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Bi-directional communication with the cumulus cells is involved in the deficiency of XY oocytes in the components essential for proper second meiotic spindle assembly

Baozeng Xu [a], Saeid Noohi [a], Jonghyun S. Shin [a], Seang Lin Tan [b,c], Teruko Taketo [a,b,c,*]

[a] Departments of Surgery and Biology, McGill University, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, QC, Canada H3A 1A1
[b] Department of Obstetrics and Gynecology, McGill University, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, QC, Canada H3A 1A1
[c] Montreal Reproductive Center, 2110 Decarie Blvd, Montreal, QC, Canada H4A 3J3

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ABSTRACT

The oocyte becomes competent for embryonic development by involving mutual communication with cumulus cells (CCs) during folliculogenesis. How this communication takes place under physiological conditions is not fully understood. Current study examined oocyte–CCs communication in the XY sex-revered female mouse. We have previously found that the XY oocyte is defective in its cytoplasm, causing abnormal MII-spindle assembly and a failure in embryonic development. Our present study showed that transcript levels of *Pfkp*, *Pkm2* and *Ldh1* involved in glycolysis were lower in the CCs surrounding XY oocytes than in those surrounding XX oocytes. ATP contents in XY oocytes were also lower than those in XX oocytes, suggesting that lower glycolytic gene expression in CCs resulted in lower ATP contents in the enclosed oocyte. Co-culture of oocytectomized CC-oocyte complexes (COCs) with denuded oocytes showed that XY oocytes were less efficient than XX oocytes in promoting glycolytic gene expression in CCs. Furthermore, both glycolytic gene expression levels in CCs and ATP contents in oocytes of XY COCs increased to similar levels to those of XX COCs after culture for 20 h in the presence of milrinone (=preincubation), which prevented spontaneous oocyte maturation. By increasing ATP levels in XY oocytes by either COC preincubation or ATP microinjection into oocytes prior to in vitro maturation, an improvement in MII-spindle assembly was observed. We conclude that the XY oocyte produces lesser amounts of paracrine factors that affect its companion CCs, which in turn make the ooplasm deficient in its components, including ATP, essential for MII-spindle assembly.

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Introduction

The oocyte is physically and metabolically coupled to its surrounding granulosa cells (GCs) throughout folliculogenesis. Upon follicular antrum formation, the GCs differentiate into two anatomically and functionally distinct populations: mural GCs lining the follicular wall and cumulus cells (CCs) enclosing the oocyte. The communication between the oocyte and its companion GCs/CCs is bi-directional and plays an essential role in the establishment of oocyte competence for embryonic development (Eppig, 2001). The oocyte depends on the supply of nutrients and metabolites from GCs during follicular growth and from CCs in antral follicles since it has a limited capacity to uptake or metabolize amino acids (Eppig et al., 2005), cholesterol (Su et al., 2008), or glucose by itself (Biggers et al., 1967; Buccione et al., 1990a; Downs and Utecht, 1999; Eppig, 1976; Sutton-McDowall et al., 2010). The oocyte, in turn, controls the proliferation, differentiation, and functions of GCs/CCs (Diaz et al., 2007; Eppig, 2001; Su et al., 2009; Sugiura and Eppig, 2005; Sugiura et al., 2007). A couple of TGF-β superfamily members, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15, also referred to as GDF9B), are known to be specifically expressed in the oocyte (Dube et al., 1998; McGrath et al., 1995) and play essential roles in follicle development and ovulation in the mouse (Dong et al., 1996; Yan et al., 2001). Consequently, the quality of oocytes may reflect into the functions of GCs/CCs; this hypothesis justifies the use of CCs for oocyte competence diagnosis in assisted reproduction technologies (Assidi et al., 2011; Assou et al., 2008; Cillo et al., 2007).

Bi-directional communication between the oocyte and its surrounding GCs/CCs has been recapitulated in a culture system. When the oocyte has been removed microsurgically from the cumulus-oocyte complex (COC) of antral follicle and the remaining oocytectomized COC (OOX–COC) is cultured, Krebs cycle activity, glycolysis, as well as the expression of genes encoding glycolytic

* Corresponding author at Urology Reserach Laboratory, Royal Victoria Hospital H6-19, 687 Pine Avenue West, Montral, QC, Canada H3A 1A1.
Fax: +1 514 843 1457.
E-mail address: teruko.taketo@mcgill.ca (T. Taketo).

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enzymes and *Slc38a3* (an amino acid transporter) are all dramatically reduced in the CCs (Eppig, 2005; Eppig et al., 2005; Sugiura et al., 2005,2007). These metabolic activities in CCs are restored when OOX–COCs are co-cultured with denuded oocytes. These results unequivocally indicate that the oocyte secretes paracrine factors (OSFs) that regulate metabolic activity in CCs. Recombinant BMP15 and other unidentified TGFβ family members have been shown to exert these effects. Furthermore, FGF8, a member of fibroblast growth factor family, is expressed in the oocyte in vivo (Valve et al., 1997), and although FGF8 itself has weak activity, it drastically enhances the activity of BMP15 and other unknown OSFs on glycolytic gene expression in CCs in vitro (Sugiura et al., 2007). More experimental models are needed to establish the role of bi-directional communication between the oocyte and its surrounding GCs/CCs in promoting the developmental competence of oocytes.

The B6.Y^{TIR} sex-reversed female mouse provides an excellent animal model for investigating the role of bi-directional communication in the establishment of oocyte competence. Despite the expression of the Y-encoded testis-determining factor SRY, more than half of B6.Y^{TIR} (XY) gonads develop into ovaries, in which germ cells enter meiosis and go through the meiotic prophase (Alton et al., 2008; Amleh et al., 2000; Taketo-Hosotani et al., 1989). The XY females with bilateral ovaries develop into anatomically normal females, but never produce offspring except for one case (Eicher et al., 1982; Taketo-Hosotani et al., 1989). We have previously reported that the infertility of the XY female mouse is intrinsic to its oocytes; all XX–XY chimeric females are fertile and produce offspring only derived from XX oocytes (Amleh and Taketo, 1998). Furthermore, the XY oocytes collected from antral follicles can reach the second meiotic metaphase (MII) in culture, but they contain abnormal MII-spindles and fail in proper chromosome segregation upon fertilization or parthenogenic activation (Amleh et al., 1996; Obata et al., 2008; Villemure et al., 2007). This spindle defects can largely be attributed to the XY ooplasm because healthy pups are produced from the nuclei of XY oocytes which have been transferred into enucleated XX oocytes (Obata et al., 2008). To identify the defective cytoplasmic components in the XY oocyte, we compared gene expression profiles in the fully-grown oocytes collected from XX and XY females by cDNA-microarray (Xu et al., 2012). The results suggested that expression of a couple of genes, in addition to many others, is altered in the XY oocyte in response to a shortage of glucose or energy supply. In the present study, we examined the metabolic cooperation between the oocyte and its surrounding CCs with respect to glycolytic gene expression and ATP production. Since we found lower ATP contents in XY oocytes than in XX oocytes, we further tested the effect of ATP contents on MII-spindle assembly in the oocytes after in vitro maturation (IVM). Our results suggest that ATP is one of the deficient components responsible for MII-spindle defects in the XY oocyte.

