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Original Article

Insulin-like growth factor 1 promotes cumulus cell expansion and nuclear maturation of oocytes via Pi3K/Akt pathway

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Abstract: Oocyte maturation is critical for successful fertilization and pregnancy. The mechanism underlying the control of this complex process remain relatively unknown, and even more so the specific mechanism through which oocytes acquire competentce to restrain apoptosis and resume meiosis. Insulin-like Growth Factor 1 (IGF-1) is a growth factor that had been frequently used in inducing oocyte maturation. However, the studies in downstream signaling are relatively inadequate. In this study, we initially established a linear model which illustrates the correlation between ratio of serum IGF-1 over oocyte number and follicular fluid (FF) IGF-1. Moreover, supplementation of IGF-1 to mouse COCs promotes cumulus expansion, oocyte nuclear, and expression of extracellular matrix (ECMs) involved in cumulus expansion, which was blocked by treatment of LY294002, indicating the implication of PI3K/Akt pathway. In conclusion, this study not only provide relatively comprehensive evidence showing the importance of IGF-1, but also elucidates the downstream signaling behind the enhance of IGF-1 to oocyte maturation.

Keywords: Oocyte maturation, cumulus cell expansion, insulin growth factor-1, PI3K/Akt pathway

Introduction

Inadequate production of mature oocytes or sperms is the most common causes of infertility [1]. Gonadotropins are considered as a critical regulator in oocyte maturation [2, 3]. In addition, steroid and non-steroid substance had been reported to locally regulate ovarian functions, which include Insulin-like Growth Factors (IGFs), Growth Hormone (GH), Epidermal Growth Factor (EGF), and Prolactin (PRL) [4-6]. They are secreted from granulosa and cumulus cells in response to gonadotropins and subsequently function to affect the maturation of oocytes via paracrine and autocrine pathways [7].

IGF-1 is a member of IGFs family, which is composed of polypeptides, receptors and binding proteins. IGF-1 and IGF-2 share a 62% amino acid sequence homology [8, 9]. IGFs act as an effective mitogen, which plays an important role in cell cycles. It stimulates synthesis of glycogen and protein via facilitating intake of amino acids and glucose, thereby mediating and augmenting the effect of gonadotropin on oophoron [10]. Multiple in vitro studies suggest the important role of IGF-1 in local function of oophorons. IGF-1 is required for the development and maturation of follicles during reproduction. At early stages of follicular development, from maturation of primordial follicles through to preantral phase, the regulation of initiation is dependent on presence of IGF-1, especially the parascrine IGF-1 [11]. In mouse, the function of GH is mediated by IGF-1 [12]. Furthermore, clinical studies also demonstrated that increased concentration f IGF-1 could result in excitement of ovary [13]. Exogenous GH treatmentlead to elevated IGF-1 levels, however, and the consequential increase of IGF-1 in turn reduce GH production. The impaired development of follicles in preantral phase due to deficiency of IGF-1 could not be reversed by gonadotropin [14]. Although IGF-1 has been intensively studied, the mechanism underlying

its enhancement on the development and maturation of oocyte remained unthorough, especially the downstream signaling during oocyte maturation.

In this study, we initially established a linear model which illustrates the correlation between ratio of serum IGF-1 over oocyte number and follicular fluid (FF) IGF-1. The FF IGF-1 level if MII phase was found significantly higher than that of MI phase. Moreover, supplementation of IGF-1 to mouse COCs promotes cumulus expansion, oocyte nuclear maturation and embryo development, as well as expression of extracellular matrix (ECMs) involved in cumulus expansion, which was blocked by treatment of LY294002, indicating the implication of PI3K/Akt pathway. In conclusion, this study not only provide relatively comprehensive evidence showing the importance of IGF-1, but also elucidates the downstream signaling behind the enhance of IGF-1 to oocyte maturation.

