[Frontiers in Bioscience 12, 1804-1813, January 1, 2007]

Luteinizing hormone receptors expression in cumulus cells closely related to mouse oocyte meiotic maturation

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1. ABSTRACT

In the process of oocyte maturation, gonadotrophins are believed as main stimulators for oocyte meiosis resumption. However, which gonadotrophin (i.e. FSH or LH) is the key hormone in this process is still unknown. This study indicated a close relationship between LH and FSH on activating meiotic maturation of oocyte *in vitro*. FSH efficiently induced oocyte meiosis at concentration of 50 IU/L, while LH alone had no effect on oocyte meiotic initiation. Using RT-PCR and *in situ* hybridization, a tight corelationship between FSH-stimulated oocyte meiotic resumption and LHR mRNA expression in cumulus cells was found. LHR mRNA was present in cumulus cells of oocyte-cumulus cell complexes. Except the expression of LHRs was present in cumulus cells surrounding all maturing oocytes, low level expression was also detected in cells associated with oocytes that were still at GV stage. Its expression was enhanced by FSH stimulation before oocyte maturation. However, LHRs did not express in cumulus cells associated with oocytes that were completely arrested at GV stage by IBMX. Furthermore, increased progesterone concentration was found in the medium in which CEOs were cultured with FSH and LH, but not in those with FSH or LH alone. LHR expression in cumulus cells increases with time in culture, and the levels of expression are enhanced in the presence of FSH. Oocyte meiotic resumption may create conditions favorable for LHR expression. LHR expression may be considered as a potential marker for oocytes maturation initiation.

2. INTRODUCTION

The characteristics of the meiotic maturation of oocytes, which are present in antral follicles, involve resumption of meiosis I, transition between meiosis I and meiosis II and arrest in metaphase II (MII) (1). However the regulatory mechanisms of this process remain poorly understood. It is now well established that the two pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are both structurally related glycoprotein, play pivotal roles in oocyte meiotic maturation and ovulation (2, 3). *In vivo* a secretory peak of LH triggers ovulation. LH surge stimulates preovulatory follicles that lead to oocyte meiotic resumption. As a consequence, most mammalian oocytes arrest in MII upon first polar body (PB1) extrusion and later follicular rupture (4). Albeit the physiological regulation of resumption of meiosis is likely dependent on the actions of LH and FSH together (5), it is not clear whether FSH treatment alone has different effect from that of LH induction on granulosa cell function in meiotic maturation of oocytes. Many *in vitro* studies highlighted that isolated cumulus-enclosed oocytes (CEOs) from medium size or large preovulatory follicles can resume meiosis by respond to FSH in physiological concentrations, whereas high concentrations of LH or human chorionic gonadotrophin (hCG), a LH analog, have no effect at all (6-10).

The actions of the two gonadotrophins are mediated by binding to and activating their individual receptors (LHRs and FSHRs), which are G protein-coupled

receptors (GPCRs) family proteins (11). It has been widely accepted that oocytes have no LHRs (12). Inefficient stimulation of LH on oocyte maturation *in vitro* is probably due to the fact that LHRs were absent or expressed at very low levels on the cumulus cells surrounding the oocyte. Although LHRs are mainly localized on thecal cells and mural granulosa cells of preovulatory follicles, its expression is perplexing and appears to be very different in cumulus cells (13-18). The accurate time of LHR appearance on cumulus cell is still unknown. Considering oocyte maturation is compromised in the LHR knockout (LHRKO) mouse because the follicles never develop to the stage where the effects of FSH can be found (19), and FSH present a positive role to initiate the maturation of oocytes *in vitro* (7), investigations of the temporal changes of LHR expression in cumulus cells and its functional relevance with FSH during the meiotic maturation of oocytes will offer a better understanding to decipher the regulatory mechanisms of LH and FSH.

To analyse oocyte meiotic resumption *in vitro*, it is critical to choose the appropriate cultural model. The cumulus oophorus is defined as a group of closely associated granulosa cells called cumulus cells in the development stage of antral ovarian follicle in which oocyte has already been in possession of the competence to resume meiotic maturation. At the same time oocyte cell cycle is arrested in prophase I because of the inhibitory environment of follicle until the appearance of preovulatory gonadotrophin peak. Many *in vitro* CEO and follicle models were established based on this fact. Expression of LHRs in these models provides a tool to investigate the maturation induction of oocyte with gonadotrophin in particular. Recently, RT-PCR and *in situ* hybridization methods were also used to study the gonadotrophin receptor mRNAs levels (13, 20-23). We have successfully developed a novel method that allows much better signal retention in oocyte-cumulus cell complex cultured *in vitro* than currently available methods. And this method overcame usual morphological maintenance problem associated with *in situ* hybridization.

In the study reported here, we utilized the *in vitro* model of cultural CEOs to investigate the LHR transcription level in cumulus cells associated with germinal vesicle (GV) stage or maturing oocytes. *In situ* hybridization result further illustrated the interrelation of FSH with LHR in oocyte meiotic resumption. Moreover, we detected the progesterone concentration in CEOs cultured with LH and / or FSH.