Materials and methods

Mouse

All animal experiments were conducted in accordance with the Guide to the Care and Use of Experimental Animal issued by the Canadian Council on Animal Care and with the approval by Animal Research Committee of McGill University. The B6.Y^{TIR} mouse was established by repeating backcrosses to place the Y chromosome originating from a variant of *Mus musculus domesticus* caught in Tirano, Italy (TIR) on the C57BL/6J (B6) genetic background (Nagamine et al., 1987). B6.Y^{TIR} male mice (N55–60 backcross generations) were crossed with B6 females (Jackson Laboratory, Bar Harbor, ME) to produce XY females and their XX littermates. The day of delivery was defined as 0 day postpartum (dpp). Upon weaning of pups at 20–25 dpp, their ear punches were taken and used for determining their genotypes by PCR amplification of the Y-linked *Zfy* gene as described previously (Amleh and Taketo, 1998).

Collection of COCs, oocytes, cumulus cells and preparation of oocytectomized COCs (OOX–COCs)

XX and XY females at 29 dpp were injected intraperitoneally each with 5 IU equine chorionic gonadotropin (Sigma, St Louis, MO), and sacrificed 45–47 h later. Fully-grown GV-stage oocytes surrounded by CCs, named COCs, were isolated by puncturing large antral follicles with a pair of 26-gauge needles. Then, oocytes were denuded of CCs by repeated pipetting through a fine glass needle, and the oocytes were either processed for further culture or stored for various assays, whereas CCs were collected and stored for assays. Only the oocytes with intact GV and no apparent sign of degeneration were collected for further studies. From some COCs, oocytes (both nucleus and cytoplasm) were microsurgically removed, leaving the zona pellucida for maintaining the three-dimensional structure, named OOX–COCs, as described previously (Buccione et al., 1990b). At the end of culture, CCs were collected after removing zona pellucida by repeated pipetting through a fine glass needle and stored at −80 °C.

Culture of COCs and OOX–COCs

COCs and OOX–COCs were cultured in MEM-α (Invitrogen Lifescience Technologies, Grand Island, NY) supplemented with 3 mg/ml bovine serum albumin, 75 mg/l penicillin G, 50 mg/l streptomycin sulphate and 10 μM milrinone (all from Sigma) at 37 °C in an atmosphere with 5% CO_2 and saturated humidity. Milrinone is an inhibitor of type 3 phosphodiestrase and prevents spontaneous resumption of meiosis in oocytes (Sugiura et al., 2005, 2007; Tsafriri et al., 1996). In the first set of experiments, a group of 40–50 COC_S was cultured in 2.5 ml medium for 20 h. In the second set of experiments, a group of 5 OOX–COCs was cultured in a 10 μl droplet of medium supplemented with or without recombinant FGF8B and with or without denuded 2, 5, or 10 oocytes under paraffin oil for 26 h. In the third set of experiments, a group of 5 COCs was cultured in a 10 μl droplet of medium supplemented with or without FGF8B and with or without 5 XX denuded oocytes for 26 h.

Gap junction permeability assay

Gap junction permeability was assessed by microinjecting 7–10 pl Alexa Fluor 594 (A10442, Invitrogen) into the oocyte within COC placed in a droplet of M2 medium containing 10 μM milrinone covered with paraffin oil, and imaging the diffusion of fluorescence into the companion CCs at 30 min in culture under a Zeiss Axiovert microscope.

Semi-quantitative RT-PCR

Total RNA was isolated from a pool of 40–50 oocytes or CCs collected from 10 to 20 COCs by using the RNeasy Micro Kit (Qiagen, Toronto, ON) according to the manufacturer's instruction. First-strand cDNA synthesis was carried out by using Maloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) and random hexamers as described previously (Lee and Taketo, 2001). Each cDNA sample was processed for PCR amplification by denaturation at 95 °C for 5 min, followed by 30 or 36 cycles at 95 °C for 25 s, 60 °C for 25 s, and 72 °C for 40 s, and final extension at 72 °C for 10 min with a thermocycler (Biometra, Model T1, Göttingen, Germany). The primers used for RT-PCR are given in Table 1. After PCR amplifications, 15 μl of each reaction mixture

Table 1
Primers used for RT-PCR analyses.

Gene symbol	Reference sequence	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Amplicon size (bp)	Amplification cycles
Amh	NM_007445	TCCTACATCTGGCTGAAGTGATATGGGAGC	CTCAGGGTGGCACCTTCTCTGCTTGGTTGA	283	30
β-actin	NM_007393	CCTAGGCACCAGGGTGTGAT	TCACGGTTGGCCTTAGGGTT	239	30 or 36
Bmp15	NM_009757	TTTGGATCTGGCCAAAGAAG	GGGAGAAGGCTTTGAGGAAC	391	36
Ffg8	NM_010205	GCGAAGCTCATTGTGGAGAC	GGCGGGTAGTTGAGGAACTC	344	36
Gdf9	NM_008110	CTGATAGGCGAGGTGAGACC	GGAGGAGGAAGAGGCAGAGT	302	36
Ldh1	NM_010699	AGCTGCTGATCGTCTCCAAT	GTAGGCACTGTCCACCACCT	323	30
Lhcgr	NM_013582	CTCGCCCGACTATCTCTCAC	AGATTAGCGTCGTCCCATTG	451	30
Pfkp	NM_019703	TGTGTCTGAAGGAGCAATCG	TTCAGGTTTCCCTCAAAACG	366	36
Pkm2	NM_011099	GTGACCTGGGCATTGAGATT	CGGAGTTCCTCGAATAGCTG	313	30
Tgfb2	NM_009367	GCAGGATAATTGCTGCCTTC	TTCGATCTTGGGCGTATTTC	265	36

was applied to 2% agarose gel electrophoresis in TAE buffer and visualized with ethidium bromide staining. The intensity of each band was quantified and the relative intensity against β-actin in the same lane was calculated by using AlphaImager 2000 system (Alpha Innotech Co., San Leandro, CA). We confirmed that the products of PCR reaction were within a linear range for each gene. PCR amplicons were sequenced to verify the amplified sequences in the Montreal Genome Center (Montreal, Quebec).

Immunoblotting

CCs collected from 50 to 100 XX or XY COCs were lysed in a SDS sample buffer by boiling for 10 min, and stored at −80 °C until use. The proteins in the lysed samples were separated by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes at 4 °C. The membranes were blocked in TBST buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4) containing 5% skim milk (TBST-SM) for 2 h at room temperature and then incubated with either polyclonal rabbit anti-PFKP (Novus Biologicals, Oakville, ON, diluted 1:500), monoclonal rabbit anti-PKM2, (Abcam, Cambridge, MA, diluted 1:1000), anti-LDH1 antibody (Abcam, diluted 1:10,000), or polyclonal goat anti-AMH (Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:1000) in TBST-SM overnight at 4 °C. After three times washing in TBST, the membrane was incubated with either HRP-conjugated goat anti-rabbit IgG or HRP-conjugated rabbit anti-goat IgG (Bio-Rad, Mississauga, ON, diluted 1:2000) in TBST-SM for 1 h at room temperature. After three times washing in TBST, the membrane was processed for the detection of HRP using the enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ). Protein loading was verified by the detection of GAPDH as follows. After ECL detection, the membrane was washed in stripping buffer (62.5 mM Tris, 100 mM β-mercatoethanol, 20% SDS, pH 6.7) for 30 min at 50–55 °C and then incubated with polyclonal rabbit anti-GAPDH (Santa Cruz Biotechnology, diluted 1:200) in TBST-SM overnight at 4 °C. The membrane was incubated with HRP-conjugated goat anti-rabbit IgG (Bio-Rad, diluted 1:5000) in TBST-SM for 1 h at room temperature and HRP was detected as described above. All experiments were repeated three times starting with independently collected CC samples. Band densities were quantified by Image J software (NIH image). Individual band intensities were normalized by those of GAPDH in each experiment.

