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In Fig. 4, parts A and B, the indicated concentrations of TGFB2 are incorrect, they should be 0, 1.0, 5.0 and 10 ng/ml.

This error does not affect the conclusions of the paper.

The authors apologise to readers for this mistake.

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Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells

Koji Sugiura[1], You-Qiang Su[1], Francisco J. Diaz[1], Stephanie A. Pangas[2], Shweta Sharma[3], Karen Wigglesworth[1], Marilyn J. O'Brien[1], Martin M. Matzuk[2,4,5], Shunichi Shimasaki[3] and John J. Eppig[1,*]

Mammalian oocytes are deficient in their ability to carry out glycolysis. Therefore, the products of glycolysis that are necessary for oocyte development are provided to oocytes by companion cumulus cells. Mouse oocytes secrete paracrine factors that promote glycolysis in cumulus cells. The objective of this study was to identify paracrine factors secreted by oocytes that promote glycolysis and expression of mRNA encoding the glycolytic enzymes PFKP and LDHA. Candidates included growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and fibroblast growth factors (FGFs). *Bmp15*$^{-/-}$ and *Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$ (double mutant, DM) cumulus cells exhibited reduced levels of both glycolysis and *Pfkp* and *Ldha* mRNA, and mutant oocytes were deficient in promoting glycolysis and expression of *Pfkp* and *Ldha* mRNA in cumulus cells of wild-type (WT) mice. Alone, neither recombinant BMP15, GDF9 nor FGF8 promoted glycolysis and expression of *Pfkp* and *Ldha* mRNA in WT cumulus cells. Co-treatment with BMP15 and FGF8 promoted glycolysis and increased expression of *Pfkp* and *Ldha* mRNA in WT cumulus cells to the same levels as WT oocytes; however, the combinations of BMP15/GDF9 or GDF9/FGF8 did not. Furthermore, SU5402, an FGF receptor-dependent protein kinase inhibitor, inhibited *Pfkp* and *Ldha* expression in cumulus cells promoted by paracrine oocyte factors. Therefore, oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells.

KEY WORDS: BMP15, FGF8, Cumulus cells, Glycolysis, Mouse, Oocytes

INTRODUCTION

Bidirectional communication between mammalian oocytes and their associated follicular somatic cells, cumulus cells, is essential for development of both cell types (Eppig, 2001). While fully grown oocytes regulate the proliferation, gene expression and function of cumulus cells, nutritional support from cumulus cells is essential for growth and development of the oocyte (Eppig, 2001). Notably, oocytes are deficient in carrying out glycolysis, and utilize glucose poorly as an energy substrate. The cumulus cells surrounding the oocyte provide products of glycolysis, such as pyruvate, to the oocyte by either secreting them or passing them through the gap junctions that couple the two cell types (Biggers et al., 1967; Donahue and Stern, 1968; Leese and Barton, 1985).

Glycolysis in cumulus cells is regulated by paracrine factor(s) secreted by oocytes (Sugiura et al., 2005). When compared with other follicular cell types, such as mural granulosa cells that line the follicular wall, cumulus cells express higher levels of mRNA encoding glycolytic enzymes, such as platelet phosphofructokinase (*Pfkp*) and lactate dehydrogenase A (*Ldha*). Removing oocytes from cumulus cell-oocyte complexes (COCs) reduces the steady-state levels of these transcripts and decreases glycolytic activity in cumulus cells, and these decreases are completely reversed by co-culturing the cumulus cells with fully grown oocytes (Sugiura and Eppig, 2005; Sugiura et al., 2005). Therefore, mouse oocytes regulate glycolysis in their companion cumulus cells, and this is achieved, at least in part, by secreted products that promote expression of transcripts encoding glycolytic enzymes.

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Mouse oocytes secrete proteins of two growth factor families: members of the transforming growth factor β (TGFβ) superfamily, including growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and TGFB2, and members of the FGF family. Before the luteinizing hormone (LH) surge, recombinant GDF9 and BMP15 suppress follicle stimulating hormone (FSH)-stimulated expression of *Lhcgr* mRNA, encoding LH/choriogonadotropin receptors in granulosa cells (Elvin et al., 1999; Otsuka et al., 2001), and stimulate cumulus expansion after the LH surge (Elvin et al., 1999; Yoshino et al., 2006). BMP15 and GDF9/TGFB2 signals in cumulus cells are mediated by phosphorylation of SMAD1/5/8 and SMAD2/3, respectively (Shimasaki et al., 2004). *Bmp15*-null mice (*Bmp15*$^{-/-}$) are subfertile because of defective cumulus cell development, and reduced ovulation and fertilization rates, an effect augmented by deficiency of GDF9 (Su et al., 2004; Yan et al., 2001). Although *Gdf9* heterozygous mutant (*Gdf9*$^{+/-}$) mice do not exhibit any obvious defects in follicular and cumulus cell development, *Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$ (double mutant, DM) mice exhibit more severe ovarian defects than those observed in *Bmp15*$^{-/-}$ mice (Su et al., 2004; Yan et al., 2001).

The other family of growth factors secreted by mouse oocytes is the FGF family, including FGF8. During embryonic development, *Fgf8* is expressed widely in a temporally and spatially regulated manner (Crossley and Martin, 1995; Heikinheimo et al., 1994; Ohuchi et al., 1994); however, detection of *Fgf8* mRNA expression in adult tissues by northern blot analysis is restricted to ovary and testis (MacArthur et al., 1995b). In adult mouse ovaries, *Fgf8* is expressed specifically in oocytes (Valve et al., 1997). Expression of *FGF8* mRNA was also reported in bovine oocytes (Buratini, Jr et al., 2005). Furthermore, expression of FGF receptors was reported in granulosa cells of human, mouse, rat and bovine (Asakai et al., 1994; Ben-Haroush et al., 2005; Berisha et al., 2004; Puscheck et al., 1997). Although these reports suggest oocyte-derived FGF8 may be important for granulosa cell development or function, no evidence for this has been presented previously.

The objective of the present study was to identify the oocyte factor(s) that regulate glycolysis and expression of transcripts encoding glycolytic enzymes, specifically *Pfkp* and *Ldha*, in mouse cumulus cells. Because ovaries of *Bmp15*$^{-/-}$ and DM mice exhibit abnormal follicular development, the role of BMP15 and GDF9 on glycolysis in cumulus cells was examined using knockout mouse models. We then examined the effects of recombinant proteins on the expression of genes encoding glycolytic enzymes and glycolysis in cumulus cells.

MATERIALS AND METHODS

Animals

Bmp15$^{-/-}$, *Gdf9*$^{+/-}$ and DM female mice on a B6/129S background were produced and genotyped at Baylor College of Medicine (Houston, TX) and maintained at the Jackson Laboratory importation facility (Bar Harbor, ME). Wild-type B6129SF1/J and B6SJLF1 mice were bred and raised in the research colony of the authors at the Jackson Laboratory. For experiments assessing steady-state mRNA expression in mutant and WT cumulus cells, and comparing the ability of mutant and WT oocytes to stimulate mRNA expression in cumulus cells, 6-month-old mutant and WT mice on a B6/129 background were used for collection of both cumulus cells and oocytes. For all other experiments, 22-day-old B6SJLF1 mice were used.

In situ hybridization

Preparation of *Ldha* RNA probe was reported previously (Sugiura et al., 2005). For the preparation of *Pfkp* and *Fgf8* RNA probes, cDNA prepared from mRNA isolated from either cumulus cells or fully grown oocytes of B6SJLF1 mice was amplified using polymerase chain reaction (PCR). The PCR primers used were 5′-AACCGAGCCCGAAAAAAG-3′ and 5′-GTAAAGCACAAGACAGTATCC-3′ for *Pfkp* (GenBank accession number NM_019703), and 5′-TGCTGTTGCACTTGCTGGTT-3′ and 5′-AGTTGTTCTCCAGCACGATCTC-3′ for *Fgf8* (GenBank accession number AK131980). Although the PCR primers for *Fgf8* amplified multiple alternative spliced isoforms of *Fgf8* mRNA (MacArthur et al., 1995a), the PCR products cloned and used for template of probe production were *Fgf8b* isoform [381 base pair (bp), GenBank accession number AK131980]. The sequence of *Fgf8* probe contained the common sequence of all alternative spliced *Fgf8* isoforms, therefore, the *Fgf8* probe used in the present study recognizes all known alternative spliced isoforms of *Fgf8* mRNA. In situ hybridization was performed as reported previously (Eppig et al., 2002), using ovaries of 22-day-old primed female mice, approximately 44-48 hours after equine chorionic gonadotropin (eCG) injection.

