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Connexin37 mRNA expression in *in vivo* and *in vitro* mouse oocyte

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Summary

To evaluate gene expression of Connexin37 (Cx37) in oocytes from *in vitro* follicles at different stages, mouse preantral follicles were isolated and cultured for 12 days *in vitro*. Compared with *in vitro* follicles, follicles grown *in vivo* were collected at day 14 (d14), d16, d18, d20, d22 and d24 with the same stages for gene expression of Cx37 in oocytes. Our results showed that Cx37 mRNA increased along with follicular development, reached the highest level at the onset of antrum cavity formation and decreased after antrum formation in both *in vivo* and *in vitro* mouse oocytes. However, Cx37 mRNA was significant higher ($p < 0.01$) in *in vitro* cultured oocytes than *in vivo* oocytes. Moreover, significantly higher levels of Cx37 mRNA were found in oocytes from *in vitro* disrupted follicles ($p < 0.01$) and non-grown follicles ($p < 0.05$) than those from normal follicles with a similar size. These data determine temporal gene expression of Cx37 in oocytes from follicules at different stages and indicate that the gene expression level of Cx37 in oocytes could be evaluated as a criterion to the regulatory mechanism of Cx37 in an *in vitro* model.

Keywords: Connexin, Follicle culture, Mouse, Oocyte development

Introduction

In mammalian ovarian follicles, oocyte and somatic follicular cells coordinately develop and function as a physiological unit by the means of intercellular communications that are mediated by an indirect endocrine and paracrine mechanism and a bi-directional contact, gap junction. Gap junctions channels connect the cytoplasm of adjacent cells through two hemichannel connexons, six oligomerized connexin (Cx) subunits, and diffuses small metabolites and molecules (molecular weight ≤1.8 kD) between closed cells (Goodenough *et al*., 1996; Goldberg *et al*., 2004; Neijssen *et al*., 2005). Gap junction couples are retained in rodent ovaries from the earliest stages of folliculogenesis (Juneja *et al*., 1999) until several hours after ovulation (Simon *et al*., 2006). Multiple connexins are expressed within the developing ovarian follicle (Kidder & Mhawi, 2002). Cx32, Cx37, Cx43 and Cx45 have been localized in mouse ovarian follicles.

It is accepted that the developmental programme in oocytes dominates the direction of differentiation and function of somatic follicular cells and their follicular development (Matzuk *et al*., 2002; Eppig, 2005; Diaz *et al*., 2007). Through gap junctions between oocyte and somatic cells, the oocyte does not only receive nutrients and regulatory molecules transferred from surrounding somatic cells (Eppig, 1991; Eppig & O'Brien, 1996), but also provides regulatory signals that regulate the development and differentiation of the follicle cells (Sugiura & Eppig, 2005). Cx37 might be the only gap junction protein secreted by oocytes to form gap junctions with somatic follicular cells (Simon *et al*., 1997). Cx37 gap junctions exclusively locate at the surface of the oocyte and surrounding follicular cells from the primary follicle stage onward (Simon *et al*., 1997; Teilmann, 2005), although Cx37 were also detected in the granulosa cells (Wright *et al*., 2001; Veitch *et al*., 2004). The physiological role of Cx37 in ovarian follicles has been defined by generating knockdown mice that lack Cx37 (Simon *et al*., 1997) and chimeric ovary (Gittens & Kidder, 2005). In ovaries that lack Cx37, premature corpi lutea were present and oocytes could not grow to their full size and

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resume meiosis, which is due to the lack of gap junction-mediated communication between oocytes and granulosa cells (Simon *et al*., 1997; Carabatsos *et al*., 2000; Gittens & Kidder, 2005). These studies confirmed the hypothesis that Cx37 gap junctions are essential to maintain oocyte growth and follicular development. However, the regulatory mechanism of Cx37 still remains unclear.

The purpose of this study was to determine the temporal expression of Cx37 mRNA in *in vitro* oocytes compared with *in vivo* oocytes and determined whether the expression level of the Cx37 gene could be a criterion for the study of the regulatory mechanism of Cx37 in an *in vitro* model.

Materials and methods

Materials and animals

All chemicals were purchased from Invitrogen unless otherwise indicated. Dishes for follicle culture were purchased from Corning/Coster Company.

C57Bl/6 mice were purchased from the Animal Centre of Xi'an Jiaotong University (Xi'an, China). Female mice were given free access to food and water and bred under controlled conditions (12 h of light, 12 h of darkness; temperature: 20–22°C).