Materials and methods

Patients

A total of 158 infertile women attending the IVF unit of the Reproductive Center of First Affiliated Hospital of Xiamen University for IVF-ET treatmentin from July, 2012 to August 2014 were recruited for study in 2014. Women that meet any one of the following criterion were excluded: 1) Received hormone treatment or operation in uterine cavity over past three months; 2) Have endometriosis, adenomyosis or endocrinopathy, such as polycystic ovarian syndrome (PCOS), hyperprolactinemia, or hypo-and-hyperthyroidism were excluded; 3) Basal Follicle-Stimulating Hormone (BFSH) >10 mU/ml; 4) Age >40; 5) Exposure to mutagenic toxics or radiations; 6) Severe mental diseases or diseases of urogenital system; 7) Smokers. Finally, 104 infertile women who meet the inclusion criteria were enrolled (**Table 1**). Written informed consents were obtained from all subjects, and this study was censored and approved by Ethics Committees of First affiliated hospital of Xiamen University (KY2014-018).

IVF-ET

The long standard protocol was adopted for treatment. On day 20 of cycle or in the middle of luteal phase, the patients were subcutaneously injected with 0.1 mg/day Triptorelin Acetate (Beaufour IPSEN, France) for 14-18 days. When the endometrium reached 5 mm and the E2 <50 pg/ml, Gonal-F (EMDSerono) was injected subcutaneously, 250-300 U/d. The follicular development was monitored by B-mode ultrasonography, and the levels of serum luteinizing hormone (LH), estradiol (E2) and progesterone (P) were measured. Administration of Gonal-F was ceased when the diameter of dominant follicle reached above 10 mm, replaced by 250 g hCG (Ovidrel, EMD Serono) at 20:00-21:00 to induc follicle maturation, and oocyte retrieval was cnducted after 36-38 h under transvaginal ultrasonographic guidance. On oocyte retrieval, 3 ml fasting venous blood was drawn and follicle fluid was collected from every patient, which was centrifuged at 2000 g for 10 min and the supernatant was subsequently stored at -80°C.

Criteria for oocyte maturation

The event of germinal vesicle breakdown (GVBD) and release of first polar body after removal of corona radiata and cumulus cells were indicator of oocyte maturation. GVBD was determined when nuclear structure was visible in the plasma. Good quality embryos were those with homogenously distributed fine grains, homogenous well-shaped blastomere, and segments below 20%.

IGF-1 quantification

IGF-1 level in serum and follicle fluid were both measured by Enzyme-Like Immuno Sorbent Assay (ELISA) (Rapidbio, USA) as per the manufacturer's instructions.

Isolation of mice cumulus-oocyte complex (COCs) and in vitro culture

All mice were purchased from Laboratory Animal Center of the Academy of Military Medical Sciences, which were maintained on a 12 L: 12 D photoperiod with rodent chow and water provided ad libitum. Upon the approval of experimental protocols by Institutional Animal Care and Use Committee and Ethics Committee of Xiamen University, the immature 22- to 25-day-old female mice were injected with 5 IU PMSG and subsequently 5 IU hCG to induce ovulation. Ovaries were extracted from mice 48 h after PMSG injection, and placed in pH7.3 MEM containing 30 mM HEPES and 1

Table 1. Clinical characteristics of studied population (n=52/52)

Characteristics	Fertile	Non-fertile	*p*-value
Age	29±3	28±2	0.0689
LH (mU/ml, mean ± SD)	0.97±0.31	1.22±0.38	P<0.01
E2 (μg/ml)	3751±273	3184±321	P<0.01
P (ng/ml)	0.66±0.05	0.67±0.07	0.2531
Oocyte count	15±7	12±5	0.05149
Fertilization rate (%)	72.3	65.4	0.4522
High quality embryos (%)	53.4	28.6	0.0008919
IGF-1 in mural granulosa cells (ng/ml)	68.3±15.3	53.2±12.1	P<0.01