Measurement of ATP contents

ATP contents in oocytes or CCs were determined by using the assay kit (Bioluminescent Somatic Cell Assay Kit, FL-ASC, St Louis, MO) based on the luciferin–luciferase reaction as previously described (Combelles and Albertini, 2003; Van Blerkom et al., 1995) with minor changes. Briefly, 40 denuded oocytes or CCs collected from 20 to 40 COCs in a group were snap-frozen in 160 μl and 180 μl water, respectively, in a microfuge tube and stored at −80 °C. For ATP assays, 50 μl of each thawed sample solution was added to 100 μl ice-cold Cell ATP-Releasing Reagent and incubated on ice for 5 min, followed by addition of 100 μl ice-cold ATP Assay Mix (1:25 diluted in assay mix buffer). The reaction mixture was then incubated for 10 min in dark at room temperature for the initial chemiluminescence flash period. The bioluminescence of each sample was measured by high-sensitivity luminometer (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) including an 11-point standard curve (containing 0 to 5.0 pmol ATP) in each assay. DNA concentrations in the remaining lysed samples of CCs were determined and used for normalizing ATP contents in each sample. ATP contents in oocytes were expressed as pmol/oocyte, while those in CCs were expressed as pmol/mg DNA.

ATP microinjection

ATP microinjection was performed by using Narishige MMN-1 micromanipulators mounted on a Zeiss Axiovert microscope. Within 30 min of COC collection, 7–10 pl of either PBS alone (control) or (space) 10 mM ATP (Sigma) in PBS was injected into each oocyte in COC placed in a droplet of M2 medium containing 10 μM milrinone covered with paraffin oil. 20–40 oocytes in a group were microinjected, and subjected to IVM as described below. Each experiment was repeated three times.

Oocyte maturation in vitro

COCs were washed thoroughly in MEM-α supplemented with 5% fetal bovine serum (GIBCO, Invitrogen), 300 ng/ml FSH, 25 μg/ml sodium pyruvate, 75 mg/l penicillin G and 50 mg/l streptomycin sulphate (all from Sigma), and cultured in the fresh medium at 37 °C in an atmosphere with 5% CO_2 and saturated humidity for 19 h. For the preincubation experiment, a group of 5 COCs was cultured in a 10 μl droplet of medium under paraffin oil whereas for the ATP injection experiment, a group of 20–30 COCs was cultured in 2.5 ml medium. At the end of culture, COCs were treated with 300 μg/ml hyaluronidase for 5 min at 37 °C, and oocytes were denuded of CCs by repeated pipetting in an M2 medium.

Immunofluorescence staining and confocal microscopy

Denuded oocytes were fixed in a solution containing 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and microtubule stabilizing buffer (100 mM Pipes pH 6.9, 5 mM $MgCl_2$, 2.5 mM EGTA, 0.5% Triton X-100, 1 μM taxol, 10 IU/ml aprotinin, and 50% D_2O) (all from Sigma) for 30 min at room temperature. Then, oocytes were blocked in PBS containing 3% BSA and 0.01% Triton X-100 (blocking solution) for 1 h at room temperature and incubated with mouse anti-Polo-like 1 (PLK1) antibody (Sigma) diluted 1:100 in the blocking solution overnight at 4 °C. After three washes in PBS containing 0.1% Tween-20 and

0.01% Triton X-100 (washing solution), the oocytes were incubated with Alexa Fluor 647-conjugated goat anti-mouse IgG (Invitrogen, diluted 1:500) for 1 h. After three washes, the oocytes were incubated in the blocking solution for 1 h and then with rabbit anti-γ-tubulin antibody (Sigma, diluted 1:500) followed by rhodamine-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL, diluted 1:500). Then, the oocytes were washed three times, and incubated with mouse anti-α-tubulin-FITC antibody (Sigma, diluted 1:400) for 1 h. After three washes, oocytes were transferred into Prolong Antifade mounting medium containing DAPI (Molecular Probe, Eugene, OR) and mounted on Plus-charged histology slides. Oocytes were observed under a confocal laser-scanning microscope (Zeiss LSM 510 META, Germany) within one week. Each experiment was repeated three times, and at least 30 oocytes were examined in each group.

Statistical analyses

Results were analyzed by one-way ANOVA followed by LSD test, Student's *t*-test, or χ^2-test.

Results

Gene expression levels and ATP contents in CCs were altered in the XY antral follicle

Our previous cDNA-microarray results suggested that gene expression in the XY fully grown oocyte has been altered by a limited supply of glucose or energy source from its neighbouring CCs (Xu et al., 2012). Since the oocyte cannot directly metabolize glucose, its surrounding CCs provide glucose intermediates to the oocyte for energy production (Biggers et al., 1967; Donahue and Stern, 1968). We hypothesized either that oocyte-CCs communication was impaired or that CCs had limited metabolic activity in the XY ovary. To test the direct oocyte-CC communication, we injected a gap junction channel-permeant fluorescent dye into the oocytes within COCs. The injected dye diffused into the surrounding CCs similarly in XX and XY COCs, indicating that junctional communication between the oocyte and CCs in the XY ovary had no obvious defects (Fig. S1).

To test the possibility that CCs in the XY ovary have limited activity of glucose metabolism, we compared the transcript levels of *Pfkp*, *Pkm2*, and *Ldh1* involved in glycolysis, as well as *Amh* and *Lhcgr*, all of which are known to be regulated at transcription levels by OSFs, in the CCs isolated from antral follicles of XX or XY ovaries by sqRT-PCR. *Pfkp* encodes an enzyme phosphofluctokinase (PFKP), which is the rate-limiting step of glucose metabolism in the pentose phosphate pathway, whereas pyruvate kinase (PKM2) is required for the conversion of phosphoenolpyruvate into pyruvate. Lactate dehydrogenase (LDH1) mediates bidirectional conversion between pyruvate and lactate and hence their homeostasis. The results indicated that the transcript levels of *Pfkp*, *Pkm2*, and *Ldh1* were significantly lower in the CCs of XY COCs compared to those of XX COCs (Fig. 1A), whereas those of the LH receptor gene *Lhcgr* were undetectable in the CCs of either XX or XY COCs (Fig. S2). On the other hand, the transcript levels of anti-Mullerian hormone gene *Amh* were significantly higher in the CCs of XY COCs. In consistent with glycolytic gene expression levels, ATP contents were significantly lower in the CCs of XY COCs than those of XX COCs (Fig. 1B). We also confirmed that protein levels of PFKP, PKM2, and AMH in CCs corresponded to their transcript levels, although no difference was found in the protein levels of LDH1 (Fig. 1C and D). These results indicate that glycolytic gene expression levels were specifically decreased, resulting in lower ATP contents, in the CCs of XY COCs.