3. MATERIALS AND METHODS

3.1. Isolation and culture of cumulus-enclosed oocytes (CEOs)

All animal procedures were approved by the Animal Care Committee of China Agricultural University. Experiments were conducted with immature female Kunming White mice (outbreed strain) weighting 14-16 g (22-24 days old) without equine chorionic gonadotrophin (eCG) priming. Mice were housed under controlled temperature and light conditions and fed ad libitum. Mice were killed by cervical dislocation, and ovaries were immediately removed and washed in handling medium, i.e. hypoxanthine (HX) medium as described below. CEOs with intact cumulus cells connection were isolated under a stereomicroscope by manual rupture of follicles using a pair of 26 gauge needles, washed three times with fresh medium, and then transferred to 35 mm culture dishes (Nunclon; Nunc, Roskilde, Denmark). Each dish contained 2 mL culture medium with 60-80 CEOs. The cultures were incubated in humidified modular incubator at 37□ and with 5% CO_2. At the end of culture period, media were collected and stored at -20□ until progesterone concentrations were measured using Progesterone Radioimmunoassay Kit (Beifang, Beijing, China).

The main culture medium used here was prepared with M199 (GIBCO-invitrogen, Carlsbad, CA, USA) which containing 4 mM HX, 3 mg/mL bovine serum albumin (BSA), 0.23 mM pyruvate, 2 mM glutamine (all from Sigma, St. Louis, MO, USA) and named "HX medium" infra. Medium termed "induction medium" was HX medium supplemented with FSH (50 IU/L, Sigma, F-2293) or LH (0.1, 1 and 10 IU/mL, Sigma, L-9773). M199 with 150 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma, I-5879) was used as alternative to HX medium in respective experiments.

3.2. RNA isolation from CEOs

Total RNA of 450 CEOs from each group was extracted using TRIzol™ Reagent (GIBCO-invitrogen) according to manufacturer's protocol and quantified by measuring the absorbance at 260 nm. The integrity of RNA was analyzed by agarose-formaldehyde gel electrophoresis.

3.3. RT-PCR analysis

All RNA samples subject to reverse transcription were treated with RNase-free DNase I (Ambion, Austin, TX, USA). Reverse transcription reaction using oligo $d(T)_{15}$ primers (TaKaRa, Otsu, Shiga, Japan) was performed for 1 h at 42□ in a total volume of 25 μl mixture with 200 units of M-MLV reverse transcriptase in reaction buffer, 125 mM dNTPs mixtures and 25 units of RNase inhibitor (all from Progema, Madison, WI, USA). The resultant cDNA mixtures were then heated at 95□ for 5 min to terminate the reverse transcription before storage at -20□. Negative controls were always performed under the same condition with no reverse transcriptase.

The primer sequences used for the PCR of LHRs were described by O′Shaughnessy (23). The PCR reaction to determine the LHR transcription level mRNA was conducted with premix *Taq* (TaKaRa) according to the manufacturer's recommendations. Beta-actin transcription level was used as internal standard using same quantity of cDNA mixture as template. Following 94□ 5 min initial denaturation, PCR was carried out for 30 cycles (94□ denaturation, annealing specific for primers (Table 1), 72□ extension, each step 1 min). The reaction was terminated with a final extension of 5 min at 72□.

Table 1. Oligonucleotide primers used for RT-PCR

mRNA	Primers 5'-3'	Sequence of oligonucleotide	Annealing temperature	Product size
LH receptor	5'-end	GGGCTGGAGTCCATTCAGACG	55°C	453 bp
	3'-end	CAGTTTATAACGACTGGTCAG		
beta-actin	5'-end	TCCAGCCTTCCTTCTTGGGTAT	55°C	557 bp
	3'-end	TTTGACCTTGCCACTTCCGC		

The PCR products density was measured by AlphaEaseFC Software (Alpha Innotech Corp., San Leandro, CA, USA). RT-PCR experiments for the LHRs and beta-actin gene were each repeated for three times.

3.4. *In situ* hybridization

The plasmid containing a cDNA fragment corresponding to nucleotides 1-591 of the mouse LHR gene was kindly provided by Dr. Segaloff D.L. (Department of Physiology and Biophysics, The University of Iowa, USA). Sense and antisense DIG labeled, single stranded RNA probes of defined length were generated using the DIG RNA Labeling Kit (Roche, Basel, Switzerland). Effectively it was carried out by *in vitro* transcription of linearized plasmids in the presence of digoxigenin-UTP, T7 RNA polymerases following digestion with *Spe* I or *Eco*R I, respectively.

CEOs cultured *in vitro* were mounted on the glass slides and washed with phosphate buffered saline (PBS), then fixed for 20 min in 4% paraformaldehyde (PFA). After washed twice with PBS to remove the PFA, CEOs were treated with 0.1 M glycine (Amresco, Solon, OH, USA), 0.4% Triton X-100 (Biomol, Plymouth Meeting, PA, USA) and 1 □g/mL Proteinase K (Merck, Whitehouse Station, NJ, USA) to increase the permeability. Cells were then subject to a further fixation for 20 min in 4% PFA. Again PFA was removed by twice PBS wash. Finally CEOs were incubated for 2.5 h in prehybridization solution at 55□. This hybridization mix contained 50% deionised formamide (Amresco), 10% dextran sulphate (Amresco), 1× Denhard's solution (Dingguo, Shanghai, China), 0.5 mg/ml salmon sperm DNA (Sigma), 0.25 mg/ml yeast tRNA (Sigma) and 2× SSC. Sense or antisense probes were added to the prehybridization buffer in which CEOs were incubated for 20 h at 55□. After hybridization, the slides were incubated with 20 □g/mL RNase A (Amresco) for 30 min at 37□ and washed with depressive concentrations of SSC (2×-0.1×) to remove remaining probes. Colorimetric detection of the digoxigenin labeled probe was performed with DIG Nucleic Acid Detection Kit (Roche).