Isolation and culture of COCs, oocytes, mural granulosa cells and oocytectomized (OOX) cumulus cells

COCs, fully grown oocytes, mural granulosa cells and cumulus cells were isolated and cultured as reported previously (Sugiura et al., 2005). Fully grown oocytes were maintained at the germinal vesicle (GV)-stage throughout experiments by addition of the phosphodiesterase inhibitor, milrinone (10 μM) (Sigma). Unless otherwise indicated in the text, cells were cultured 20 hours before collection for isolation of mRNA or measurement of glycolytic activity. OOX cumulus cells were produced by microsurgically removing oocytes, but not the zona pellucida, from the COCs collected from 22-day-old eCG-primed B6SJLF1 mice as described previously (Buccione et al., 1990).

In some experiments, OOX cumulus cells were treated with recombinant human TGFB2 (Leinco Technologies, St Louis, MO), human GDF9 (Liao et al., 2004), human BMP15 (Otsuka et al., 2000), mouse FGF8B (Sigma), dimethyl sulfoxide (DMSO) or SU5402 (Calbiochem, La Jolla, CA) at several concentrations. In some experiments, a fine-glass tube (diameter, 75-100 μm) was placed as a barrier bisecting the drop of culture medium and preventing contact between denuded oocytes from OOX cumulus cells while still allowing the passage of potential paracrine factors. For culturing mural granulosa cells, the culture medium was supplemented with 100 ng/ml FSH (Organon, Roseland, NJ) to induce expression of *Lhcgr* (Eppig et al., 1997).

Assessing cumulus expansion

To induce cumulus expansion in OOX cumulus cells, the culture medium was supplemented with 5% fetal bovine serum and 10 ng/ml of epidermal growth factor (EGF; BD Biosciences, San Jose, CA) with various

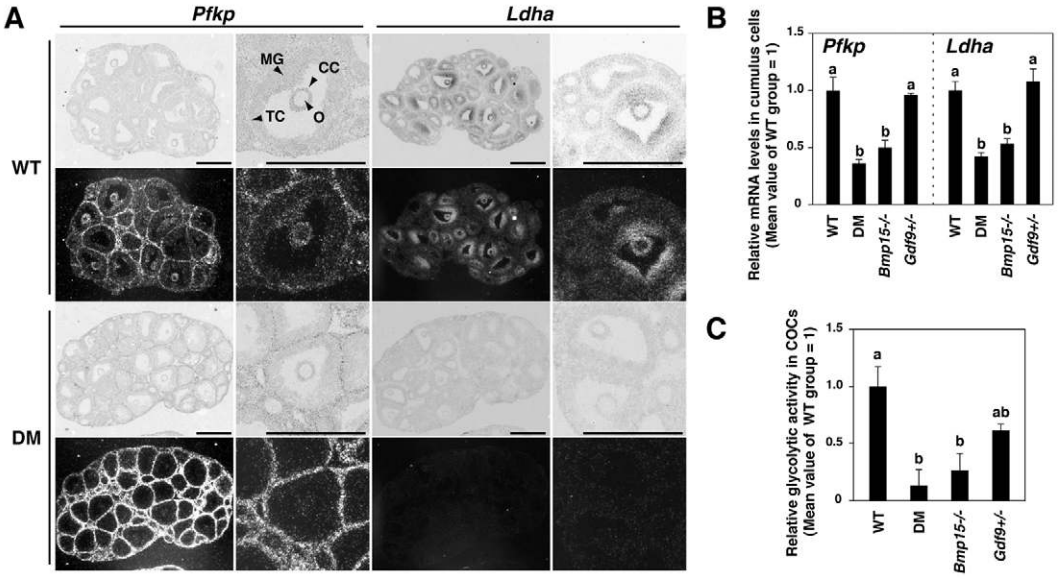

Fig. 1. Expression *of Pfkp* and *Ldha* mRNA and glycolysis in *Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$ (double mutant, DM) and *Bmp15*$^{-/-}$ cumulus cells. (**A**) Localization of *Pfkp* and *Ldha* mRNA was detected by in situ hybridization using 22-day-old eCG-primed, either WT or DM, mice. CC, cumulus cells; MG, mural granulosa cells; O, oocytes; TC, theca cells. (Top) Brightfield images. (Bottom) Darkfield images. Scale bar: 500 μm. (**B**) Relative mRNA levels of *Pfkp* and *Ldha* in cumulus cells of WT, DM, *Bmp15*$^{-/-}$ or *Gdf9*$^{+/-}$ mice were examined using real-time PCR. (**C**) Relative glycolytic activity in COCs of WT, DM, *Bmp15*$^{-/-}$ or *Gdf9*$^{+/-}$ mice was measured as their ability to metabolize [5-^{3}H]-glucose to $^{3}H_2O$. Mean±s.e.m. The values indicated by different letters (a and b) are significantly different (P<0.05).

concentrations of the recombinant proteins or with oocytes (2 oocytes/μl). Cumulus expansion indexes were assessed as reported previously (Eppig et al., 1993).

Real-time PCR

Expression levels of a housekeeping gene, ribosomal protein L19 (*Rpl19*), were used as internal control. Total RNA isolated using the RNeasy Micro Kit (Qiagen, Valencia, CA) was reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen). The real-time PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) with ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The PCR primers used were 5′-ATTTAC-CAAGTCTTGCAGGAGCAT-3′ and 5′-CTAGTTGCTGTGATGTCAA-ATTCCA-3′ for *Bmp6*, 5′-TGTGGCAGACTTGGCTGAGA-3′ and 5′-CTGAGGAAGACATCCTCATTGATTC-3′ for *Ldha*, 5′-GGATA-GAAGCTAATGCCTTTGACAAC-3′ and 5′-TAAAAGCACCGGGTTC-AATGTATAG-3′ for *Lhcgr*, 5′-GCCGTGAAACTCCGAGGAA-3′ and 5′-GTTGCTCTTGACAATCTTCTCATCAG-3′ for *Pfkp*, 5′-CTGAAG-GTCAAAGGGAATGTGTTC-3′ and 5′-TGGTCAGCCAGGAGCTT-CTTG-3′ for *Rpl19*, and 5′-GCTTGCAAAACCCCAAAGC-3′ and 5′-GCTGGGTGGGAGATGTTAAGTCT-3′ for *Tgfb2*. The PCR primers used to amplify *Bmp15*, *Gdf9* and *Fgf8* are reported previously (Su et al., 2007). The results were presented as the relative expression levels to the transcript amount of a standard sample, as indicated in the text, after normalization to the expression levels of *Rpl19* by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). To avoid false-positive signals, dissociation-curve analyses were performed at the end of analyses. Moreover, the PCR products were applied to agarose gel electrophoresis to confirm the sizes, and were sequenced to verify sequence identity as preliminary experiments. The reactions were performed at least in duplicate.

Measurement of glycolytic activity

Glycolytic activity was estimated as the ability of cells to metabolize [5-^{3}H]-glucose to ^3H_2O as reported previously (Sugiura et al., 2005). Either three COCs or three OOX cumulus cell complexes were used to measure glycolytic activities as indicated in the text.