ATP contents were significantly lower in XY oocytes than in XX oocytes

Low glycolytic gene expression levels and/or ATP contents in CCs may be reflected in ATP contents of the enclosed oocyte. Accordingly, we measured ATP contents in the oocytes collected from XX or XY COCs. The results show that ATP contents in XY oocytes were significantly lower than those in XX oocytes (Fig. 1B). These data suggest that ATP contents in the oocyte correlate with glycolytic gene expression levels as well as ATP contents in its neighboring CCs in the ovary.

XY oocytes were less efficient than XX oocytes in promoting glycolytic gene expression in CCs

In order to determine whether or not the XY oocyte is responsible for low glycolytic gene expression levels in CCs, we cultured OOX–COCs from XX ovaries with or without XX or XY denuded oocytes at different dosages in the medium containing milrinone for 26 h, and examined the transcript levels of glycolytic genes in CCs after culture. We used milrinone to maintain the oocytes at the GV-stage during culture since metabolic activity of both oocytes and CCs drastically change when oocytes undergo spontaneous meiotic resumption (Downs, 1995; Yu et al., 2010). As shown in Fig. 2, the removal of oocyte from COC (oocytectomy) alone resulted in significantly lower transcript levels of all the glycolytic genes tested, compared to those in intact COCs ($n=4$, $P<0.05$). Co-culture of OOX–COCs with XX oocytes at 0.2 or 0.5/μl dosage significantly increased the transcript levels of glycolytic genes, compared to the oocytectomy control without co-culture ($n=4$, $P<0.05$). XY oocytes at 0.5/μl dosage had no effect ($n=4$), whereas those at 1.0/μl dosage significantly increased the transcript levels of glycolytic genes, compared to the oocytectomy control without co-culture ($n=4$, $P<0.05$). No difference was found between co-culture with XY oocytes at 1.0/μl and that with XX oocytes at 0.2/μl ($n=4$ each). The transcript levels of *Amh* and *Lhcgr* were under the detection limit in all tested groups (Fig. S3). These results suggest that XY oocytes were less efficient than XX oocytes in producing the OSF activity that regulates glycolytic gene expression in CCs.

Expression of the genes encoding oocyte-secretory factors in the XY oocyte

To identify the OSFs that are deficient in XY oocytes, we examined the transcript levels of *Ffg8* and *Bmp15*, known to promote glycolysis gene expression in CCs (Sugiura et al., 2007), in the fully-grown oocytes isolated from XX or XY ovaries (Fig. 3). Significantly lower levels in XY oocytes were found for *Ffg8* ($n=3$, $P<0.05$) but not for *Bmp15* ($n=3$).

Addition of FGF8 to the culture medium synergistically enhanced the effects of either XX or XY oocytes on glycolytic gene transcript levels in CCs

Since FGF8 is known to synergistically enhance glycolytic gene expression in CCs with other OSFs in vitro (Sugiura et al., 2007), we tested whether lower expression of *Ffg8* in XY oocytes could contribute to lower glycolytic gene transcript levels in CCs. Of eight possible alternative spliced isoforms of *Fgf8* (MacArthur et al., 1995), *Fgf8b* is the most abundant isoform expressed in the mouse oocyte (Sugiura et al., 2007). We co-cultured XX OOX–COCs with or without denuded XX or XY oocytes in the medium supplemented with recombinant FGF8B. We chose the dosage 50 ng/ml of FGF8B, which alone had no or little effect ($n=4$), whereas FGF8B at 100 ng/ml and 200 ng/ml moderately increased

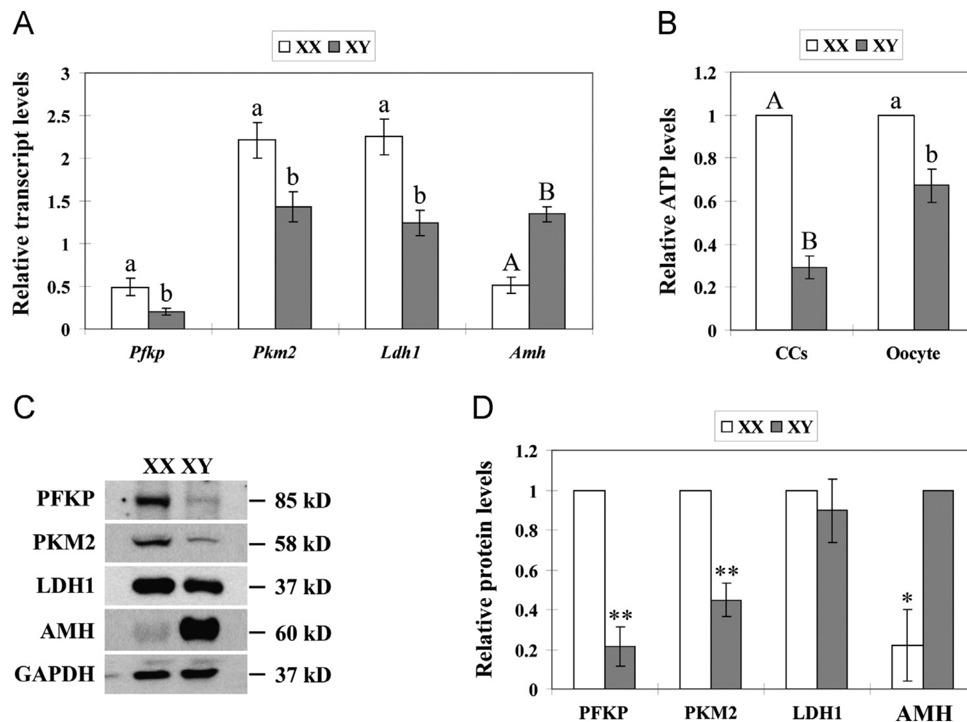

Fig. 1. Gene expression levels and ATP contents in CCs associated with ATP contents in the enclosed oocytes within XX and XY COCs. (A) Transcript levels of each gene ($n=4$) were normalized against those of β-actin. (B) ATP contents in CCs ($n=3$) and the enclosed oocytes ($n=6$) of XY COCs were normalized against those of XX COCs. Each column indicates the mean ± SEM. Different low case and capital letters above columns indicate statistical differences at $P<0.05$ and 0.01, respectively, by paired Student's *t*-test. (C) Protein levels in CCs were compared by immunoblotting and normalizing to those of GAPDH in each lane. (D) Protein levels of PFKP, PKM2, and LDH1 in XY CCs were normalized to those in XX CCs whereas protein levels of AMH in XX CCs were normalized to those in XY CCs in each experiment ($n=3$). Each column indicates the mean ± SEM. * and ** above columns indicate statistical differences at $P<0.05$ and 0.01, respectively, by unpaired Student's *t*-test.