3.5. Statistical analysis

All experiments were performed at least three times, and quantitative results here were exhibited as mean +/- SEM. This was obtained by adoption of t-test or one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (SigmaStat; Systat Software, Inc., Richmond, CA, USA). All relevant percentage data were transformed by arcsine square root before analysis. P less than 0.05 was considered to indicate a significant difference.

4. RESULTS

4.1 Effect of FSH on oocytes maturation in CEOs

50 IU/L FSH is sufficient to induce oocytes maturation in CEOs *in vitro* as described previously (24). To monitor the exact timing of oocyte maturation, CEOs were treated in induction medium for 0 h, 10 h, 12 h and 24 h respectively. All oocytes tested were arrested at GV stage when CEOs were induced for less than 10 h. After 12 h culture, 8% oocytes started to undergo breakdown of germinal vesicle (GVBD). In 24 h cell culture, 47% oocytes developed to undergo GVBD in a FSH dependent manner, comparing to 10% of that in control (P less than 0.05) (Figure 1). To verify the dependent of FSH function in oocyte maturation on LHR expression in cumulus cells, CEOs were stimulated with 50 IU/L FSH in 150 μM IBMX medium. The resumption of meiosis was not found even after 24 h incubation (data not shown).

4.2. FSH but not LH induces oocytes maturation in CEOs

To test if LH also induced oocyte maturation, CEOs were cultured with 0.1, 1 or 10 IU/mL LH for 24 h. No significant differences in %GVBD occurrence (11.8%, 14.8% or 12.3%) were observed compared to the control cells (9.9%). However, addition of 50 IU/L FSH to HX treated cells induced 44.4% oocytes to undergo GVBD. This percentage was significantly higher than that of the control. There is almost no difference in GVBD occurrence when treated with FSH only or FSH/LH combination medium (42.0%). (Figure 2)

4.3. RT-PCR analyses of LHR mRNA expression

To determine the LHR expression level upon FSH treatment, we measured its mRNA level by RT-PCR analyses. CEOs were cultured with 50 IU/L FSH for a series of different time periods with 0 h, 12 h and 24 h. After cultivation, the mRNA expression level of LHRs in each CEOs culture time point was examined using RT–PCR with housekeeping gene beta-actin mRNA yielding a 557bp product as a control. Gel electrophoresis of each group revealed bands of 453bp length, corresponding to the regions of the LHR genes (Figure 3A). This confirmed the presence of LHR mRNA in cumulus cell of CEOs cultured in all induction media with FSH for 12 h (Capital D), 24 h (Capital E) or HX medium for 24 h (Capital F). Compared with group F, significantly abundant expression of LHR mRNA was detected in group E. The culture in medium with FSH for 0 h (Capital A) or in HX medium for 12 h (Capital B) did not exhibit any LHR expression. Meanwhile, 50 IU/L FSH in the IBMX medium could not stimulate LHR expression either (Capital C) after 24 h culture. (Figure 3A and B)

4.4. Location of LHR mRNA in oocyte-cumulus cell complexes

Using *in situ* hybridization, LHR mRNA expression could not be detected in oocyte-cumulus cell complexes collected directly (0 h). After 12 h and 24 h culture, all the cumulus cells of CEOs which had