Immunoblot

Immunoblotting was performed as reported previously (Diaz et al., 2006). Briefly, samples were prepared from 40 OOX complexes cultured alone or with WT or *Bmp15*$^{-/-}$ oocytes (2 oocytes/μl) for 15 hours. Samples were simultaneously denatured by boiling in 1× loading buffer for 5 minutes followed by quenching on ice for 5 minutes. Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to polyvinylidene fluoride membrane. Membranes were blocked in 1× blocking buffer (Odyssey blocking buffer; Licor Bioscience, Lincoln, NE) for 1 hour with shaking at room temperature, followed by incubation with specific anti-phospho-SMAD2 antibody (1:1000; Invitrogen, Carlsbad, CA), anti-phospho-SMAD1/5/8 antibody (1:1000; Cell Signaling Technology, Danvers, MA) or β-actin (ACTB) antibody (1:6000; Sigma) diluted in blocking buffer with 0.1% Tween-20 for 12 hours at room temperature. Following incubation, blots were washed three times for 10 minutes each with wash buffer [phosphate-buffered saline (PBS), 0.1% Tween-20]. Fluorescently labeled secondary antibodies (IRDye 800 anti-mouse or anti-rabbit; Rockland Immunochemicals, Gilbertsville, PA) were

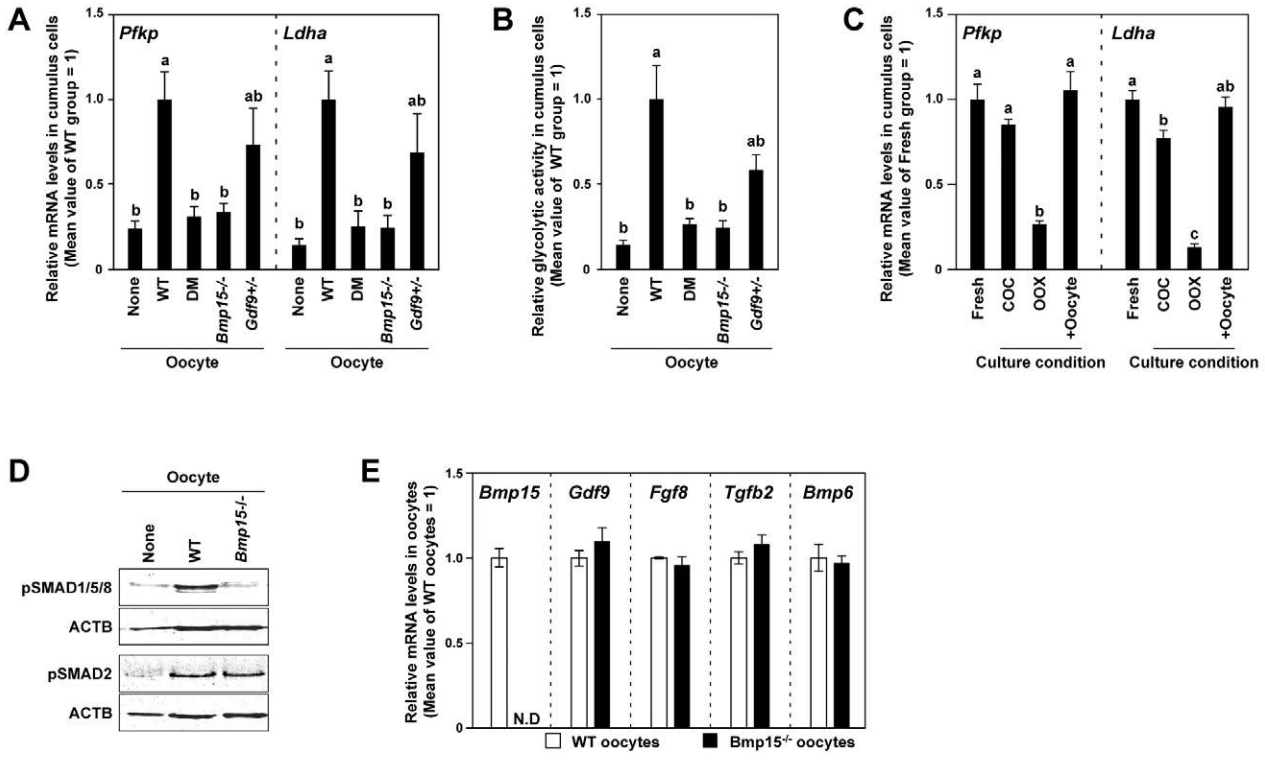

Fig. 2. Effect of oocytes in promoting expression of *Pfkp* and *Ldha* mRNA and glycolytic activity in WT OOX cumulus cells. OOX cumulus cells of WT mice were co-cultured with oocytes of WT, DM, *Bmp15*$^{-/-}$ or *Gdf9*$^{+/-}$ mice for 20 hours; (**A**) relative mRNA levels of *Pfkp* and *Ldha*, and (**B**) glycolytic activity in the OOX cumulus cells of WT mice were examined. (**C**) Expression levels of Pfkp and Ldha mRNA in freshly isolated cumulus cells (Fresh), COCs and OOX cumulus cells cultured without (OOX) or with (+Oocyte) oocytes for 20 hours were assessed by real-time PCR analysis. Mean±s.e.m. The values indicated by different letters (a, b and c) are significantly different (P<0.05). (**D**) OOX cumulus cells of WT mice were co-cultured with oocytes of WT or *Bmp15*$^{-/-}$ mice, and phosphorylation status of SMAD1/5/8 and SMAD2 in the OOX cumulus cells of WT mice was examined. β-actin (ACTB) was used as a loading control. (**E**) Expression levels of *Bmp15*, *Gdf9*, *Fgf8*, *Tgfb2* and *Bmp6* mRNA were compared between WT and *Bmp15*$^{-/-}$ oocytes. N.D., not detected. Mean±s.e.m.

diluted at 1:5000 and incubated with the blots for 1 hour at room temperature. Blots were washed as above with an additional final wash in PBS without Tween-20. Detection was accomplished with an infrared scanner (Licor Bioscience).

Reverse transcriptase (RT)-PCR of *Fgf8* mRNA

Messenger RNA was isolated from GV-stage oocytes using Micro-FastTrack 2.0 Kit (Invitrogen), and was reverse transcribed into cDNA using SuperScript II and oligo-dT primer (Invitrogen). The PCR reaction was performed with 30 to 36 cycles as indicated. Each cycle consisted of 1 minute of denaturation at 94°C, 1 minute of annealing at 60°C and 3 minutes of extension at 68°C, followed by a final extension cycle of 10 minutes at 68°C. PCR primers used were 5′-TTGCACTTGCTGGTTCTCTGC-3′ and 5′-TAGAGCTGGTAGGTCCGGATGA-3′. The PCR products were separated on a 3% agarose gel, and visualized with ethidium bromide staining. Some of the bands were gel purified (Qiagen), and directly sequenced using above primers from both directions. Also, PCR products were cloned into pCRII–TOPO plasmid using TOPO TA Cloning Kit (Invitrogen), and individual clones were isolated and digested using *Eco*RI restriction enzyme. After agarose gel electrophoresis, clones that exhibited different insert sizes were subjected to sequencing from both directions. A total of 96 clones were examined.

Statistical analysis

All experiments were repeated at least three times. Statistical analyses were performed using computer software JMP (SAS Institute, Cary, NC). Tukey-Kramer honestly significant difference tests were used to compare multiple groups. For paired comparison, a standard *t*-test was used. A P value <0.05 was considered statistically significant.

RESULTS

Bmp15$^{-/-}$ and DM oocytes are deficient in promoting expression of *Pfkp* and *Ldha* and glycolysis in cumulus cells

To test whether BMP15 or GDF9 is required for the elevated expression of transcripts encoding glycolytic enzymes in cumulus cells, in situ hybridization was performed to evaluate relative steady-state levels of *Pfkp* and *Ldha* mRNA in ovaries of either WT or DM mice. In WT ovaries, neither *Pfkp* nor *Ldha* mRNA were detected in oocytes, but the highest levels of expression were found in the cumulus cells and the mural granulosa cells that were located near the antrum (Fig. 1A). Relatively high expression of *Pfkp* was also noted in theca cells in WT ovaries. These expression patterns in WT ovaries agree well with previous results (Sugiura et al., 2005). However, in DM ovaries, a high level of *Pfkp* mRNA was found in theca cells; only weak expression of either *Pfkp* or *Ldha* mRNA was detected in cumulus cells or mural granulosa cells (Fig. 1A). To further assess expression levels of these transcripts in cumulus cells, real-time PCR analyses were performed using cumulus cells of WT, *Bmp15*$^{-/-}$, DM and *Gdf9*$^{+/-}$ mice (Fig. 1B). *Gdf9*$^{-/-}$ mice were not used because follicular development in ovaries of the *Gdf9*$^{-/-}$ mouse is blocked at the primary follicle stage when cumulus cells have not yet developed (Dong et al., 1996). As shown in Fig. 1B, expression levels of both *Pfkp* and *Ldha* mRNA in freshly isolated cumulus cells of either *Bmp15*$^{-/-}$ or DM mice were significantly less than that in cumulus cells of WT mice. Expression levels of *Pfkp* and *Ldha*