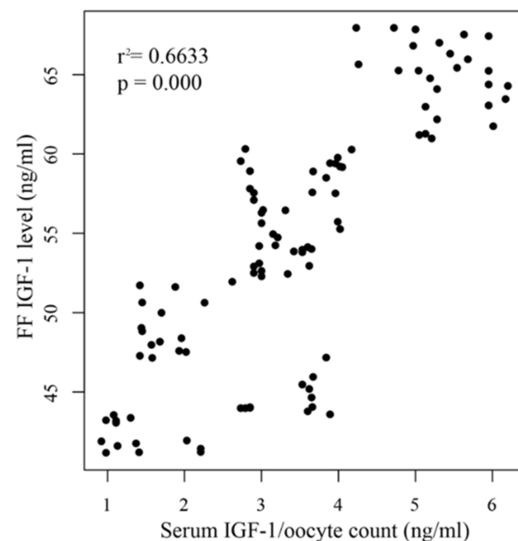

Figure 1. Correlation between ratio of serum IGF-1/oocyte number and FF IGF-1. y-axis represent the IGF-1 level in follicle fluid, and the x-axis stands for the ratio of serum IGF-1/oocyte number.

mg/ml BSA. Compact COCs were released from antral follicles by puncturing the large ovarian follicles with a syringe. Under stereomicroscope, the grade 1 COCs were selected, which were then washed twice with M-199 supplemented with 25 mM HEPES and 0.6% (w/v) BSA. In vitro culture medium was M-199 supplemented with 6 μg/ml FSH, 6 μg/ml LH, 1 μg/ml oestradiol, 30 μg/ml gentamycin, and 0.6% (w/v) fatty acid-free BSA. COCs were incubated for 24 h at 38.5°C under 5% CO_2 in humidified air.

Assessment of cumulus cell expansion and determination of nuclear maturation

Partially expansion was defined as those with outer layer cells loosened, and fully expansion with all cumulus cells loosened. Degree of cumulus expansion was assessed under a stereomicroscope after 18 h of maturation. At the end of maturation period, the denuded oocytes were fixed on a slide with vaseline/paraffin mixure (40:1) and then dimmed with acetic acid:methanol fixative (1:3 v/v). After 45 hours, aceto-orcein (45:1 acetic acid:orcein) was used to stain the oocytes. Morphology of nuclear material can be visible under phase contrast microscopy, which served to determining the stages of nuclear maturation.

cDNA preparation and real-time PCR analysis

Total RNA extracted from with Trizol and treated with DNase I to prevent from DNA contamination. Complementary DNA was synthesized using random hexamer primers as described by manufacturer (Ambion Cells-to-cDNA II kit from Life Technologies, USA). PCR-mastermix containing specific primers for Has2, Ptgs2, Ptx3, and Tnfaip6, Hot-Star Taq DNA polymerase (Qiagen, Valencia, USA) and SYBR-Green PCR buffer were added. Real-time quantification was performed by SYBR-green real-time reverse transcription PCR assay using Icycler (Bio-Rad Laboratories, USA). The result of mRNA expression were normalized to Rp119 mRNA expression. Primers were as follows: Has2-forward: 5'-CGAGTCTATGAGCAGGAGCTG-3'; Has2-reverse: 5'-GTGATTCCGAGGAGGAGAGACA-3'; Ptgs2-forward: 5'-AAAGACGTACAGACCAGACACG-3'; Ptgs2-reverse: 5'-AGAAGGACACTTGGCTTCCTC-3'; Ptx3-forward: 5'-AATGCATCTCCTTGCGATTC-3'; Ptx3-reverse: 5'-TGAAGTGCTTGTCCCATTCC-3'; Tnfaip6-forward: 5'-GCAGCTAGAGGCAGCCAGAA-3'; Tnfaip6-reverse: 5'-ACTCTACCCTTGGCCATCCA-3'; Rp119-forward: 5'-CATGAGTATGCTTAGGCTACAGAAG-3'; Rp119-reverse: 5'-GCGATTTCGTTGGTTTCATT-3'.