Isolation and culture of preantral follicle

The female pups, 12 days of age, were killed by cervical dislocation. In non-sterile conditions, ovaries were isolated and placed into petri dishes containing phosphate-buffered saline (PBS). Ovaries were trimmed under a dissecting microscope, then preantral follicles were isolated by 1 ml syringe needles without using any enzyme. Isolated preantral follicles were collected and transferred into culture dishes containing culture medium (tissue culture medium (TCM) 199 supplemented with 3 mg/l BSA, 0.22 mg/l sodium pyruvate, 0.5 IU/ml insulin–transferrin–selenium (ITS), 100 IU/ml penicillin and 100 IU/ml streptomycin). Follicle diameter was measured with an ocular micrometer under inverted phase-contrast microscopy (Olympus). Only follicles with a normal morphology and a diameter of 80–100 μm (Fig. 1) were selected and cultured in 96-well plates in CO_2 incubator (37°C, 5% CO_2, 100% humidified). Each well contained 10 follicles and 100 μl culture medium covered with mineral oil. Half the volume of culture medium was replaced with the fresh medium every another day.

The viability of follicles was microscopically evaluated according to the morphological criteria: the viable follicles had intact basement membranes and oocytes with smooth outer layer and even a zona pellucida plus homogeneous cytoplasm; the degenerated follicles contained retracted oocytes with deformed zona pellucida and contracted cytoplasm and/or were surrounded by disorganized granulosa cells.

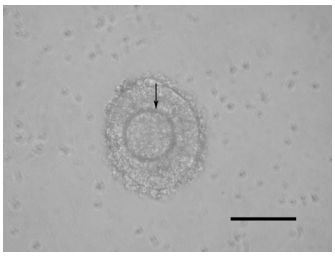

Figure 1 Photomicrograph of a freshly isolated mouse preantral follicle. Arrow indicates an oocyte inside the follicle. Bar = 50 μm.

Collection of oocytes

Follicles cultured *in vitro* were collected on days 2, 4, 6, 8, 10, 12 (days 2–12; *in vitro* group). As compared with the *in vitro* group, *in vivo* follicles at the same stage were collected from each mouse at 14, 16, 18, 20, 22, or 24 days old (days 14–24; *in vivo* group) respectively under a dissecting microscope. Then, somatic cells surrounding the oocyte were removed by repeatedly pipetting with 0.11% hyaluronidase. After washing three times with PBS, oocytes in groups of five were transfered into eppendorf tubes, each containing 11 μl of lysis buffer and stored at –80°C for later processing.

Synthesis of cDNA

The oocyte cDNA was synthetized with Cells-Direct cDNA Synthesis System for qPCR kit (Invitrogen) according to manufacturer's instruction. In brief, oocytes were lysed in lysis buffer; DNase I and DNase I buffer were added to digest genomic DNA; then RT-Reaction Mix and Enzyme Mixture were used to generate double-stranded cDNA and mRNA; eventually single-stranded cDNA was obtained by digestion with RNase H and stored at –20°C for subsequent real-time PCR.

Quantitative analysis of oocyte Cx37 mRNA

Specific primers for the internal standard β-actin (146 bp products) and gene Cx37 (135 bp products) were designed using Primer 5.0 (Applied Biosystems) based on the GenBank database. The β-actin primers (GenBank accession no. NM007393) were: sense primer 5′-CCCATCTACGAGGGCTAT-3′ and antisense primer 5′-ATGTCACGCACGATTTCC-3′; the Cx37 primers (GenBank accession no. NM008120) were: sense primer 5′-CGGTTGCGGCAGAAAGAG-3′ and antisense primer 5′-CCCACGAATCCGAAGACG-3′.

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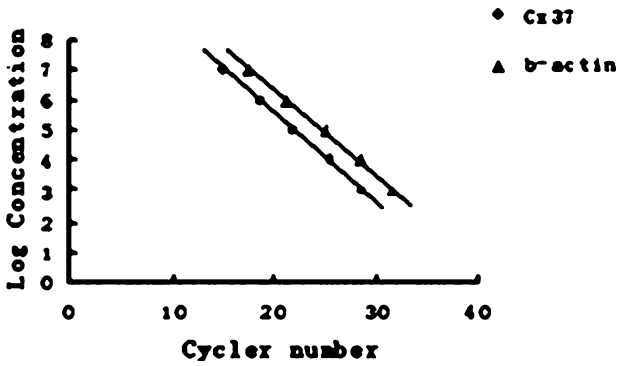

Figure 2 Curves obtained with several dilutions of β-actin and Cx37 from crossing points (cycler numbers) plotted against the log concentration of the serial dilution.