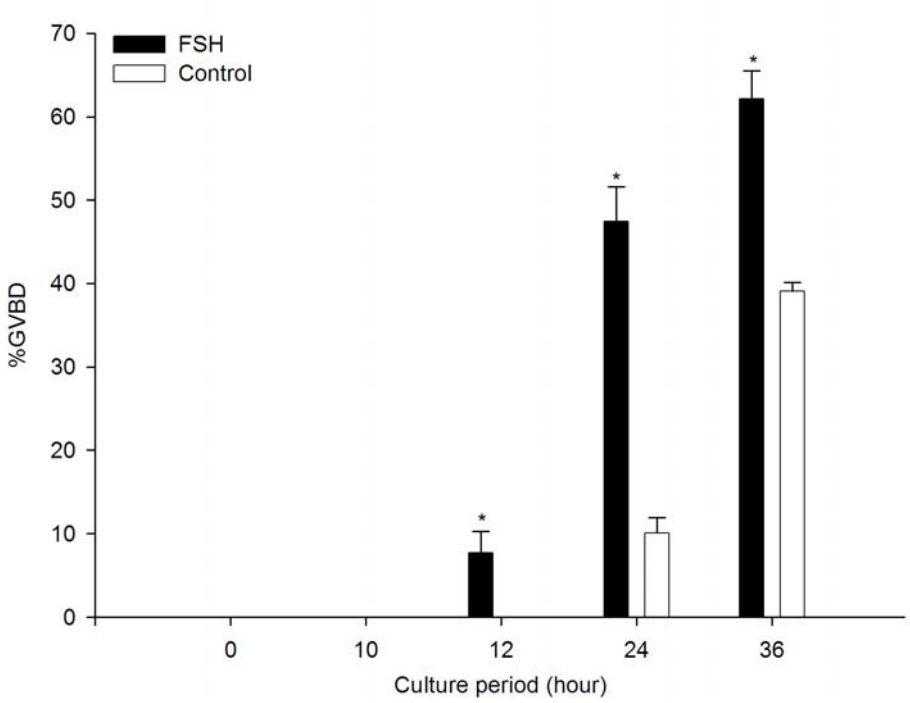

Figure 1. Effect of FSH on oocytes maturation in CEOs. Intact CEOs were cultured in induction medium with 50 IU/L FSH for 0 hour, 10 hour, 12 hour, 24 hour and 36 hour respectively. There are no bars shown at 0 hour and 10 hour, i.e. %GVBD = 0. Data are presented as mean percentage of GVBD +/- SEM of three independent experiments. Columns labeled with asterisk exhibit significant difference compared to the control group (P less than 0.05).

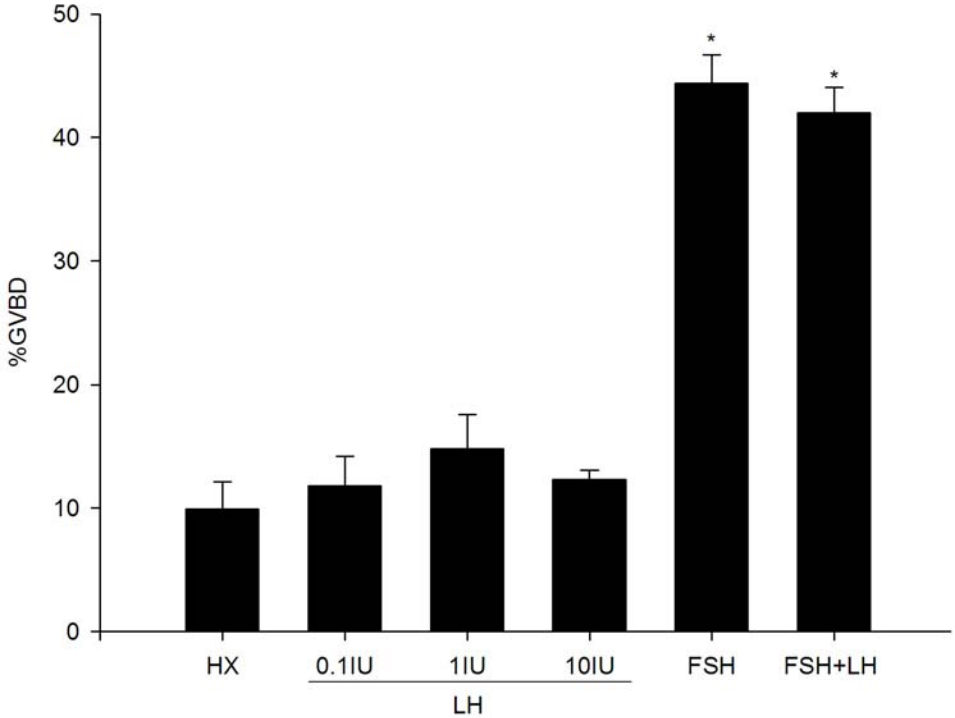

Figure 2. Effect of FSH and LH on the maturation of CEOs after 24 hour culture. Intact CEOs were cultured in induction medium with 0.1, 1, 10 IU/mL LH alone, or 50 IU/L FSH alone, or 50 IU/L FSH together with 1 IU/mL LH for 24 hour respectively. Data are presented as mean percentage of GVBD +/- SEM of three independent experiments. Columns labeled with asterisk exhibit significant difference compared to the HX group (P less than 0.05).