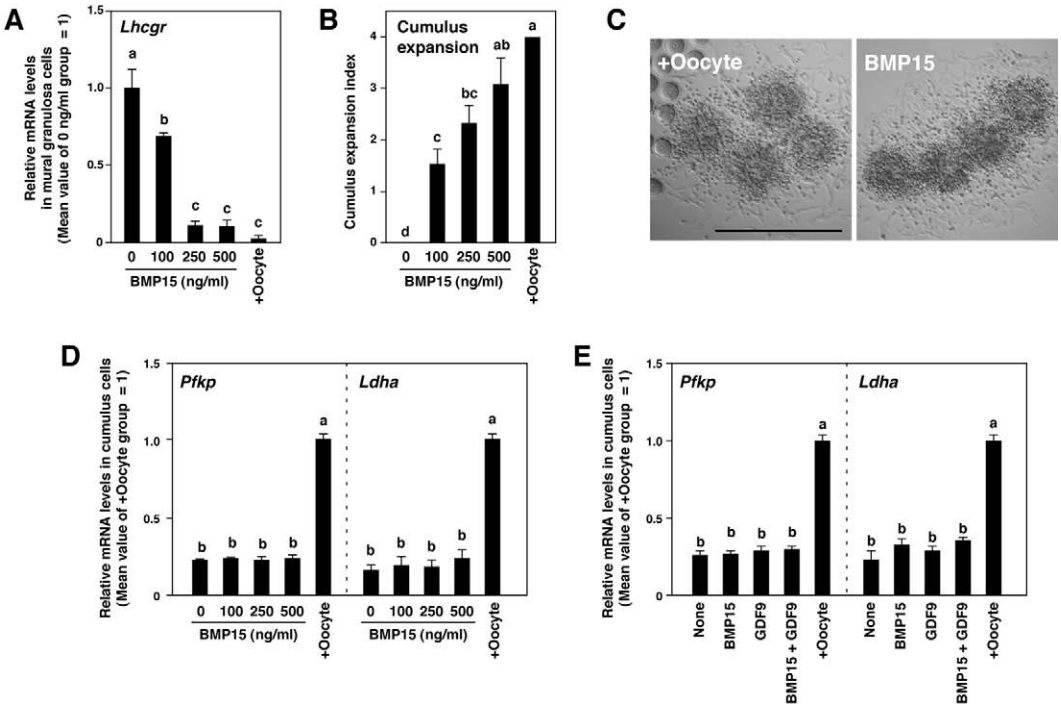

Fig. 3. Inability of recombinant BMP15 alone, or in combination with GDF9, to stimulate expression of *Pfkp* or *Ldha* mRNA in cumulus cells. Recombinant BMP15 suppressed FSH-induced *Lhcgr* mRNA expression in mural granulosa cells and promoted cumulus expansion, but did not promote expression of *Pfkp* and *Ldha* mRNA in OOX cumulus cells. (**A**) Mural granulosa cells were cultured with recombinant BMP15 or oocytes (2 oocytes/μl) in the presence of FSH, and *Lhcgr* mRNA expression in the mural granulosa cells was examined. (**B**) OOX cumulus cells were treated with recombinant BMP15 or oocytes (2 oocytes/μl) in the presence of EGF, and degree of cumulus expansion was examined. (**C**) Representative photographs of expanded OOX cumulus cells treated with oocytes (left) or recombinant BMP15 (right). Scale bar: 500 μm. (**D**) OOX cumulus cells were treated with recombinant BMP15 or oocytes (2 oocytes/μl) and expression levels of *Pfkp* and *Ldha* mRNA in OOX cumulus cells were examined. (**E**) OOX cumulus cells were treated with recombinant BMP15 (500 ng/ml), GDF9 (500 ng/ml) or both, or co-cultured with oocytes (2 oocytes/μl) and expression levels of *Pfkp* and *Ldha* mRNA in OOX cumulus cells were examined. Mean±s.e.m. The values indicated by different letters (a, b, c and d) are significantly different (P<0.05).

mRNA in cumulus cells of *Gdf9*$^{+/-}$ mice were comparable to levels in cumulus cells of the WT mice. Furthermore, glycolytic activities in freshly isolated COCs of *Bmp15*$^{-/-}$ and DM mice were significantly lower than that in COCs of WT mice (Fig. 1C).

Although the above results suggest that BMP15 is essential for the higher expression of *Pfkp* and *Ldha* mRNA and high glycolytic activity in cumulus cells, these could be the consequence of the chronic absence of oocyte-secreted BMP15 throughout follicular development in the mutant mice. Therefore, to address more direct effects of the absence of oocyte-secreted BMP15 on glycolysis in cumulus cells, OOX cumulus cells of WT mice were co-cultured with oocytes of either WT or mutant mice, and *Pfkp* and *Ldha* mRNA levels, as well as glycolytic activity, were examined. As shown in Fig. 2A,B, OOX cumulus cells cultured without oocytes exhibited lower levels of *Pfkp* and *Ldha* mRNA and reduced glycolytic activity when compared with OOX cumulus cells co-cultured with WT oocytes, as previously reported (Sugiura et al., 2005). However, co-culturing WT OOX cumulus cells with DM or *Bmp15*$^{-/-}$ oocytes failed to maintain elevated *Pfkp* and *Ldha* mRNA expression and glycolytic activity, and levels of these transcripts were comparable to those of OOX cumulus cells cultured without WT oocytes. Therefore, oocytes of *Bmp15*$^{-/-}$ and DM mice were not able to sustain normal levels of *Pfkp* and *Ldha* mRNA and glycolysis in WT cumulus cells.

The culture conditions used in these experiments could have promoted elevated *Pfkp* and *Ldha* mRNA levels in cumulus cells. To test this possibility, levels of *Pfkp* and *Ldha* mRNA in cumulus cells of cultured intact COCs, OOX cumulus cells and OOX cumulus cells co-cultured with WT oocytes were compared with levels in freshly isolated cumulus cells. Culture did not increase the levels of expression of *Pfkp* or *Ldha* mRNA in intact COCs above those of freshly isolated cumulus cells. Sustaining levels of expression of these transcripts in cultured cumulus cells that were equivalent to freshly isolated cumulus cells was dependent upon the presence of WT oocytes (Fig. 2C).

Signals from the oocyte-secreted TGFβ superfamily members in cumulus cells are mediated by phosphorylation of SMAD families (Shimasaki et al., 2004). BMP15 and GDF9/TGFB2 stimulate phosphorylation of SMAD1/5/8 and SMAD2/3, respectively (Moore et al., 2003; Vitt et al., 2002). Therefore, to assess the possibility that production of not only BMP15, but also other factors, such as GDF9 and TGFB2, are reduced in *Bmp15*$^{-/-}$ oocytes, the phosphorylation status of SMADs in co-cultured WT OOX cumulus cells was examined (Fig. 2D). When OOX cumulus cells were co-cultured with WT oocytes, both SMAD1/5/8 and SMAD2 were phosphorylated, indicating that both SMAD pathways in cumulus cells are activated by oocyte-derived members of the TGFβ superfamily in vitro. However, *Bmp15*$^{-/-}$ oocytes did not stimulate SMAD1/5/8 phosphorylation in WT OOX cumulus cells, consistent with the absence of BMP15 in *Bmp15*$^{-/-}$ oocytes. However, *Bmp15*$^{-/-}$ oocytes did promote SMAD2 phosphorylation in WT OOX cumulus cells, suggesting that other relevant members of the TGFβ superfamily are produced by *Bmp15*$^{-/-}$ oocytes (Fig. 2D). In fact, expression levels of *Gdf9*, *Fgf8*, *Tgfb2* and *Bmp6* mRNA in *Bmp15*$^{-/-}$ oocytes were comparable to those in WT oocytes (Fig. 2E). Therefore, the failure of *Bmp15*$^{-/-}$ oocytes to stimulate *Pfkp* and *Ldha* expression in OOX cumulus cells is due directly or indirectly to the absence of BMP15.