Real-time PCR was performed using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) with Smart Cycler (Cepheid). Each real-time PCR reaction was carried out in 25 μl of reaction mixture containing 12.5 μl Platinum SYBR Green qPCR SuperMix-UDG, 0.5 μl of 0.2 μM sense primer, 0.5 μl 0.2 μM antisense primer, 4 μl of template and 7.5 μl distilled water. Reaction conditions were as follows: 50 °C for 2 min (UDG incubation), 95°C for 2 min, followed by 45 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 10 s. For the negative control, the temple was substituted with distilled water. After cycling, melting curve analysis was undertaken to verify the amplification of the specific target. Each reaction was performed three times. Standard curves used in this study were established with several dilutions of plasmid including β-actin and Cx37 (Fig. 2).

Statistical analysis

All experiments were replicated three to five times. Real-time PCR data were analysed by double standard curve method. Data were analysed with the software package SPSS. Difference was considered statistically significant when $p < 0.05$.

Results

Morphological changes of *in vitro* cultured follicles

In present study, morphological changes of cultured follicles were observed everyday during *in vitro* culturing of preantral follicles. The number of granulosa cells and volume of oocytes obviously increased during the first 6 days in culture. Antrum-like cavities were observed in the centre of cultured follicles at d6–8 and increased with growing follicles. After 12 days in culture, mature oocytes, were obtained from *in vitro* follicles culture following simulation with HCG. Degenerated and disrupted follicles (Fig. 3) were observed mostly at d4–6.

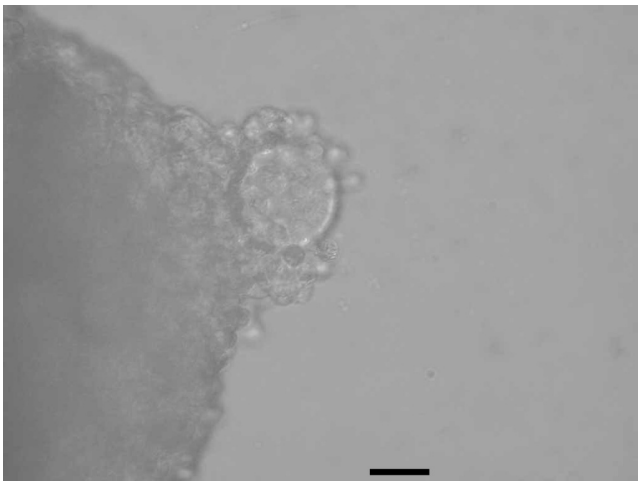

Figure 3 Oocyte in cultured follicles. Bar = 50 μm.

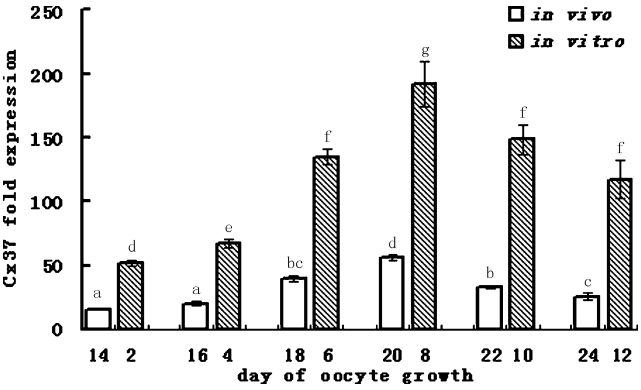

Figure 4 Expression of Cx37 mRNA of oocytes in *in vivo* and *in vitro* follicles grown to different stages. The data are means ± S.E.M. Different superscripts denote significantly different values ($p < 0.05$). Relative Cx37 mRNA abundance was normalized to constitutively expressed β-actin and expressed in arbitrary units relative to the expression of Cx37 mRNA in oocytes from *in vivo* follicles.