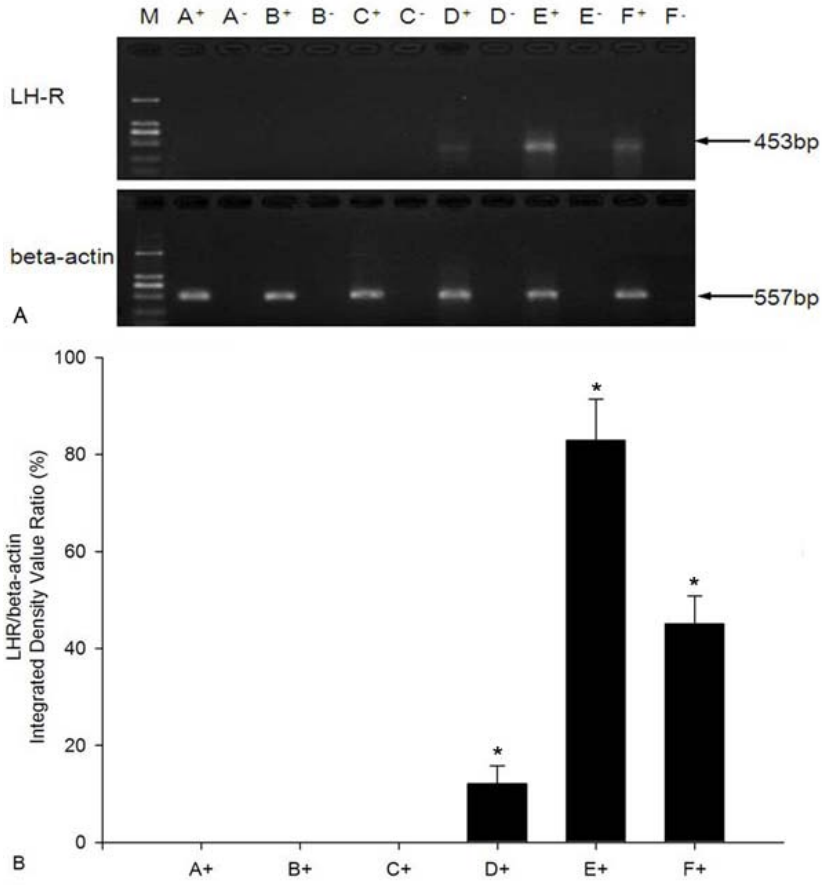

Figure 3. RT-PCR amplification of LH receptor mRNA in mice CEOs *in vitro*. LHR mRNA were present in cumulus cell of CEOs cultured in induction medium with FSH for 12 h (Capital D) and 24 h (Capital E) and in HX medium for 24 h (Capital F). No LHR signal was detected in culture with FSH for 0 h (Capital A), in HX medium for 12 h (Capital B) and in IBMX medium containing 50 IU/L FSH for 24 h (Capital C). The ratio between the intensity of the bands is reported as the mean +/- SEM of the three experiments performed. Columns labeled with asterisk exhibit significant difference (P less than 0.05). Arrows point RT-PCR products. M: D2000 molecular weight marker; The label "-": negative control without reverse transcriptase.

undergone GVBD or/and extruded PB1 (first polar body) showed positive LHR mRNA staining, whether they were incubated in HX medium or induction medium with 50 IU/L FSH. In the CEOs treated with FSH, when oocytes maintained at GV stage, the antisense probe produced intense signals in cumulus cells of some CEOs (Panel B), whilst others were found no staining (Panel A). The signal was significantly stronger in cumulus cells when oocyte started to undergo GVBD (Panel C) or/and extrude PB1 (Panel D). No staining was observed with the control sense probe (Panel E, F). (Figure 4).

4.5. FSH increases LHR appearance prior to oocyte meiotic maturation

20 CEOs were mounted on each glass slide and probed with digoxigenin-labeled RNA. The experiments were repeated three times and the number of CEOs as well as the hybridization signal in different oocyte stage (GV, GVBD/PB1) from each group were scored (Table 2). Because a few CEOs were lost during the experiment procedure and some CEOs were not performed *in situ* hybridization for significant cumulus cells expansion, we could not obtain data as accurate as *in vitro* culture. However, the results showed that after 12 h of culture, the proportion of LHR expression positive cells in media with FSH was 36% (21 of 58), but no cell showed any level of LHR without FSH. After 24 h FSH plus culture, the overall proportion of hybridization positive CEOs (84%; 48 of 57) was significantly higher than that in media without FSH (55%; 30 of 55) (P less than 0.05) (Figure 5A).

4.6. LHR appearance at GV stage is closely related to oocyte meiotic maturation

In FSH-induced media, the proportions of CEOs still at GV stage with LHR expression were 28% (16 of 58) in 12h culture and 47% (27 of 57) in 24h culture, respectively. They were in response to the change of GVBD rate followed by 12 h continuous culture, i.e. from 12 h (8%) to 24h (47%) or from 24h to 36 h (62%). In the same way, in HX-arrested culture, the percentage of CEOs with positive hybridization signals in cumulus cells surrounding oocytes at GV stage in 24 h culture (40%; 22 of 55) was closely related to the increasing GVBD from 24 h (10%) to 36 h (39%) culture. (Figure 5B)

Table 2. Expression of LHR mRNA in cumulus cells of CEOs *in vitro* culture *in situ* hybridization

Culture time (hr)	Medium	GV		GVBD/PB1	GV[1] (%)	GVBD (%)[3]
0	NA	19[2]	0	0	NA	
12	induction medium with FSH	37[2]	16[1]	5[1]	28	8
	HX medium	55[2]		0	0	0
24	induction medium with FSH	9[2]	27[1]	21[1]	47	47
	HX medium	25[2]	22[1]	8[1]	40	10
36	induction medium with FSH	NA	NA	NA	NA	62
	HX medium	NA	NA	NA	NA	39

20 CEOs were mounted on each glass slide with some lost in the experiment. Some CEOs were not selected for *in situ* hybridization because of significant cumulus cells expansion. NA: not available, GV: germinal vesicle, GVBD: breakdown of germinal vesicle, PB1: first polar body, [1]: hybridization signal, [2]: no hybridization, [3]: data from Figure 1