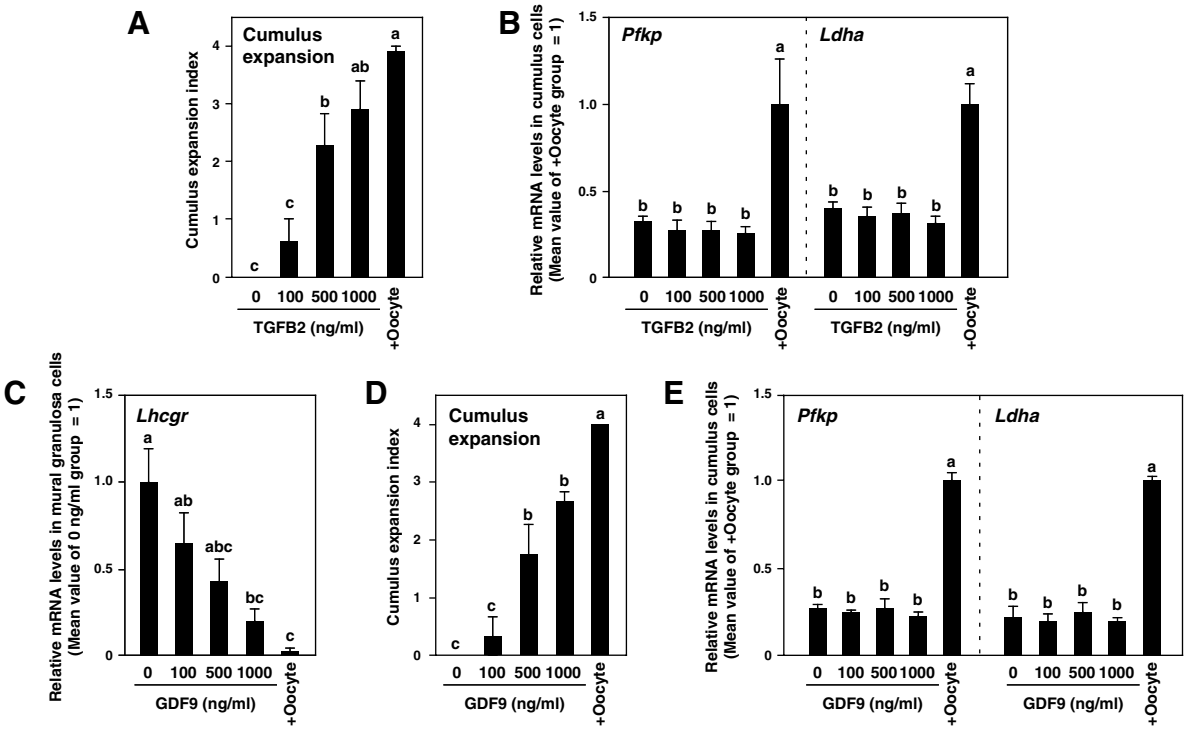

Fig. 4. Inability of recombinant TGFB2 and GDF9 to affect *Pfkp* and *Ldha* mRNA levels in cumulus cells. Although recombinant TGFB2 and GDF9 promoted cumulus expansion and recombinant GDF9 suppressed *Lhcgr* mRNA expression in mural granulosa cells, they did not promote expression of *Pfkp* and *Ldha* mRNA in OOX cumulus cells. OOX cumulus cells were treated with recombinant TGFB2 (**A**,**B**), GDF9 (**D**,**E**) or oocytes (2 oocytes/μl) and (A,D) degree of cumulus expansion and (B,E) expression levels of *Pfkp* and *Ldha* mRNA in OOX cumulus cells were examined. (**C**) Mural granulosa cells were cultured with recombinant GDF9 or oocytes (2 oocytes/μl), and *Lhcgr* mRNA expression in the mural granulosa cells was examined. Mean±s.e.m. The values indicated by different letters (a, b and c) are significantly different (P<0.05).

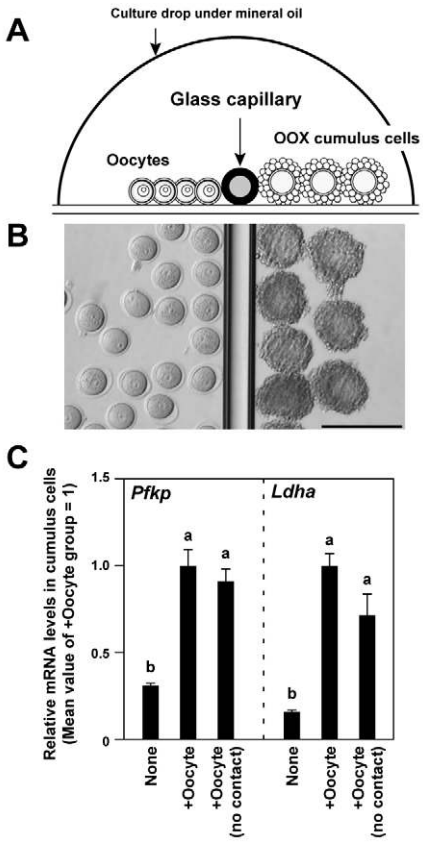

Fig. 5. Effect of blocking contact between oocytes and cumulus cells on the elevated *Pfkp* and *Ldha* mRNA expression in OOX cumulus cells cultured with oocytes in vitro. (**A**) Contact between OOX cumulus cells and oocytes was prevented with a glass capillary. (**B**) A representative photograph of the culture condition. Scale bar: 200 μm. (**C**) Relative mRNA levels of *Pfkp* and *Ldha* in cumulus cells cultured alone (None), with oocytes (+Oocyte), or with oocytes but with contact prevented with the glass capillary [+Oocyte (no contact)]. The glass capillary was present in all cultures, even when there were no oocytes (None) or contact was allowed (+Oocyte). Mean±s.e.m. The values indicated by different letters (a, b) are significantly different ($P<0.05$).

Recombinant BMP15 alone is not sufficient to promote expression of transcripts encoding glycolytic enzymes in cumulus cells

To further evaluate whether BMP15 is an oocyte-derived factor that induces cumulus cell glycolysis, OOX cumulus cells were cultured with recombinant BMP15, or with oocytes (2 oocytes/μl), and levels of *Pfkp* and *Ldha* mRNA in the OOX cumulus cells were assessed (Fig. 3). Surprisingly, culturing OOX cumulus cells with recombinant BMP15 did not promote expression of these transcripts or glycolysis in OOX cumulus cells (Fig. 3D, Fig. 8B). The inability of BMP15 to stimulate *Pfkp* and *Ldha* mRNA expression in OOX cumulus cells was not because of poor activity of BMP15, because this preparation of recombinant BMP15 suppressed FSH-induced *Lhcgr* mRNA expression in mural granulosa cells, and promoted cumulus expansion in a dose-dependent manner (Fig. 3A-C) as described previously (Otsuka et al., 2001; Yoshino et al., 2006). The effect of either recombinant TGFB2 or GDF9 was also assessed (Fig. 4). Although both recombinant TGFB2 and GDF9 stimulated cumulus expansion (Fig. 4A,D) and the recombinant GDF9 suppressed *Lhcgr* expression in mural granulosa cells (Fig. 4D), the recombinant proteins did not promote the expression of *Pfkp* and *Ldha* mRNA in OOX cumulus cells (Fig. 4B,E). Taken together, these results strongly suggest that some other factor(s), in addition to BMP15, is also required for promoting elevated expression of transcripts encoding glycolytic enzymes and glycolytic activity in cumulus cells.

To assess whether contact between cumulus cells and oocytes may participate in oocyte-mediated elevation of the levels of *Pfkp* and *Ldha* mRNA in cumulus cells, contact between OOX cumulus cells and oocytes was prevented by placing a fine-glass capillary between them during culture (Fig. 5A,B). As shown in Fig. 5C, high *Pfkp* and *Ldha* mRNA levels were maintained even when contact between cumulus cells and oocytes was prevented. Therefore, contact between cumulus cells and oocytes is not required for oocytes to promote expression of these transcripts in cumulus cells in vitro; however, it is possible that contact, in addition to paracrine factors, may function in some way to promote expression in vivo.

Because synergistic actions of BMP15 and GDF9 have been reported (McNatty et al., 2005a; McNatty et al., 2005b; Su et al., 2004; Yan et al., 2001), it was possible that the factor required for stimulation of cumulus cell glycolysis in addition to BMP15 might be GDF9. Therefore, the effects of co-treatment of BMP15 and GDF9 on glycolysis in cumulus cells were examined (Fig. 3E). Treatment of OOX cumulus cells with BMP15, GDF9, or both had no effect on the expression levels of *Pfkp* and *Ldha* mRNA in OOX cumulus cells (Fig. 3E).