Expression of Cx37 mRNA in oocytes of *in vitro* follicles

Cx37 mRNA expression varied with the follicles grown *in vitro* (Fig. 4). Cx37 mRNA increased gradually and was signficantly higher at d2–8 ($p < 0.01$) before antrum-like formation. The highest level of Cx37 mRNA was detected during follicular antrum formation. Subsequently, Cx37 mRNA declined ($p < 0.05$). In addition, compared with morphologically normal follicles (MNFs), the level of Cx37 mRNA was significantly higher in the oocytes from abnormal

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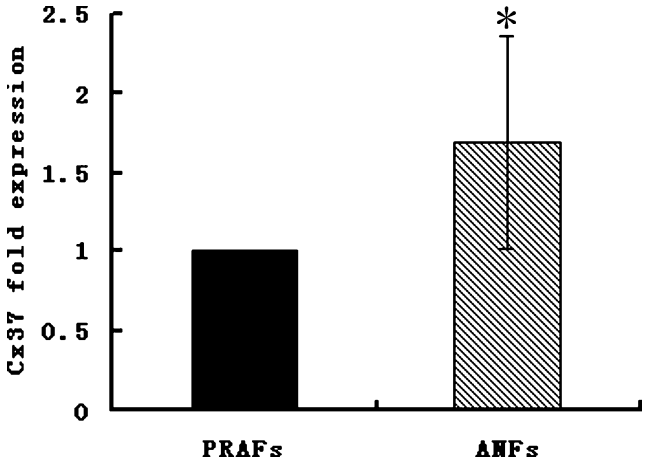

Figure 5 Expression of Cx37 mRNA in *in vitro* oocytes of PRAFs and ANFs with the same size. PRAF indicates preantral follicle; ANF indicates antral follicle The data are fold expression. $^{*}p < 0.05$ significant differences. The expression of Cx37 is normalized on the basis of β-actin expression.

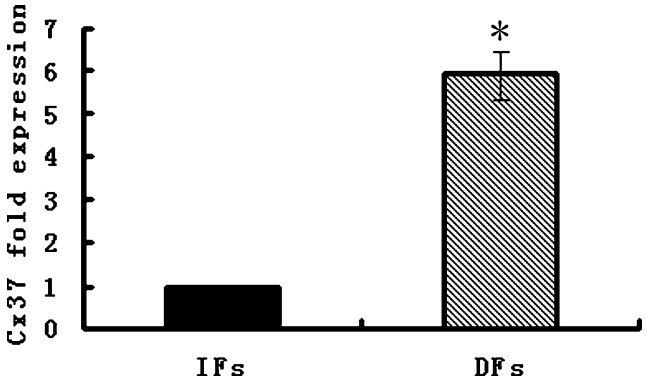

Figure 6 Expression of Cx37 mRNA in *in vitro* oocytes of DFs and IFs with the same size. IF indicates intact preantral follicle; DF indicates disrupted preantral follicle. The data are fold expression. $^{*}p < 0.05$ = significant differences. The expression of Cx37 is normalized on the basis of β-actin expression.

developed follicles, such as follicles with no cavities ($p < 0.05$, Fig. 5) and disrupted follicles ($p < 0.01$, Fig. 6).

Expression of Cx37 mRNA in oocytes of *in vivo* follicles

As shows in Fig. 4, the growth curve of Cx37 mRNA from *in vivo* oocytes varied similarly with that of *in vitro* oocytes, but the Cx37 mRNA expression was significant lower ($p < 0.01$) in *in vivo* cultured oocytes than in *in vivo* oocytes at the different stages.

Discussion

This study investigated the temporal expression patterns of Cx37 mRNA in mouse oocytes during *in vivo* and *in vitro* follicle development. In both *in vivo* and *in vitro* follicles, mRNA expression of Cx37 in oocytes increased gradually with follicle development, reached the highest ($p < 0.01$) level at the onset of antrum form and significantly decreased after follicular antrum formation. However, significantly higher ($p < 0.01$) levels of Cx37 mRNA were detected in oocytes from *in vitro* follicles compared with *in vivo* follicles at the same stage. In addition, significantly higher levels of Cx37 mRNA were detected in oocytes from *in vitro* follicles in which oocyte-to-somatic cell contacts disrupted and no antrum formed.