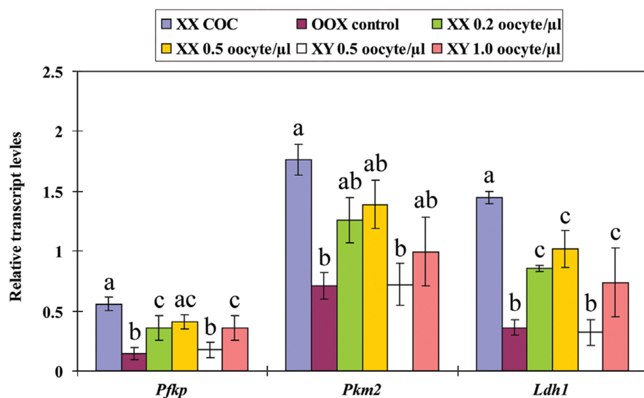

Fig. 2. Effects of XX and XY oocytes on the transcript levels of glycolytic genes in CCs. Oocytes had been eliminated from XX COCs (OOX), which were then cultured with or without denuded oocytes for 26 h in the presence of 10 μM milrinone. Transcript levels were normalized against those of β-actin. Each column indicates the mean ± SEM ($n=4$). Different low case letters above columns indicate statistical differences at $P<0.05$ by one-way ANOVA followed by LSD test.

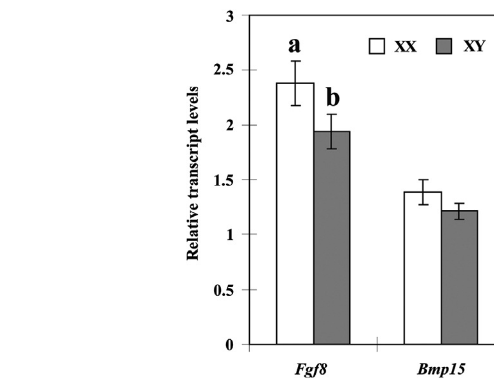

Fig. 3. Transcript levels of *Fgf8* and *Bmp15* genes in XX and XY oocytes collected from antral follicles. Transcript levels were normalized against those of β-actin. Each column indicates the mean ± SEM ($n=3$). (a) and (b) above columns indicate statistical differences at $P<0.05$ by paired Student's *t*-test.

Pkm2 transcript levels in a dose-dependent manner (Fig. 4). We also chose the dosage of XX oocytes at 0.2/μl and XY oocytes at 1.0/μl because they showed similar effects on the transcript levels of *Pfkp*, *Pkm2* and *Ldh1* in CCs (Fig. 2). The results showed that the addition of FGF8B to the culture medium dramatically increased the transcript levels of all the tested glycolytic genes in CCs when OOC–COCs were co-cultured with either XX or XY denuded oocytes ($n=4$, $P<0.05$) (Fig. 5). When the dosage of XY oocytes for co-culture was decreased to 0.5/μl, however, no or little increase was observed despite FGF8B supplementation (Fig. S4). These results suggest that low *Fgf8* transcript levels are not sufficient to explain the low OSFs activity of XY oocytes, while XY oocytes are deficient in an OSF(s), other than FGF8, which synergistically increases glycolytic gene expression in CCs with FGF8.

ATP contents in the XY oocyte paralleled glycolytic gene transcript levels in its companion CCs after culture

Since co-culture with denuded oocytes and FGF8 showed a drastic increase in glycolytic gene expression levels in CCs, we asked whether the same treatment of COCs would increase ATP contents in the enclosed oocyte. However, the CCs of XY COCs are of the XY genotype, which may be intrinsically dysfunctional. To test this possibility, we cultured the COCs isolated from XX or XY ovaries for 20 h in the medium containing milrinone. The results showed that at the end of culture, the transcript levels of *Pfkp*, *Pkm2* or *Ldh1* in the CCs of XY COCs were no longer different from

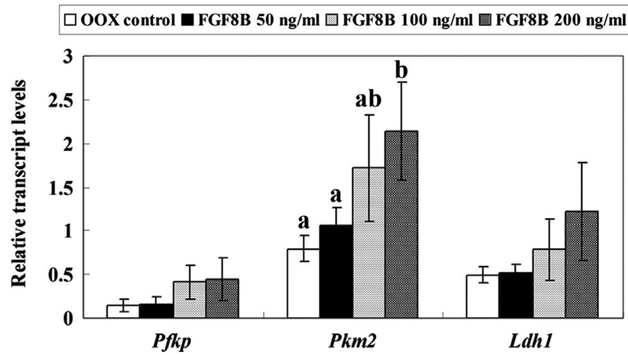

Fig. 4. Effects of recombinant FGF8B on the transcript levels of glycolytic genes in CCs. OOX–COCs from XX ovaries were cultured in the medium supplemented with 10 μM milrinone and FGF8B at the dosages of 0 ng/ml, 50 ng/ml, 100 ng/ml, or 200 ng/ml for 26 h. Transcript levels were normalized against those of β-actin. Each column indicates the mean ± SEM ($n=3$). Different low case letters above columns indicate statistical differences at $P<0.05$ by one-way ANOVA followed by LSD test.

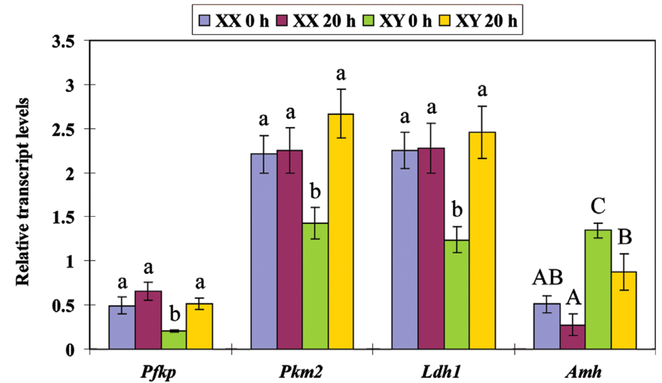

Fig. 6. Relative gene transcript levels in the CCs of XX and XY COCs before and after culture in the presence of 10 μM milrinone for 20 h. Transcript levels were normalized against those of β-actin. Each column indicates the mean ± SEM ($n=4$). Different low case and capital letters above columns indicate statistical differences at $P<0.05$ and 0.01, respectively, by one-way ANOVA followed by LSD test.

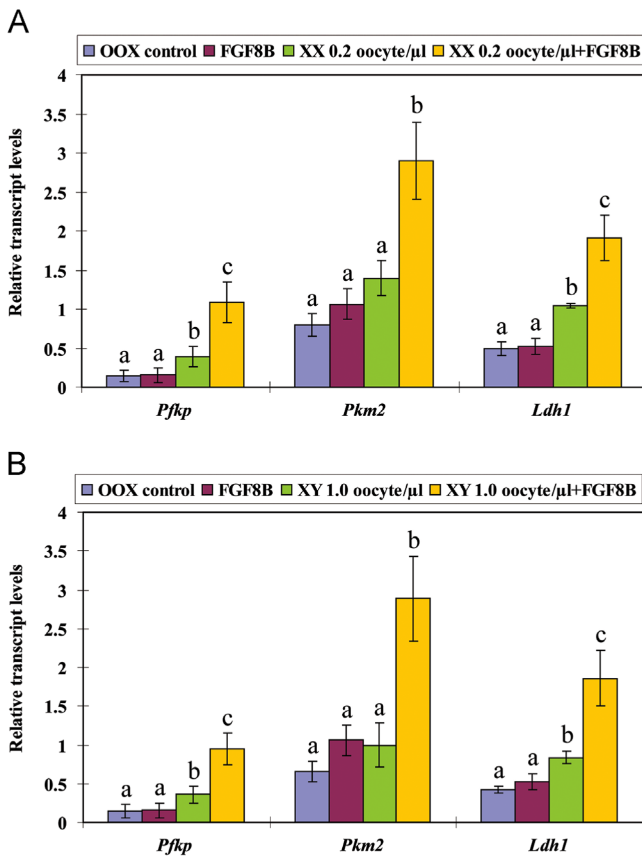

Fig. 5. Synergistic effects of FGF8 with co-cultured oocytes on the transcript levels of glycolytic genes in CCs. XX OOX–COCs were cultured with or without denuded (A) XX oocytes at 0.2/μl or (B) XY oocytes at 1.0/μl in the medium supplemented with 10 μM milrinone and with or without 50 ng/ml FGF8B for 26 h. Transcript levels were normalized against those of β-actin. Each column indicates the mean ± SEM ($n=4$). Different low case letters above columns indicate statistical differences at $P<0.05$ by one-way ANOVA followed by LSD test.

those of XX COCs (Fig. 6). These results suggest that glycolytic gene expression in CCs was repressed within the antral follicles of XY ovaries in vivo, but isolation and culture of COCs relieved the CCs from this repressive effect. By contrast, the transcript levels of *Amh* remained higher in the CCs of XY COCs than those of XX COCs after culture.