FGF activity is required for elevated expression of *Pfkp* and *Ldha* in OOX cumulus cells

As shown above, in addition to BMP15, some other factor(s), other than GDF9, secreted by oocytes, is also required for stimulating cumulus cell glycolysis. One of the other growth factors secreted by oocytes is FGF8 (Valve et al., 1997). Using in situ hybridization, we confirmed specific expression of *Fgf8* mRNA in oocytes of secondary to large antral follicles (Fig. 6A). Because cooperation between BMP and FGF signals has been observed during embryonic development (Hayashi et al., 2003; Hayashi et al., 2001; Nakamura et al., 2005; Reinhold et al., 2004; Warren et al., 2003), it was possible that FGF8 secreted by oocytes might cooperate with BMP15 to promote glycolysis in cumulus cells.

There are eight possible alternative spliced isoforms of *Fgf8* (Fig. 6B) (MacArthur et al., 1995a). To assess which isoforms of *Fgf8* mRNA are expressed in mouse oocytes, PCR analysis was performed to amplify oocyte cDNA using *Fgf8*-specific primers indicated in Fig. 6B. After agarose electrophoresis, eight bands, whose sizes are well-correlated with the expected PCR product sizes of each *Fgf8* isoforms, were detected (Fig. 6C). Identities of bands 8a, 8b and 8e were confirmed by sequencing; however, expression of the other *Fgf8* isoforms in oocytes was not confirmed (see Materials and methods).

Because *Fgf8b* appeared to be the most abundant isoform expressed in mouse oocytes, effects of recombinant FGF8B on the expression levels of *Pfkp* and *Ldha* mRNA in OOX cumulus cells were tested. As shown in Fig. 7A, when OOX cumulus cells were treated with recombinant FGF8B alone, there were no significant effects on the expression levels of *Pfkp* and *Ldha* mRNA. However, when OOX cumulus cells were co-cultured with a low density of oocytes (0.1 oocytes/μl, 20-fold lower than the previous oocyte co-culture experiments, i.e. Figs 2-5), FGF8B stimulated cumulus cell expression of *Pfkp* and *Ldha* transcripts in a dose-dependent manner (Fig. 7A). This FGF8B effect is not because of the additive effect of recombinant FGF8B and

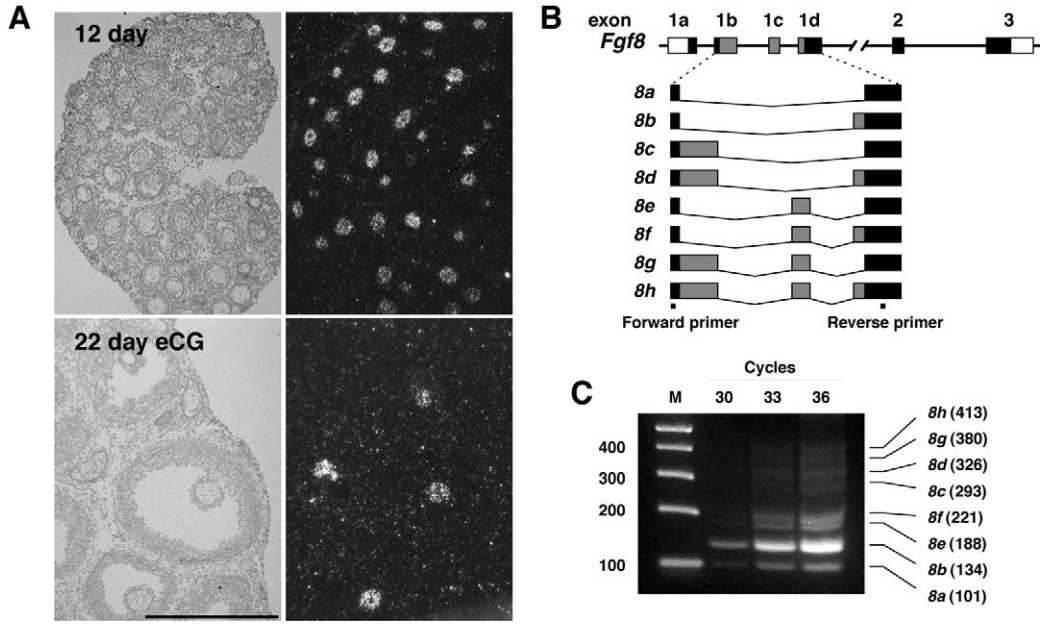

Fig. 6. Expression of *Fgf8b* mRNA in oocytes. (**A**) *Fgf8* mRNA was detected in oocytes in ovaries of both 12-day-old (12 day) and 22-day-old eCG-primed (22 day eCG) mice by in situ hybridization. (Left) Brightfield images. (Right) Darkfield images. Scale bar: 500 μm. (**B**) Schematic diagram of eight different *Fgf8* isoforms, *Fgf8a-h* [modified from MacArthur et al. (MacArthur et al., 1995a)]. Positions of PCR primers used are indicated in the expanded region. (**C**) Oocyte cDNA were amplified with PCR and the products were separated by agarose electrophoresis. A representative gel photograph after ethidium bromide staining is shown. Approximate sizes are shown on the *y*-axis (bp). The numbers in parentheses indicate expected size of PCR products (bp).

endogenous oocyte-secreted FGF8B, because higher concentrations of FGF8B (up to 1 μg/ml) did not promote expression of these genes to levels comparable to those in OOX cumulus cells co-cultured with oocytes (2 oocytes/μl) (data not shown). Therefore, these results strongly suggest that FGF8B is capable of inducing *Pfkp* and *Ldha* mRNA expression in cumulus cells, but some other oocyte-secreted factor(s) is also required.

To further test whether FGF signaling is necessary for the upregulated *Pfkp* and *Ldha* mRNA in cumulus cells, an inhibitor of FGF receptor-dependent protein kinase, SU5402 (Mohammadi et al., 1997), was used. Concentrations of this inhibitor below 60 μM have no cross-reactivity between platelet-derived growth factor, EGF or insulin signaling (Mohammadi et al., 1997). When OOX cumulus cells co-cultured with oocytes (0.5 oocytes/μl) were treated with SU5402 for 24 hours, *Pfkp* and *Ldha* mRNA in the OOX cumulus cells was decreased in a dose-dependent manner compared with OOX cumulus cells treated with only the solvent, DMSO (Fig. 7B). The effect of SU5402 is reversible, because when OOX cumulus cells treated with SU5402 (25 μM) for 24 hours were washed and cultured for an additional 20 hours with oocytes, elevated levels of *Pfkp* and *Ldha* mRNA were restored (Fig. 7C). These results provide supporting evidence that FGF signals are necessary for increased expression of transcripts encoding glycolytic enzymes in cumulus cells.

Co-treatment with recombinant FGF8B and BMP15 promotes *Pfkp* and *Ldha* mRNA expression and glycolysis in OOX cumulus cells

Effects of co-treatment with recombinant BMP15 and FGF8B on glycolysis by OOX cumulus cells were examined (Fig. 8). Although recombinant FGF8B alone had no effect on levels of *Pfkp* and *Ldha* mRNA by OOX cumulus cells, co-treatment with BMP15 and FGF8B promoted expression of these transcripts by OOX cumulus cells to levels comparable to those promoted by oocytes (2 oocytes/μl) (Fig. 8A). Co-treatment with FGF8B and GDF9 seemed to have slight effects on the expression of *Ldha* mRNA, however, the difference was not significant and the mRNA level was not comparable to that stimulated with BMP15 and FGF8B together. Furthermore, the glycolytic activity of OOX cumulus cells treated with both BMP15 and FGF8B was significantly greater than that of cells treated with either FGF8B alone or co-treated with FGF8B and GDF9, or cultured without oocytes (Fig. 8B).