To our knowledge, few study have investigated the frequency curve of Cx37 mRNA expression in the follicle. This present study showed the frequency curve of Cx37 mRNA in *in vivo* and *in vitro* mouse oocytes, which was consistent with the trend of protein levels shown in previous research (Teilmann, 2005). In mice, Cx37 levels increased along with follicle development and decreased significantly at the onset of antrum formation and reached the highest point in mouse antrum follicles. Increase of Cx37 occurred along with oocyte growth before antrum formation, During this process, oocytes achieved plentiful RNA (Sternlicht & Schultz, 1981; Wassarman & Albertini, 1994) and protein synthesis (Heller *et al*., 1981; Wassarman & Albertini, 1994) for later cytoplasm maturation (Eppig & O'Brien, 1996) and completed most of their growth phase (Wassarman & Albertini, 1994), which depends on essential nutrient and signal materials transferred from surrounding granulosa cells to the growing oocyte through gap junctions made by oocytes Cx37 (Eppig, 1991; Veitch *et al*., 2004). Cx37 declined after mouse oocytes completing full growth. This drop was ever explained by a reduced number of gap junction-forming transzonal projections (TZPs) after antrum formation. No observable drop of Cx37 gap junctions was observed in bovine oocytes, which maintained slight growth after antrum formation (Nuttinck *et al*., 2000). This discrepancy may be due to the different mechanisms of oocytes development in different species.

Overexpression of connexins in *in vitro* oocyte and somatic follicular cell co-culture was shown in previous research (Veitch *et al*., 2004). A similar phenomenon appeared in this study in which significantly ($p < 0.01$) higher levels of Cx37 mRNA were observed in *in vitro* cultured oocytes compared with *in vivo* oocytes. Moreover, our result showed that significantly higher levels ($p < 0.01$) of Cx37 mRNA were detected in oocytes from *in vitro* follicles in which oocyte-to-somatic cell contacts were disrupted. One can

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hypothesize that oocytes could autoregulate Cx37 secretion by means dependent on certain intercellular contact with surrounding follicular cells.

As described above, the secretion of Cx37 in oocytes may be tuned based on oocyte development. Oocytes themselves have a low ability to incorporate and use materials from the extracellular environment and general materials in oocyte were supplied by follicular cells and transferred through gap junction channels. Before reaching full size, oocytes accept nutritional materials (Brower & Schultz, 1982; Herlands & Schultz, 1984) from surrounding somatic cells through gap junction channels by secreting Cx37. Subsequently, Cx37 declines (Teilmann, 2005) and oocytes accept only little signal materials to maintain meiotic arrest. Under *in vitro* conditions, cytoskeleton changes or other factors induce the lost of cell-to-cell contacts. Taking this into account; when the contacts with follicular cells were disrupted, materials transferred to oocytes declined. Then oocytes try to form more gap junctions by overexpressing Cx37 to accept sufficient materials for their own developmental needs. Veitch (2004) indicated that there is a specific cell–cell contact event that mediates Cx37 recruitment and stabilization and that explained why Cx37 is able to stabilize at the surface of granulosa cells that contact the oocyte and not between other granulosa cells. Why Cx43 is not recruited in the same way, however, was not explained. Our data reconfirmed his opinion to some extent and could explain why Cx37 gap junctions in bovine oocytes did not decline after antrum formation. Conversely, the functional foundation of gap junction depends on the stabile expression of connexin and a stable cytoskeleton (Shaw *et al*., 2007), which is the structural foundation of cell-to-cell contacts. Under *in vitro* conditions, the stability of oocyte-to-somatic cell contacts was broken to different degrees, especially in oocytes that showed a significantly higher level of Cx37 mRNA in the present study. Cytoskeletons were evenly distribute at the surface of *in vivo* cell membranes, whereas were not in *in vitro* suspension conditions (Baluska *et al*., 2004). This change in the cytoskeleton affects the nuclear matrix and induces transcription of the corresponding gene in chromatin (Folkman & Moscona, 1978). Thus, we can speculate that the overexpression of Cx37 in *in vitro* oocytes may be due to changes in the cytoskeleton. This opinion is supported by the report that artificial disruption of the cytoskeleton *in vitro* induced rapid assembly of gap junctions at the surface of mouse epidermal cell (Tadvalkar & Silva, 1983). Though we did not test whether Cx37 mRNA from follicles induced the accumulation of active Cx37, present data suggest that rapid gap junction assembly *in vitro* shown in a previous report (Tadvalkar & Silva, 1983) maybe regulated by increasing gene transcription for Cx37. This present study indicated that expression of the *Cx37* gene depended on the stage of oocyte development and on cell-to-cell contact dependent upon the cytoskeleton's situation.

In summary, our data demonstrated that expression of Cx37 mRNA increased gradually in preantral follicles, then reached the peak at onset of antrum formation thereafter decreased along with antrum growth. In addition, the present study indicated that the regulatory mechanism of Cx37 in an *in vitro* model could not be evaluated by the expression level of Cx37 mRNA in oocytes. Further study may optimize more criteria for investigating the regulatory mechanism of Cx37 in follicular development in the *in vitro* model.

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