We next cultured XX and XY COCs in the presence of denuded XX oocytes (0.2/μl), 50 ng/ml FGF8 and milrinone for 26 h. As shown in Fig. 7A, the transcript levels of *Pfkp*, *Pkm2* and *Ldh1* significantly increased in the CCs of both XX and XY COCs by the co-culture ($n=4$, $P<0.05$). By contrast, the transcript levels of *Amh* in the CCs of XY COCs decreased by the co-culture, but still remained higher than those in the CCs of XX COCs ($n=4$, $P<0.05$). In consistent with glycolytic gene transcript levels, ATP contents in the CCs of both XX and XY COCs became equal after culture alone and were further increased by the co-culture ($n=3$, $P<0.05$) (Fig. 7B). ATP contents in the enclosed oocytes also increased in both XX and XY COCs ($n=3$, $P<0.05$); however, the increase in XY oocytes was significantly smaller than that in XX oocytes ($P<0.05$). These results demonstrate that ATP contents in the oocyte are tightly associated with the transcript levels of glycolytic genes and ATP contents in its neighbouring CCs, whereas the XY oocyte may have a limited capacity of producing ATP when the supply of glycolysis intermediates from CCs is high.

Preincubation of COCs in the presence of milrinone, followed by IVM, improved MII-spindle assembly in XY oocytes

Our previous studies have shown that when COCs are collected from XY antral follicles and immediately subjected to IVM, the enclosed oocytes reach the MII-stage but their meiotic spindles are morphologically abnormal, resulting in a zygotic developmental failure (Villemure et al., 2007; Obata et al., 2008). Our current studies indicated that while ATP contents in fully grown XY oocytes were lower than those in XX oocytes when collected from antral follicles, they increased to comparable levels after COCs were incubated in the presence of milrinone for 20 h (preincubation). Accordingly, we tested whether the preincubation improves MII-spindle assembly in the oocytes from XY females after IVM. We analyzed MII-spindles as to spindle morphology, chromosome alignment, and spindle orientation as shown in Fig. 8A. Spindle morphology was assigned into four groups; Type I with two pointing poles which were narrower than the equator; Type II with one or two spread poles as wide as the equator; Type III with one spread pole wider than the equator; others with no or fuzzy spindles. Some oocytes were positioned with their spindles vertically on histology slides, hence the structure of their spindles could not be identified, and such oocytes were excluded from the analysis. MII-chromosome alignment was judged normal when they were compact in one area along the equator. Spindle orientation was judged normal when it was positioned parallel to the ooplasmic membrane. As summarized in Fig. 8B, a significantly larger percentage of oocytes from XY females showed abnormal MII-spindles in every parameter examined, compared to

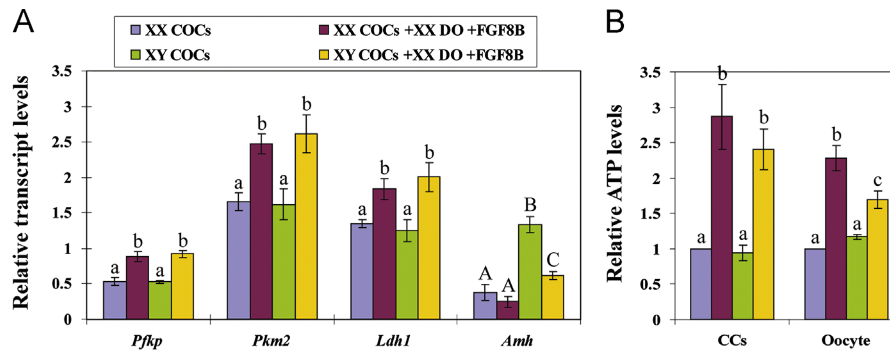

Fig. 7. Synergistic effects of OSFs and FGF8 on glycolytic gene expression levels and ATP contents in CCs and ATP contents in the enclosed oocytes of XX and XY COCs. XX or XY COCs were co-cultured with denuded XX oocytes at 0.5/μl in the medium supplemented with 50 ng/ml FGF8B and 10 μM milrinone for 26 h. (A) Relative gene transcript levels in CCs ($n=4$). Transcript levels were normalized against those of β-actin. (B) Relative ATP contents in CCs and oocytes ($n=3$). ATP contents were normalized against those in the CCs or oocytes of XX COCs. Each column indicates the mean ± SEM. Different low case and capital letters above columns indicate statistical differences at $P<0.05$ and 0.01, respectively, by one-way ANOVA followed by LSD test.

those from XX females, when they had been subjected to IVM without preincubation. By contrast, preincubation of COCs prior to IVM significantly increased the percentages of oocytes from XY females with either normal spindle morphology or MII-chromosome alignment, but not those with normal spindle orientation, while little change was seen in the oocytes from XX females.

ATP injection into the oocytes within COCs, followed by IVM, improved MII-spindle assembly

To test whether beneficial effects of preincubation on MII-spindle assembly can be attributed to higher ATP contents, we microinjected ATP or PBS (control) into the oocytes within COCs prior to IVM. As summarized in Fig. 8C, while microinjection of ATP or PBS into XX oocytes had little effect on their MII-spindle assembly, microinjection of PBS into XY oocytes lowered all the three parameters of MII-spindle assembly, suggesting that XY oocytes were more vulnerable to mechanical manipulations. Nonetheless, microinjection of ATP into XY oocytes improved MII-spindle assembly compared to the control in all the three parameters tested including spindle orientation. The overall improvement in either spindle morphology or chromosome alignment by ATP injection did not reach the levels observed with preincubation (Fig. 8B).

Discussion

Our current results demonstrate that the XY oocyte produces lesser activity of OSFs that enhances glycolytic gene expression in its neighbouring CCs, compared with the XX oocyte. We predicted that the CCs in turn provide either a lower supply of glycolysis intermediates or the end-product ATP to the enclosed XY oocyte. In fact, we found that ATP contents were lower in XY oocytes than in XX oocytes, in consistent with ATP contents in their companion CCs. Moreover, incubation of XY COCs in the presence of milrinone to maintain the oocyte at the GV-stage for 20 h increased both glycolytic gene expression levels in CCs and ATP contents in the enclosed oocytes. By raising ATP contents in the XY oocyte by either preincubation or ATP injection prior to IVM, MII-spindle defects were partially corrected. Taken together, the XY oocyte appears to become defective in its cytoplasm by involving mutual communication with its companion CCs. It has been reported that low ATP contents in the mouse and human oocytes are closely associated with MII-spindle defects and its poor outcome following IVF (Ata et al., 2012; Battaglia et al., 1996; Van Blerkom et al., 1995, 1998; Wang et al., 2009; Wilding et al., 2001; Zhang et al., 2006). Our results suggest that the oocyte-cumulus cell regulatory loop contributes to determining the ATP contents in the oocyte and, hence, developmental competence under physiological conditions. These results support the idea that the gene expression profile in CCs can be used for the diagnosis of oocyte competence in assisted reproduction technologies.