Western blot

Western blot analysis was conducted to measure the Akt and phosphorylated Akt (p-Akt). Briefly, cells were lysed in lysis buffer containing 1% Triton X-100, 1 mM EDTA, 2 mM NaF, 2 mM sodium orthovanadate, 1 mM EGTA, 150 mM NaCl, 20 mM Tris-HCl (pH7.4), and Complete TM Protease Inhibitor Mix (Roche Applied

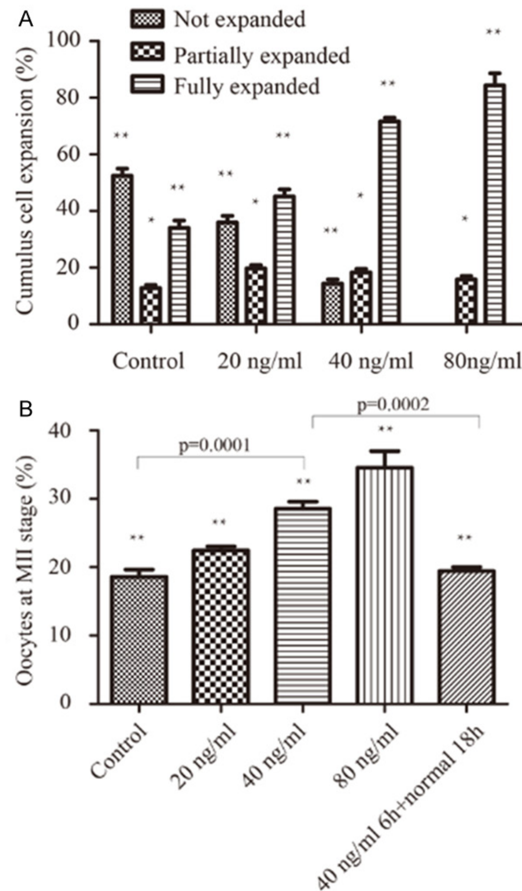

Figure 2. A. The effect of different concentration of IGF-1 supplementation to the oocyte maturation media on the cumulus expansion of mouse COCs after 24 h. Difference between multiple groups were analyzed via one-way ANOVA. Error bars represent standard error, and *: P<0.05, **: P<0.01. B. The effect of different concentration of IGF-1 on nuclear maturation of mouse oocyte. y-axis represent the percentage of MII-phase oocytes. Difference between multiple groups were analyzed via one-way ANOVA. Error bars represent standard error, and *: P<0.05, **: P<0.01. Difference of Control vs. 40 ng/ml IGF-1 treatment and 40 ng/ml IGF-1 treatment vs. 40 ng/ml 6 h+normal 18 h were analyzed by Student's t-test.

Science, Germany) for 25 min on ice and then centrifuged at 12,000 rpm, 4°C. Proteins were resolved on a 10% SDS PAGE gel, transferred onto nitrocellulose membranes, and blocked by 5% fat-free dry milk in TBST containing 100 mM NaCl, 10 M Tris-HCl and 0.05% Tween 20. Subsequently, primary antibody were added to the mixture and incubated at room temperature with shaking for 48 hours. After washing with PBS, secondary antibody conjugated with horseradish peroxidase were added to the membrane for incubation for 5 minutes. Blots were visualized using an enhanced chemiluminescence-Western blotting detection system (Thermo Fisher Scientific, USA).

Statistical analysis

Data were from at least three independent repeats. Results are expressed as mean values ± std., and a Student's t-test was used for testing statistical significance between two groups, and one-way ANOVA was applied to test the difference between multiple groups. P<0.05 is considered as significant, whereas P<0.001 represents extreme significance.