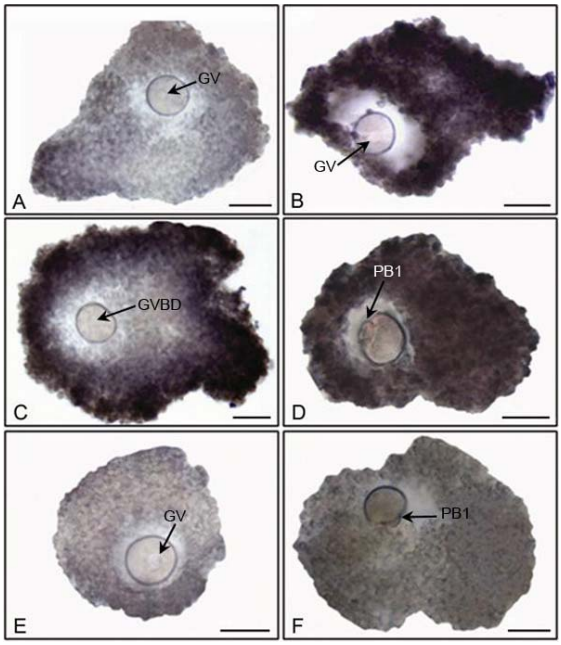

Figure 4. Expression of LHR mRNA in cumulus cells surrounding oocytes from different development stage. CEOs cultured with FSH for 12 hour were mounted on the glass slides and probed with digoxigenin-labeled RNA. When oocytes were maintained at GV stage, the antisense probe produced intense signals in cumulus cells of some CEOs (Panel B), whilst others exhibit no staining (Panel A). The signal was significantly stronger in cumulus cells in the case of oocyte undergoing GVBD (Panel C) or extruding PB1 (Panel D). No staining was observed with the control sense probe (Panel E, F). Arrows point to oocyte at different development stage. GV: germinal vesicle; GVBD: breakdown of germinal vesicle; PB1: first polar body. Bar = 100 μm.

4.7. Progesterone production in CEOs

We collected four different media in which CEOs had been cultured for 24 h: HX medium (control, no FSH), induction medium with FSH (50 IU/L), induction medium with LH (1 IU/mL), induction medium containing both FSH (50 IU/L) and LH (1 IU/mL). Progesterone levels were significantly higher in the medium with FSH than in the control group (P less than 0.05), however, no significant difference was observed between LH treatment group and the HX medium. Furthermore, increased concentration of progesterone was observed in the medium containing both FSH and LH than that with FSH or LH alone (P less than 0.05). (Figure 6)

5. DISCUSSION

Both FSH and LH are able to induce oocytes maturation *in vivo* (25-29). However, which gonadotrophin (i.e. FSH or LH) acts as the key maturation induction hormone is still unknown. *In vivo*, LHRs appearing in mural granulosa cells response to the LH peak in preovulatory follicles. The junctional complexes among the mural granulose layer, cumulus cells, and oocytes play an important role in propagating the LH signal to induce oocyte maturation (30, 31). *In vitro,* cumulus cells of mouse CEOs respond to FSH, while LH has no effect on oocyte maturation (32). *In vivo*, the oocyte is exposed to the intrafollicular environment which maintains oocyte meiotic arrest, but *in vitro* culture, the follicle environment is simulated by HX supplement in serum-free medium (33-35). Different from a complete intact follicle complex *in vivo,* no mural granulosa cells are cultured under *in vitro* experiments. Thus the fact that LH alone cannot induce oocyte maturation *in vitro* is probably due to the absence of LHR in cumulus cells under specific *in vitro* experimental conditions.

However, whether there is LHR expression in cumulus cells remains largely unknown Some studies reported very few LHRs are indeed expressed in cumulus cells (13, 16). In their reports, LHRs expression is so low, and only at one tenth the concentration of mural cells in the rat cumulus (13). Therefore, Peng *et al* (13) report offers one explanation that it is indeed lack of LHR make LH alone not able to induce oocyte maturation *in vitro.* However, a number of other studies revealed the appearance of LHRs in cumulus cells in many species (14, 15, 17, 18). These results of LHR expression in cumulus cells and its roles on oocyte maturation are contradictory to each other. It is not clear whether this is due to different species, such as porcine (18), bovine (17), rat (13, 14) and mouse (15, 16), or different experimental conditions such as technique and culture system used (14-16). However, this discrepancy could also to be due to different oocyte maturation stages, which has not yet been tested. Both protein and mRNA levels of LHRs are very low or undetectable in cumulus cells when oocyte meiosis resumption of intact complexes are arrested (16). On the other hand, cumulus cells expressed LHRs when the cumulus expansion was elicited with FSH *in vitro* (15). Cumulus expansion is regarded as one definite response in the progress of oocyte meiotic resumption (36). So far, it is technically difficult to detect the LHR