The XY oocyte is deficient in one or more OSFs that increases glycolytic gene expression in CCs

Our results suggest that the XY oocyte produced lesser activity of OSF(s) that synergistically enhanced glycolytic gene expression in CCs with FGF8. The transcript levels of *Fgf8* in the XY fully-grown oocyte were slightly but significantly lower than those in the XX oocyte. However, FGF8 itself was unlikely the major OSF deficient in the XY oocyte since 5 XY oocytes were required for exerting the same effects as one XX oocyte in promoting glycolytic gene expression in CCs. Another OSF known to increase glycolysis in CCs is BMP15 (Sugiura et al., 2007). X-linked *Bmp15* may be expected to be lower in XY oocytes than XX oocytes since both X chromosomes are activated in XX oocytes. However, we found that *Bmp15* transcript levels were comparable between XY and XX oocytes. Our previous study has shown that many more X-linked genes have equal transcript levels in XX, XO, and XY fully-grown oocytes despite their different gene dosages, suggesting that the storage of mRNAs in fully grown oocytes is well adjusted (Xu et al., 2012). We predict that OSFs other than BMP15 or FGF8 may be deficient in XY oocytes.

ATP contents in the oocyte are tightly associated with glycolytic gene expression levels in its neighbouring CCs

The importance of bi-directional communication between the oocyte and its neighbouring CCs has been well documented (Buccione et al., 1990a; Simon et al., 1997; Su et al., 2009; Sugiura and Eppig, 2005; Van Blerkom et al., 1998). However, the role of this communication in ATP contents in the oocyte is less understood. In the present study, we found that ATP contents in the oocyte were tightly correlated with glycolytic gene expression levels in its companion CCs under various conditions both in vivo and in vitro. As expected, ATP contents in CCs were also tightly associated with glycolytic gene expression levels. Therefore, in theory, any of glycolysis intermediates and products in CCs can be transported into the enclosed oocyte, resulting in low ATP contents in the oocyte. It is generally accepted that the most critical metabolite is pyruvate, which is the direct substrate for oxidative

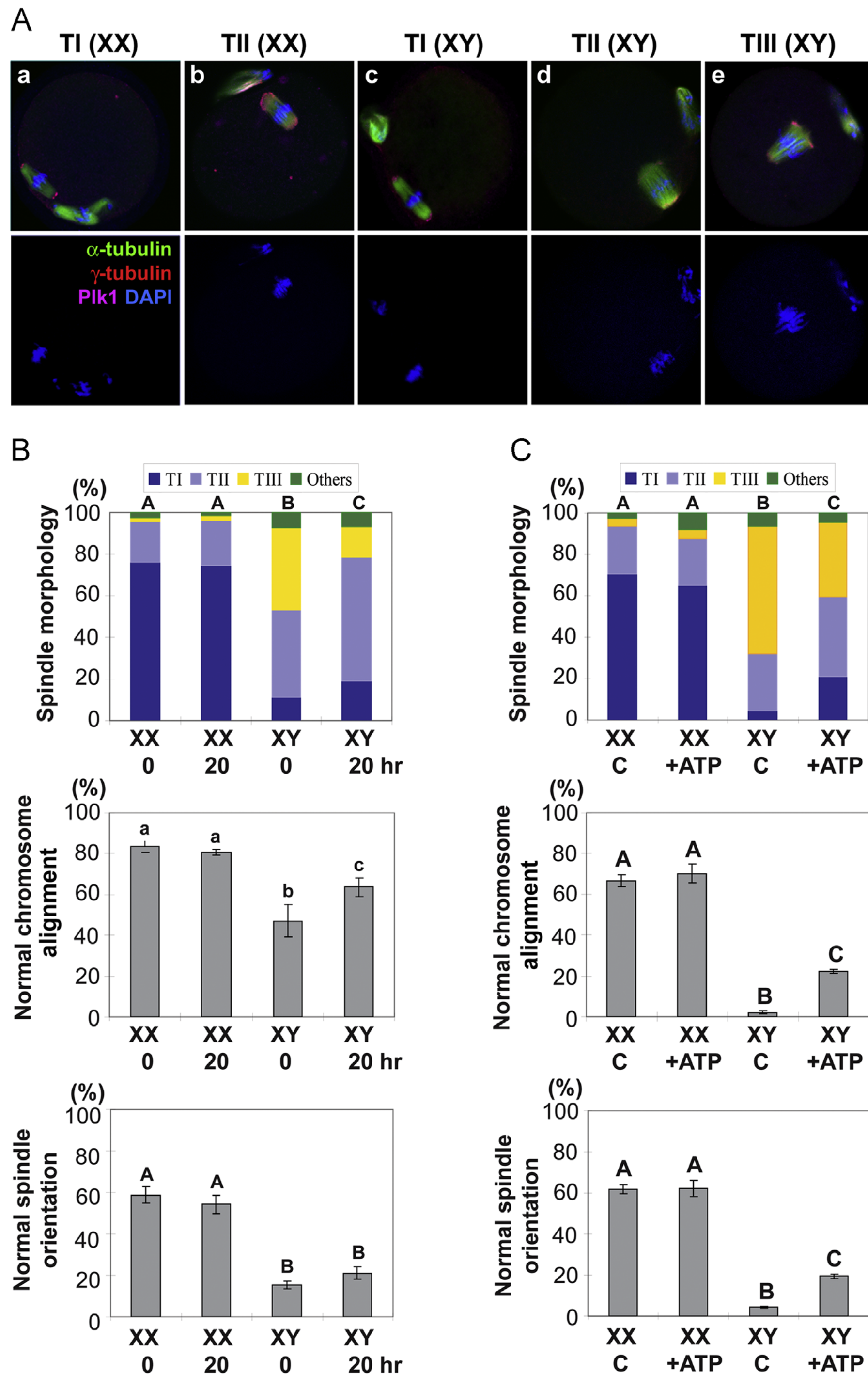

Fig. 8. Effects of ATP contents in fully grown oocytes on the MII-spindle assembly after IVM. (A) Examples of MII-spindles, immunofluorescence stained for α-tubulin (green), γ-tubulin (red), and PLK1 (pink), counterstained for DAPI (blue). Merged images are shown in the top panel and DAPI alone in the bottom panel. (a) XX oocyte with a normal MII-spindle, i.e., in a slim shape with pointing poles (Type I) in a parallel position to the ooplasmic membrane (normal orientation). MII-chromosomes are compactly aligned along the equator. (b) XX oocyte with a spindle, the poles of which are spread as wide as the equator (Type II) and in a perpendicular position. MII-chromosome alignment is normal. (c) XY oocyte with normal spindle morphology, orientation, and chromosome alignment. (d) XY oocyte with Type II spindle in a perpendicular position. MII-chromosomes are loosely aligned around the equator. (e) XY oocyte with a spindle, which has one pole spread wider than the equator (Type III) and in the perpendicular position. MII-chromosomes are spread in a larger area around the equator, compared to normal alignment ((a)–(c)). (B) MII-spindle assembly in the oocytes immediately subjected to IVM (0 h) or first incubated in the presence of milrinone (20 h) and then subjected to IVM for 19 h. (C) MII-spindle assembly in the oocytes injected with either PBS (C; control) or ATP, followed by IVM for 19 h. Each column and bar indicate the mean ± SEM (n=3). Different low case and capital letters above columns indicate statistical differences at $P<0.05$ and 0.01, respectively, by χ^2 test.