Results

Clinical characteristics of studied population (n=52/52)

According to the pregnancy outcome, the patients were divided into two groups: fertile group and non-fertile group, each containing 52 cases. The average age for two groups were respectively 29±3 and 28±2, which did not present significant differences. In progesterone, oocyte count, fertilization rate, two groups did not show statistically significant difference, while in LH, E2, high quality embryos and IGF-1 level in mural granulosa cells, significant differences were observed (**Table 1**).

The ratio of serum IGF-1/oocyte number is correlated with follicular fluid IGF-1

From the clinical characteristics of the studied population, we noticed that the individuals with 0higher serum IGF-1 usually displayed higher IGF-1 in follicular fluid, which is associated with oocyte numbers. Thus, we attempted to examine if the correlation between serum IGF-1 and FF IGF-1 is statistically significant. Taking oocyte number into account, we established a linear model indicating the correlation between ratio of serum IGF-1/oocyte number and FF IGF-1 (P=0.000, r=0.6633; **Figure 1**).

Supplementation of IGF-1 to mouse COCs promotes cumulus expansion and oocyte nuclear maturation

To investigate the effect of IGF-1 in oocyte nuclear maturation and cumulus cell expansion, we supplemented 0 ng/ml, 20 ng/ml, 40 ng/ml and 80 ng/ml IGF-1 to COCs for 24 hours, which produced a dose-dependent increase in

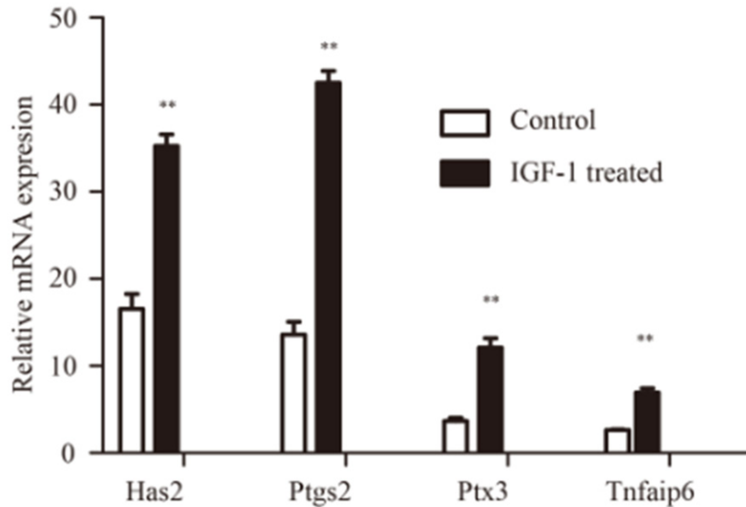

Figure 3. The effect of IGF-1 treatment (40 ug/ml) on mRNA level of ECMs including Has2, Ptgs2, Ptx3, and Tnfaip6. Difference between control and IGF-1 treated group was analyzed by t-test. Error bars represent standard error, and *: P<0.05, **: P<0.01.

percentage of COCs that were undergoing full expansion, compared with 52.48% in control (**Figure 2A**). The MII phase oocytes constituted a relatively higher portion in the group supplemented with IGF-1. While supplemented with 40 ng/ml IGF-1 for 6 hours and then transferred to normal maturation media, the MII phase oocyte counts were significantly lower than the fully supplemented COCs (**Figure 2B**).

IGF-1 increased ECM (Has2, Ptgs2, Ptx3, and Tnfaip6) expression

We further examined the expression of cumulus expansion-related genes Has2, Ptgs2, Ptx3, and Tnfaip6. As shown in **Figure 3**, the mRNA levels of Has2, Ptgs2, Ptx3, and Tnfaip6 in IGF-1 treated group were significantly increased compared with control group (P<0.05), suggesting the promotive effect of IGF-1 on expression of ECMs involved in cumulus expansion.