DISCUSSION

Four lines of evidence presented here indicate that oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in companion cumulus cells before the LH surge. First, cumulus cells of *Bmp15*$^{-/-}$ and DM mice exhibited reduced glycolysis and expression of transcripts encoding the glycolytic enzymes PFKP and LDHA. Second, oocytes isolated from these mutant mice were deficient in promoting glycolysis and expression of *Pfkp* and *Ldha* mRNA in cumulus cells of WT mice. Third, neither recombinant BMP15, GDF9 nor FGF8 alone were able to promote glycolysis in WT cumulus cells. However, co-treatment with BMP15 and FGF8 promoted glycolysis and increased expression of *Pfkp* and *Ldha* mRNA in WT cumulus cells to the same levels as WT oocytes, but the combinations of BMP15/GDF9 or GDF9/FGF8 did not. Fourth, an FGF receptor-dependent protein kinase inhibitor suppressed *Pfkp* and *Ldha* mRNA expression in cumulus cells promoted by oocyte-derived factors.

Although the finding that *Bmp15*$^{-/-}$ oocytes did not promote the expression of *Pfkp* and *Ldha* mRNA levels and glycolysis in cumulus cells is strong evidence for the role of BMP15, it could be argued that defective oocyte-cumulus cell interactions throughout

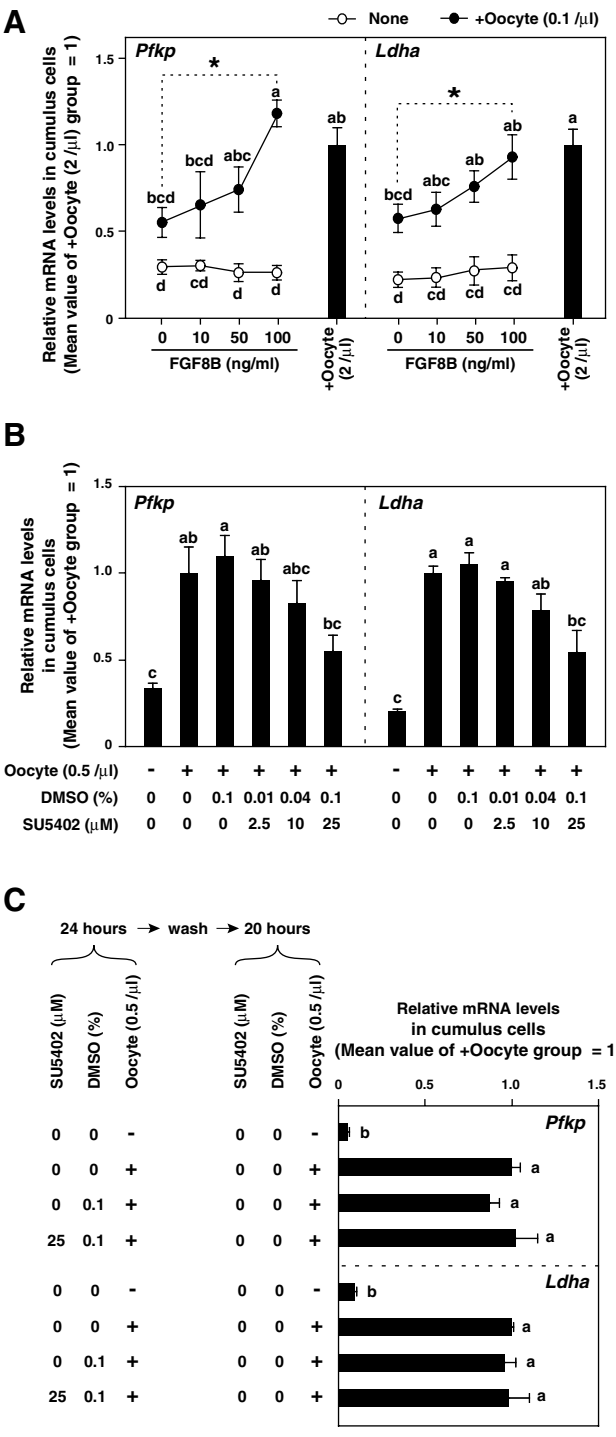

Fig. 7. Requirement for FGF signals to promote the expression of *Pfkp* and *Ldha* mRNA in OOX cumulus cells. (**A**) Dose-response of FGF8. OOX cumulus cells were cultured with (black circles) or without (white circles) oocytes (0.1 oocytes/μl) and treated with recombinant FGF8B, and expression levels of *Pfkp* and *Ldha* mRNA in the OOX cumulus cells were examined. As a control group, *Pfkp* and *Ldha* mRNA expression in OOX cumulus cells co-cultured with oocytes (2 oocytes/μl) is shown (black bars). (**B**) Effect of SU5402. OOX cumulus cells were co-cultured with oocytes (0.5 oocytes/μl) in the presence of either DMSO or SU5402 for 24 hours, and relative mRNA levels of *Pfkp* and *Ldha* in OOX cumulus cells were examined. (**C**) Reversibility of the effect of SU5404. OOX cumulus cells were co-cultured with oocytes (0.5 oocytes/μl) in the presence of either DMSO or SU5402. After 24 hours of culture, the OOX cumulus cells were washed thoroughly and cultured in a fresh medium for an additional 20 hours with freshly isolated oocytes (0.5 oocytes/μl), and relative mRNA levels of *Pfkp* and *Ldha* in the OOX cumulus cells were examined. Mean±s.e.m. The values indicated by different letters (a, b, c and d) are significantly different ($P<0.05$).

follicular development could have impaired development of *Bmp15*$^{-/-}$ oocytes and resulted in deficiencies in production of other factors that could influence cumulus cell glycolysis. However, this does not appear to be the case. In collaboration with Drs H. Pan and R. M. Schultz (University of Pennsylvania), we compared the transcriptomes of WT and DM oocytes by microarray analysis. The overall gene expression patterns were similar between these groups of oocytes, except that *Bmp15* mRNA was not detected and approximately half the amount of *Gdf9* mRNA was detected in DM oocytes as expected (H. Pan, R. M. Schultz, M.M.M. and J.J.E., unpublished). Furthermore, we have confirmed that levels of transcripts encoding other known oocyte-secreted factors, *Bmp6*, *Gdf9*, *Tgfb2* and *Fgf8*, were not significantly changed between *Bmp15*$^{-/-}$ and WT oocytes. Moreover, the finding that *Bmp15*$^{-/-}$ oocytes stimulated SMAD2 phosphorylation in co-cultured WT cumulus cells strongly suggests that *Bmp15*$^{-/-}$ oocytes develop normally to the extent that they can produce ligands that stimulate SMAD2 phosphorylation in co-cultured WT cumulus cells. Therefore, the absence of BMP15 probably accounts for the inability of DM and *Bmp15*$^{-/-}$ oocytes to stimulate glycolysis in cumulus cells.

Recently, Yoshino et al. reported that bioactive BMP15 is not detectable in mouse oocytes before the LH surge, and that active BMP15 is markedly increased just before ovulation (Yoshino et al., 2006). Moreover, Gueripel et al. have reported that although mature BMP15 protein is present in mouse GV oocytes before LH surge, it is not secreted (Gueripel et al., 2006). Consistent with these reports, Gilchrist et al. suggest that mouse oocytes are not capable of activating the SMAD1/5/8 pathway in monolayer cultured mural granulosa cells using the reporter gene assay system (Gilchrist et al., 2006). By contrast, the present results showed that WT GV-stage oocytes promoted SMAD1/5/8 phosphorylation in co-cultured OOX cumulus cells. Moreover, *Pfkp* and *Ldha* mRNA levels and glycolytic activity in *Bmp15*$^{-/-}$ cumulus cells were lower when compared with those in WT mice before LH surge. Therefore, although the levels of secreted BMP15 are low before the LH surge, the BMP15 that is present plays a biologically significant role during follicular development. The discrepancy in SMAD1/5/8 activation might be because of the different cell types used (e.g. OOX cumulus cells versus monolayer cultured mural granulosa cells), the methods used to detect activation of the SMAD1/5/8 pathway (e.g. immunoblot versus reporter gene assay), or the concentration of oocytes used (2 versus 0.24 oocytes/μl).