phosphorylation by mitochondria (Biggers et al., 1967; Buccione et al., 1990a; Downs and Utecht, 1999; Eppig, 1976; Sutton-McDowall et al., 2010). Mouse oocytes can use pyruvate, but not glucose, as a major energy source, whereas GCs/CCs actively metabolize glucose to pyruvate and rely predominantly on glycolysis for their energy production in follicles (Biggers et al., 1967;

Donahue and Stern, 1968; Downs and Utecht, 1999; Leese and Barton, 1985). However, it has been reported that the female mouse with an depletion of *Phda1* in the oocyte during the growth phase allows for oocyte survival and maturation (Johnson et al., 2007). *Phda1* encodes a subunit of pyruvate dehydrogenase complex, which plays important roles in oxidative metabolism of pyruvate in mitochondria. These findings suggest that pyruvate is not the only metabolite to support ATP production in the oocyte during follicular growth or in COCs. In our current study, pyruvate in the culture medium may have contributed to the increase in ATP contents in the XY oocytes within COCs during preincubation. However, further increase in ATP contents in the XY oocytes after co-culture with denuded XX oocytes and FGF8 cannot be explained simply by the presence of pyruvate in the culture medium. We do not exclude the possibility that ATP in CCs were directly transported into the enclosed oocyte.

Impairment of mitochondrial function in the oocyte is often blamed for low ATP contents. While the mitochondrial pathway is much more efficient than glycolysis for ATP production, follicular environment is known to be hypoxic (Gosden and Byatt-Smith, 1986), and the oocyte contains numerous mitochondria showing the characteristic of inactive forms (Piko and Chase, 1973; Wilding et al., 2009). Nonetheless, it has been shown that mitochondrial ATP production is essential for oocyte maturation and developmental competence (Dumollard et al., 2008). We have confirmed that the number and distribution of mitochondria in XY oocytes at the GV-stage or during maturation in vitro did not differ from that in XX oocytes by MitoTracker staining (Fig. S5). However, our results showed that ATP contents in the oocytes within XY COCs after co-culture with both XX denuded oocytes and FGF8B did not increase as much as those in XX COCs even though the transcript levels of glycolytic genes and ATP contents in CCs increased to comparable levels in XY and XX COCs. We speculate that mitochondria in XY oocytes were less efficient than XX oocytes in ATP production when the supply of glycolysis intermediates was high. Further studies are needed to clarify the metabolic coupling between the oocyte and CCs as well as mitochondrial functions in the XY oocyte.

Low glycolytic gene expression in the CCs of XY COCs is reversible

The transcript levels of *Pfkp*, *Pkm2*, and *Ldh1* were significantly lower in the CCs collected from antral follicles of XY COCs than those of XX COCs. However, after COCs had been cultured in the presence of milrinone for 20 h or 26 h, the glycolytic gene transcript levels were no longer different between XX and XY CCs. These results indicate that glycolytic gene expression in CCs was impaired in the follicular microenvironment in the XY ovary; however, once COCs had been released from follicles and cultured, the CCs of XY COCs recovered from the repressive status. These observations are consistent with our previous finding that XY somatic cells support XX oocytes for producing healthy pups in XX–XY chimeric females (Amleh and Taketo, 1998).

By contrast, the transcript levels of *Amh* remained higher in the CCs of XY COCs compared to those of XX COCs after preincubation. These results suggest that the CCs of XY COCs were not fully normalized after culture. Anti-Mullerian hormone (AMH or MIS) is a TGF-β family member and known to inhibit the recruitment of primordial follicles into the pool of growing follicles and to decrease the responsiveness of granulosa cells to FSH in growing follicles (Durlinger et al., 1999,2001,2002). In human ART, the serum level of AMH has been used as an indicator of follicle numbers in reserve in women with poor oocyte retrieval (Broekmans et al., 2008; Visser et al., 2012). Our current results show that *Amh* expression levels in CCs was little to do with glycolysis gene expression or ATP production. Nonetheless, abnormally higher *Amh* expression levels may suggest a permanent impairment of GCs/CCs in the XY ovary and may be associated with the incompetence of the enclosed oocyte, in addition to low ATP contents.

Increasing ATP contents in the XY fully grown oocyte partially rescues the oocyte from MII-spindle assembly defects

Our previous studies have shown that the MII-oocytes from XY females are defective in their cytoplasm, which causes abnormal MII-spindle assembly and blocks subsequent embryonic development (Obata et al., 2008). These defects can be overcome by transferring the nucleus of the XY oocyte into an enucleated XX oocyte, more efficiently when the nuclear transfer is performed at the GV-stage than at the MII-stage. Our current results indicate that ATP is one of the components deficient in XY oocytes and partially responsible for the MII-spindle assembly defect. By increasing ATP contents in XY oocytes by preincubation of COCs prior to IVM, both MII-spindle morphology and chromosome alignment significantly improved. However, spindle orientation did not improve; 60% of oocytes from XX females were seen with MII-spindles positioned in parallel to the ooplasmic membrane while only 15–20% of oocytes from XY females were found in parallel position. Therefore, the regulation of MII-spindle orientation appears to differ from that of spindle morphology or chromosome alignment. It must be noted that MII-oocytes of many mammalian species including humans normally contain spindles in perpendicular orientation (Goud et al., 2004; Sun and Schatten, 2006). We need further studies to determine whether parallel orientation of MII-spindles is essential for successful chromosome segregation in the mouse.

Microinjection of ATP into the oocytes within XY COCs prior to IVM also improved MII-spindle assembly including not only spindle morphology and chromosome alignment but also spindle orientation. However, overall improvement in MII-spindle assembly by ATP injection was not as efficient as that by preincubation. As microinjected ATP is expected to be short-lasting, its influence on MII-spindle assembly after culture for 19 h suggest that ATP contents in the fully grown oocyte have a long-lasting effect, probably by altering other ooplasmic components. Sufficient ATP levels are required for many cellular events including cell cycle kinase activity, microtubule polymerization, motor protein movement, and molecular trafficking (Dumollard et al., 2008; Stojkovic et al., 2001; Wadsworth and Salmon, 1988; Zeng et al., 2007). Nonetheless, MII-spindle assembly, particularly spindle pole organization and orientation, is most sensitively affected by low ATP levels in the oocytes from XY female mice. This is consistent with the reports in human oocytes (Van Blerkom et al., 1995; Zeng et al., 2007; Zhang et al., 2006). Further studies are needed to clarify the role of ATP levels in MII-spindle assembly and subsequent chromosome segregation in oocytes.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.11.004.

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