Treatment of LY294002 blocks the cumulus expansion and oocyte nuclear maturation promoted by IGF-1

Since IGF-1 was highly implicated in Pi3k/Akt pathway, we further examined whether Pi3k/Akt pathway contributes to the effect of IGF-1 on nuclear maturation of oocytes. COCs were treated with 10 μM LY294002 and/or 40 μM IGF-1. After 24 hours, p-Akt and Akt were extracted and analyzed by western blot. Results showed that the cumulus cell expansion was significantly re-duced when supplemented with LY294002 (**Figure 4A**), so was the percentage of oocytes at MII stage, and the increase and decrease in cumulus cell expansion and MII-phase oocytes were corresponding to the phosphrylation of Akt.

Discussion

IGF-1 is abundant in various tissues of animals, which could promote mitosis, stimulate synthesis of RNA and DNA, thereby accelerating cell proliferation or inhibiting apoptosis [15-17]. Studies of past few years suggest that IGF-1 is an important regulator in multiple biological processes, which is involved in many aspects of reproductive activity [18]. The major role of IGF-1 in regulating reproductive ability resides in promoting proliferation and differentiation and inhibiting apoptosis of granulosa cells, promoting the synthesis of steroid hormones, and synergistically acting with gonadotropin to increase activity of aromatase [19]. It was demonstrated that the follicles were arrested at an early stage and ovulation was ceased in IGF-1 knock-out mice [20]. This indicated that IGF-1 might serve as an initiator for folliculogenesis. Functional studies addressing the role of IGF-1 in follicular development, selection of dominant follicles, ovulation and atresia had also been conducted [21-24]. However, there's still a scarcity in studies investigating the effect of IGF-1 on oocyte maturation. In the present study, we observed significantly lower IGF-1 in follicle fluid in infertile patients. The correlation of FF Igf-1 and serum Igf-1 was subsequently established when taking account of oocyte numbers, indicating that increased FF IGF-1 might be concurrent with oocyte maturation.

Cytoplasmic maturation and developmental competence is the most critical component for oocyte quality, in which cell communication between oocytes and cumulus cells, as well as cumulus expansion occur [25, 26]. Cumulus expansion emcompasses changes in morphology