Some of the defects in *Bmp15*$^{-/-}$ mice could be explained by lower glycolysis in the cumulus cells. In vivo, resumption of meiosis in *Bmp15*$^{-/-}$ oocytes is slower when compared with that in *Bmp15* heterozygous mutant (*Bmp15*$^{+/-}$) oocytes, and less than half of the *Bmp15*$^{-/-}$ oocytes reach meiotic metaphase II stage at ovulation, whereas more than 90% of *Bmp15*$^{+/-}$ oocytes reach meiotic metaphase

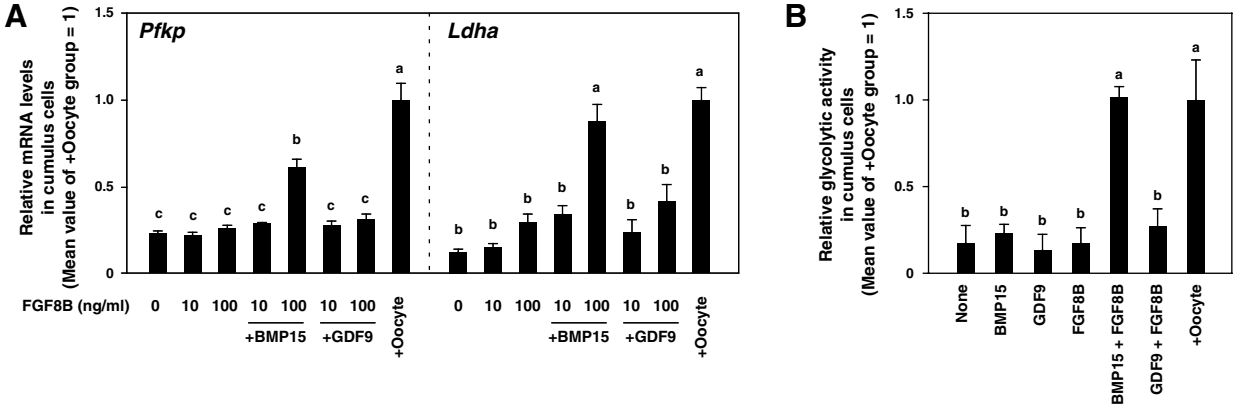

Fig. 8. Co-treatment of recombinant BMP15 and FGF8B promoted expression of *Pfkp* and *Ldha* mRNA and glycolytic activity in OOX cumulus cells. OOX cumulus cells were treated with recombinant FGF8B (0, 10 or 100 ng/ml) with or without either BMP15 (500 ng/ml) or GDF9 (500 ng/ml), or co-cultured with oocytes (2 oocytes/μl), and (**A**) expression levels of *Pfkp* and *Ldha* mRNA and (**B**) glycolytic activity in OOX cumulus cells were examined. Mean±s.e.m. The values indicated by different letters (a, b and c) are significantly different (P<0.05).

II stage (Su et al., 2004). Because oocytes require pyruvate, which is produced by glycolysis in cumulus cells, for their resumption of meiosis (Biggers et al., 1967; Eppig, 1976), it is possible that lower glycolysis in *Bmp15*$^{-/-}$ cumulus cells results in reduced availability of pyruvate in the *Bmp15*$^{-/-}$ follicles, and this accounts for the slower resumption of meiosis in *Bmp15*$^{-/-}$ oocytes. In fact, when *Bmp15*$^{-/-}$ oocytes were cultured in a medium containing pyruvate, they matured at the same rate as WT oocytes (Su et al., 2004). Furthermore, lower developmental ability of the *Bmp15*$^{-/-}$ oocytes (Su et al., 2004) could also be attributed to the lower nutritional support from cumulus cells during their development.

The relative importance of BMP15 during follicular development differs among species. Compared with the subfertile phenotype of *Bmp15*$^{-/-}$ mice, mutations of the *BMP15* gene in the human and sheep exhibit much more severe effects on fertility (Di Pasquale et al., 2004; Galloway et al., 2000; Hanrahan et al., 2004). Whether glycolysis in cumulus cells before the LH surge is regulated by BMP15 and FGFs also in women or ewes remains to be determined. However, LH promotes increased glycolytic activity in bovine COCs but not in OOX cumulus cells (Zuelke and Brackett, 1992), suggesting that factors derived from oocytes regulate glycolysis in cumulus cells after the LH surge in the bovine. Moreover, apoptosis of bovine cumulus cells is prevented by oocyte-derived BMP signals, including BMP15 (Hussein et al., 2005). Because glycolytic enzymes participate in anti-apoptotic pathways (Kim and Dang, 2005), it is possible that BMP15 prevents apoptosis of cumulus cells through promoting expression of genes encoding glycolytic enzymes in the bovine.

FGFs produced by oocytes probably function redundantly in promoting glycolysis in cumulus cells. In bovine oocytes, expression of multiple *FGF* mRNA species, including *FGF8* mRNA, were detected by microarray analysis, and expression of *FGF8*, *FGF10*, *FGF17*, *FGF18* and *FGF22* transcripts was confirmed using RT-PCR (Zhong et al., 2006). Moreover, our microarray analysis also detected transcripts encoding multiple FGF family members, including *Fgf8*, *Fgf17* and *Fgf18*, in mouse oocytes (Su et al., 2007). FGF family members are classified into seven subfamilies, and within subfamilies each member possesses similar receptor specificity (Zhang et al., 2006). Because FGF8, FGF17 and FGF18 are classified in the same FGF8 subfamily, FGF8 is probably not the only oocyte-derived FGF family member that regulates cumulus cell glycolysis. Consistent with this idea, neutralizing antibodies against FGF8 did not efficiently inhibit cumulus cell expression of genes encoding glycolytic enzymes induced by oocytes (data not shown), whereas the FGF receptor-dependent protein kinase inhibitor, SU5402, did.

Recent studies have focused on the role of oocyte-secreted TGFβ superfamily members, especially BMP15 and GDF9, because these paracrine factors are specifically expressed by oocytes and because informative knockout mutations have been produced (Dong et al., 1996; Su et al., 2004; Yan et al., 2001). Interestingly, several FGF mutant mice also exhibit reproductive deficiency. For example, whereas *Fgfr2*-knockout mice die before birth (Xu et al., 1998), mutant mice that carry an activating mutation of *Fgfr2* survive, but are infertile (Chen et al., 2003). By contrast, *Fgfr3*- and *Fgfr4*-knockout mice are fertile (Colvin et al., 1996; Deng et al., 1996; Weinstein et al., 1998), however, mice that carry a point mutation in *Fgfr3* or double-homozygous mutants of *Fgfr3* and *Fgfr4* are infertile (Amsterdam et al., 2001; Wang et al., 1999; Weinstein et al., 1998). Null mutations of *Fgf8* produce embryonic lethality with embryo resorption by E10.5 (Meyers et al., 1998). Despite these observations, no studies have focused on the importance of oocyte-derived FGF signaling on the development of granulosa cells and follicles. Therefore, the results presented here are the first showing a requirement of oocyte-secreted FGFs in the regulation of granulosa cell function.

How do BMP15 and FGF signals cooperate to regulate glycolysis in cumulus cells? During development, there are several organs in which BMP and FGF signals cooperate to regulate cell differentiation. For example, during calvarial suture osteogenesis, FGF2 augments the BMP4 signal by suppressing expression of *Nog* mRNA, encoding the BMP antagonist noggin (Warren et al., 2003). A similar mechanism was observed during chondrogenesis, where FGF18 facilitates the BMP2 signal by suppressing *Nog* mRNA expression (Reinhold et al., 2004). Other examples of cooperation of BMP and FGF signals were reported in nervous system and ectopic bone formation (Hayashi et al., 2003; Hayashi et al., 2001; Nakamura et al., 2005). During FGF-induced differentiation of pheochromocytoma-derived PC12 cells, BMP2 facilitates FGF signaling by promoting expression of *Fgfr1* mRNA, encoding FGF receptor 1 (Hayashi et al., 2003; Hayashi et al., 2001). Moreover, during ectopic bone formation, low-dose

FGF2 augments BMP2-induced ectopic bone formation by stimulating expression of *Bmpr1b*, encoding BMP receptor, type 1B in the bone-forming progenitor cells (Nakamura et al., 2005). These reports suggest the possibility that either BMP15 or FGFs modulate the expression of antagonists and receptors for FGFs or BMP15 in cumulus cells. Involvement of expression of BMP and FGF antagonists and receptors is currently under investigation. Further research on these mechanisms will clarify our knowledge of oocyte-cumulus cell communication as well as oocyte and follicular development.

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