44 h		6.01±0.19
CYP19A/GAP DH	JC 22 h	Gr 22 h	0.73±0.52
	PC 22 h		0.83±0.73
	JC 44 h	Gr 44 h	1.3±0.09
	PC 44 h		2.3±0.24*

*Values compared to the same arbitatory group are significant when $P < 0.05$.

The results of OSFs coincide with the variable number of granulosa cells that was found after the end of maturation period (Table 2); in JC group, granulosa cell number significantly decreased than the PC group which reflect the mitogenic action of both BMP15 and GDF9 on granulosa cells[22,23].

In addition, different patterns of steroidogenesis and steroids output from cocultured granulosa cells (Table 3, Fig. 4) resulted from the changes in OSFs expression; progesterone level and *β-HSD* expression showed significant increase in JC group which accompanied with a decrement of *BMP15 and GDF9* expression which reflects the potent inhibitory role of OSFs on progesterone production[23-25]. On the other hand, estradiol showed significant increase in PC group than JC reflecting the selective regulatory effect of OSFs on steroidogensis[26]. Studies have demonstrated that some members of the TGF-β superfamily (*e.g.,* oocyte-derived BMP-15 and BMP-6) also inhibit FSH-stimulated progesterone production without changing estradiol production in rat granulosa cells[27,28]. However, GDF9 alone can increase follicular estradiol production *in vitro*[29].

The GJC CX43 showed up regulation in JC group (Fig. 3), supporting the idea that mechanical rupture

of the COC from its granulosa cell layer triggers CX43 up-regulation[30]. However, the persistent increase in CX43 expression in JC group might be due to the direct contact of oocytes and/or the high concentration of progesterone in that group[31]. In the normal IVM either in porcine[30] or bovine[32] species, CX43 expression increases during the first hours of IVM followed by decrease with the GVBD and oocyte maturation. But in the current study, the oocyte condition is different. Weight of evidence indicates that CX43 does not contribute to the gap junctions connecting oocytes and granulosa cells[6,26]. However, one of the ways that connexins have been proposed to contribute to developmental or physiological processes is via undocked connexons residing in non-junctional plasma membrane regions. It has been suggested that regulated opening of such gap junction "hemichannels" could allow small signaling molecules to exit the cell to the extracellular space where they can interact with receptors of nearby cells. For example, there is accumulating evidence that ATP released via opening of gap junction hemichannels can activate purinergic receptors on neighbouring cells, providing a pathway for the propagation of intercellular Ca^{2+} waves[33]. This could have relevance for signaling within developing follicles since extracellular ATP is known to act through P2 purinergic receptors to induce Ca^{2+} release within granulosa cells[34], an effect that in turn leads to a Ca^{2+} increase in the oocyte[35]. These considerations led to the hypothesis that the essential function of CX43 in oocyte and follicle development is to enable intercellular signaling via hemichannel release of ATP rather than (or in addition to) via gap junctional communication[36]. The choice of CX43 for gap junction communication does not mean that it is responsible for the juxtacrine contact because CX43 is solely expressed in granulosa cells unlike CX45 and CX60 which are the major connexins in porcine oocytes[37]

In conclusion, this new approach describes the difference in the interplay between the porcine denuded oocytes and granulosa cells in a spatial (juxtacrine or paracrine) and temporal model. The vast difference in the oocyte quality and granulosa cell functions between the two experimental groups provides scope for further study of different ovarian cellular interaction *in vitro*.

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