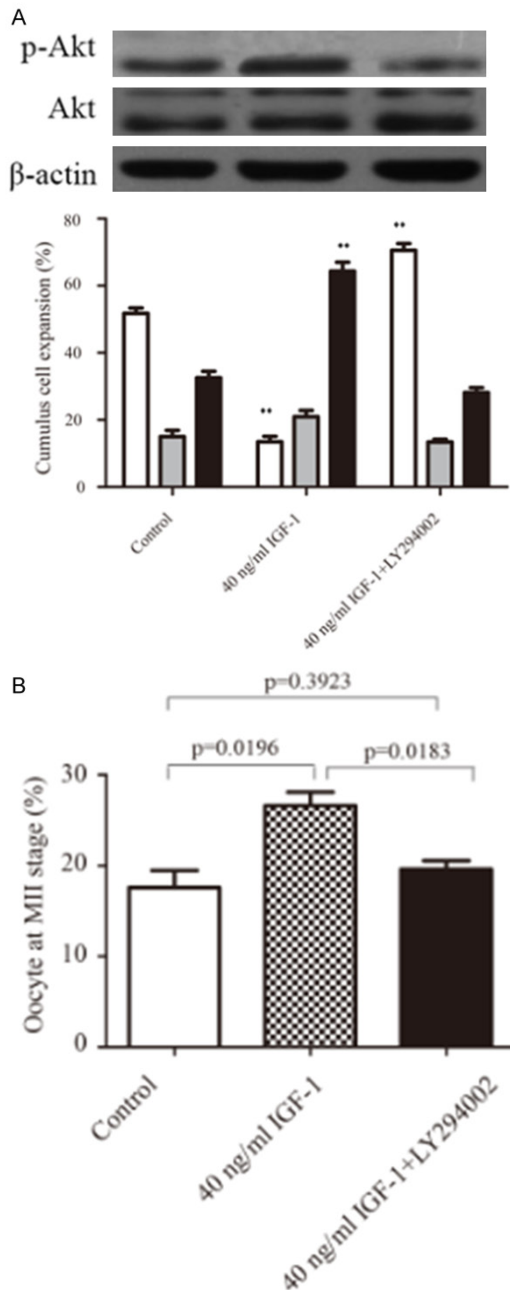

Figure 4. A. The efffect of IGF-1 and LY294002 on cumulus cell expansion of mouse oocytes. Upper panel shows the western blot of Akt and p-Akt, and lower panel shows the percentage of cumulus expansion after treatment of IGF-1 and/or LY294002. B. The efffect of IGF-1 and LY294002 on nuclear maturation of mouse oocytes. Difference between control and IGF-1 (and LY294002) treated group was analyzed by t-test.

and metabolic activity, synthesis of lots of extracellular matrix, and enlargement of cumulus-oocyte complexes (COCs). In vivo, cumulus expansion occur followed by ovulation, while in vitro, it is an important event in meiotic maturation. Chen et al. revealed that successful development to 2-cell stage was correlated with the quantity and quality of the expanded cumulus mass, and the absence of cumulus, whether by spontaneous loss or mechanical removal could result in complete infertility even cultured in maturation medium [27, 28]. Similarly, Schoevers et al. showed that inadequate number of cumulus cell layers may not necessarily indicate impaired oocyte maturation, but could substantially affect early embryonic development [29]. In the present study, we observed that the percentage of fully grown oocytes was substantially elevated by increased concentration of IGF-1. This effect was significantly smaller when the culture was tranferred to IGF-1 free mediums. These findings suggest that IGF-1 is conducive to the oocyte maturation. Moreover, we also determined a bunch of ECMs, Has2, Ptgs2, Ptx3, and Tnfaip6, were upregulated after addition of IGF-1, which had been highly implicated in the processes of oocyte maturation. Yokoo et al. had reported that HA served as both structural and signal molecule in reproductive physiology, which regulated oocyte meiotic maturation, cumulus expansion, ovulation, and fertilization [30, 31]. It is reasonable to deduce IGF-1 up-regualted HAS, an enzyme that catalyzes HA synthesis, thereby promoting oocyte maturation. PTGS2 selective inhibitor NS398, PTGS2-specific siRNA or PTGER2-receptor antagonist AH6809 could reduced nuclear maturation by 20~25% [32]. The elevation of PTX3, an angiogenin and regulator of G-protein signalling 2 (RGS2), is associated with oocytes with stronger developmental competence [33]. In addition, TNFAIP6 was reported as an indicator of adequate cumulus expansion thus improved embryonic development [34]. The promotive effect of IGF-1 on the expression of those ECMs may underly the mechanism of improving embryonic development.

PI3K/Akt pathway is a well-established downstream signalling process of IGF-1. Yumi and Eimei demonstrated that phosphatidylinositol 3-kinase (PI3K) participates in follicle-stimulating hormone (FSH)-induced mouse meiotic maturation [35]. Therefore, we assumed that cumulus expansion enhanced by IGF-1 may be associated with PI3K/Akt pathway. We used

LY294002, a specific inhibitor of PI3K, to block phosphorylation of Akt in the presence of IGF-1. The cumulus expansion was remarkably suppressed when LY294002 was added, compared to control, and was shown to reverse the effect of IGF-1. In oocyte nuclear maturation and embryo development, similar phenomenon were indicated. Akt is a kinase regulated by second messenger and participates in many crucial cellular processes, including glucose metabolism, transcription, apoptosis and migration [36]. In the present investigation, we found that LY294002 also diminished the effect of IGF-1 on the regulation of ECMs. Considering the roles of ECMs in cumulus expansion, completion ofcytoplasmic maturation, and acquisition of developmental competence, we presumed that IGF-1 may exert its effect through PI3K/Akt pathway.

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Disclosure of conflict of interest

None.

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