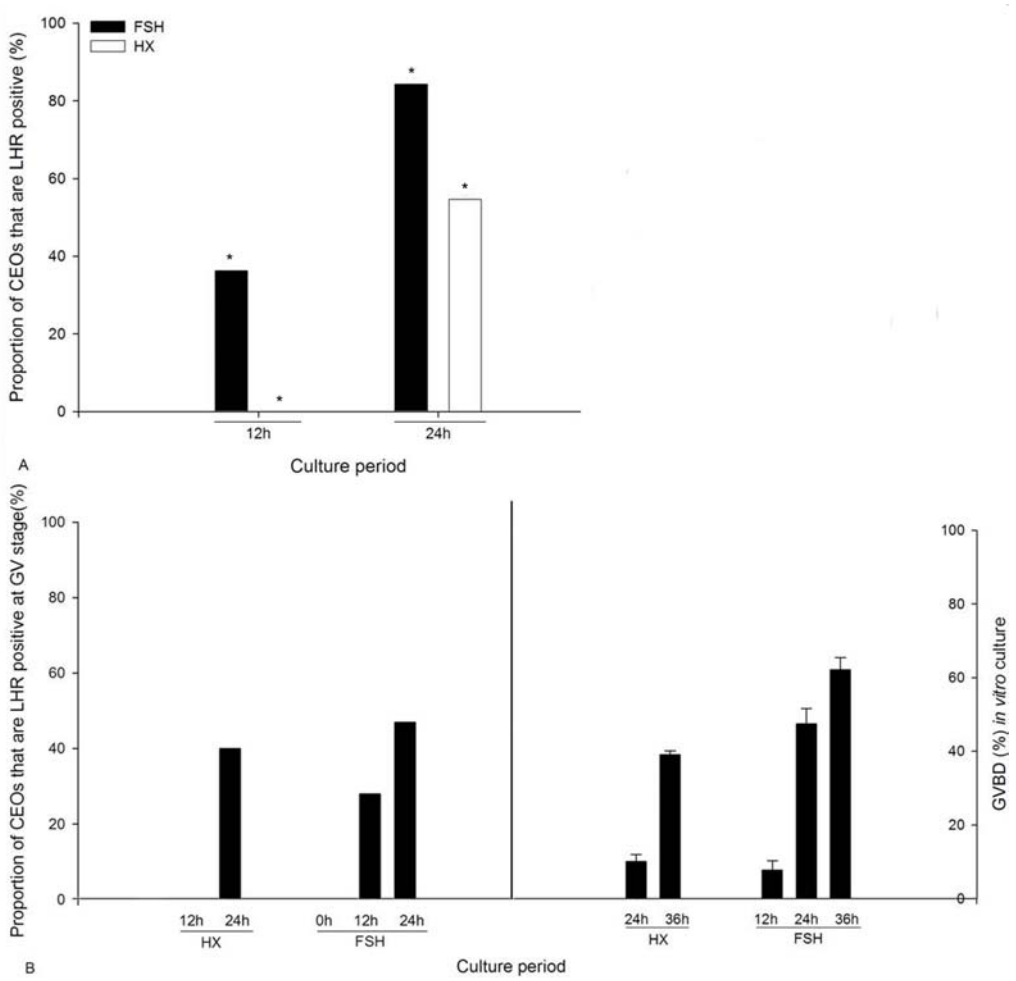

Figure 5. LHR expression correlates oocyte maturation *in vitro*. (A): the proportion of CEOs that are LHR positive; (B, left): the proportion of CEOs that are LHR positive at GV stage; (B, right): the GVBD rate in oocyte maturation *in vitro* culture. LHR appearance at GV stage is positively related to oocyte meiotic maturation. Each graph based on the data from Table 2. There are no bars shown at 12 hour HX-arrested culture in (A) and (B, left), i.e. the proportion is zero. There are no error bars in (A) and (B, left) for data from *in situ* hybridization. Columns labeled with asterisk exhibit significant difference (P less than 0.05).

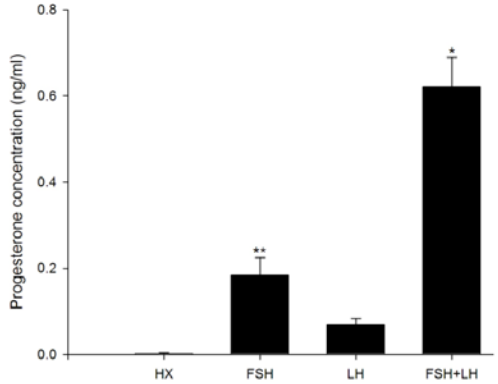

Figure 6. Progesterone production in CEOs. Four different media were selected to detect progesterone concentrations: HX medium (control group); induction medium with FSH (50 IU/L); induction medium with LH (1 IU/mL) and induction medium containing both FSH (50 IU/L) and LH (1 IU/mL). Data are presented as mean concentration of progesterone +/- SEM of three independent experiments. One askterisk indicates progesterone levels were significantly higher in the medium containing both FSH and LH than that in HX medium, induction medium with FSH or LH alone (P less than 0.05). Two asterisks indicates progesterone levels were significantly higher in the medium with FSH than in the control group (P less than 0.05). No significant difference was observed between LH treatment group and the HX medium (P more than 0.05).

expression in cumulus cells enclosed oocytes at different development stage *in vivo*. But *in vitro*, the rate of oocyte maturation upon gonadotrophins stimulation can be assessed based on the fact that the most striking event of the reinitiation of oocyte meiosis is GVBD (36). Judged on CEOs morphology, we removed the ovaries from non-gonadotrophin treated mice and maintained in serum-free medium supplemented with HX to investigate the functional role of LHR expression in cumulus cells in the progress of meiotic resumption of mouse oocytes. The selected immature oocytes grow and become competent to resume meiosis but arrest at GV stage, and finally undergo GVBD. We investigated the correlation between the time-dependent changes of LHR expression in cumulus cells and the specific status of oocyte maturation. Our result in mice is consistent with a recent report that FSH induces LHR expression on porcine cumulus cells (37). However, we also observed LHR formation in HX-treated CEOs control group that was no exposed to FSH for 24 h culture. HX was used here to maintain meiotic arrest and to mimic the inhibitory environment within the follicle (33-35). A leaky meiotic arrest is sometimes possible, although HX maintains oocytes in the GV stage (38). FSH is sufficient for resumption of oocyte meiosis by overcoming HX inhibition (6-10). Although FSH stimulation is able to increase LHRs expression in cumulus cells during oocyte maturation, our results suggest that the more likely possibility is that oocyte maturation creates a condition that initiates LHR expression, not FSH treatment itself *per se*.

To further study the correlation of LHR expression on oocyte maturation, we focused on the LHR expression in CEOs after 12 h culture. According to present report on *in vitro* culture, 12 h is the precise timing of events in FSH-stimulated maturation of CEOs. Some immature oocytes had just developed into the start stage to undergo GVBD. Our most interesting observation was that, by *in situ* hybridization, LHR expression was found in cumulus cells surrounding all maturing oocytes (spontaneous or FSH-induced), whereas expression was sporadic in cells associated with oocytes that are still GV-stage. It provided evidence that the induction of LHR expression preceded closely upon the oocyte maturation. In mice, the GV-stage oocytes suppress the expression of LHR in the cumulus cells (16). Consistent with this, we did not find any LHR synthesis when intact complexes are treated in IBMX inhibitory milieu. Different from to the leaky arrest of HX, IBMX completely blocks meiosis (38). We believed that when LHR mRNA is expressed in cumulus cells enclosed GV-stage oocytes, the arrest of these oocytes can be removed and cells are ready for meiotic resumption. To support this, we showed that the proportions of CEOs still at GV stage with LHR expression was tightly correlated to the increasing of GVBD rate followed by 12 h continuous culture. This strongly suggests that with LHR mRNA expression in cumulus cells, the enclosed oocytes can overcome the meiotic arrest and followed by GVBD occurrence. Therefore, we propose that the expression of LHRs can be considered a potential marker for initiation of oocytes maturation.

Through association with their individual receptors, FSH and LH are known to increase granulosa cell progesterone production in many species (18, 39-43). In this study, RT-PCR and *in situ* hybridization results provided solid evidence at the transcriptional level on LHR expression, while the FSH- and LH-stimulated secretion of progesterone indicated at the protein level that functional LHRs were induced by FSH in cumulus cells in the process of oocyte meiotic maturation. Using our specific *in vitro* culture, significant progesterone concentration increasing was observed in the medium in which CEOs were cultured with LH and FSH for 24 h. Furthermore, the progesterone secretion was much higher in response to both LH and FSH than to FSH or LH alone. The fact that treatment with FSH/LH combination could not increase the oocyte maturation rate compared with FSH only treatment suggested that the presence of LHR mRNA in cumulus cells is not an essential event for oocyte maturation initiation, but may play important roles in combination with the later events following oocyte maturation.

The LHR expression in cumulus cells surrounding the oocytes at specific developmental stage provides a key signal of oocyte maturation, especially the developmental competence of *in vitro*-matured oocyte. Introduction of oocytes with LHR expression in cumulus cells is a very beneficial way to reach a high blastocyst rate *in vitro* fertilization (18, 44). Although the presence of LHR transcripts in cumulus cells is not the key characteristic to judge the developmental competence of oocytes, judging LHR mRNA expression level offers a valuable tool to determine oocyte developmental competence and there is no need to destroy gamete here.

In conclusion, our study presented solid results of a close relationship of LH and FSH on meiotic maturation of mouse cumulus cell-enclosed oocytes *in vitro* culture. LHR expression in cumulus cells increases with time in culture, and the levels of expression are enhanced in the presence of FSH. We believed oocyte meiotic resumption creates conditions favorable for LHR expression. The induction of LHR expression in cumulus cells tightly correlates the oocyte maturation, thus its expression may be considered as a potential marker for oocytes maturation initiation. Further investigations will be required to characterize the functional role and the mechanism of oocytes maturation under the influence of LH and FSH, especially, LHRs in cumulus cells.

6. ACKNOWLEDGMENTS

This research was supported by National Natural Science Foundation of China (No. 30571358) and Beijing Natural Science Foundation (No. 5052017).

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Abbreviations: CEOs: cumulus-enclosed oocytes, CG: chorionic gonadotrophin, FSH: follicle-stimulating hormone, GPCRs: G protein-coupled receptors, GV: germinal vesicle, GVBD: breakdown of germinal vesicle, HX: hypoxanthine, IBMX: 3-isobutyl-1-methylxanthine, LH: luteinizing hormone, LHRKO: LHR knockout, MII: metaphase II, PB1: first polar body

Key Words: LH, FSH, Receptor, Meiosis, Cumulus Cell, Cell division

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