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An Antisense Oligodeoxynucleotide to the LH Receptor Attenuates FSH-induced Oocyte Maturation in Mice*

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ABSTRACT : It has been recently shown that expression of the LH receptor (LHR) in cumulus cells is related with FSH-induced meiotic resumption of mouse cumulus enclosed oocytes (CEOs). However, to date, it is still unclear whether LHR expression in cumulus cells plays a key role during FSH-induced oocyte maturation. The purpose of this study was to characterize the functional role of LHRs in cumulus cells. CEOs were isolated from eCG-primed preovulatory follicles and cultured in hypoxanthine (HX) arrested medium. LHR protein expression in cumulus cells was time-dependent increasing during the process of FSH-induced oocyte maturation. While the sense oligodeoxynucleotide (ODN) had no effect, antisense ODN inhibited FSH-induced LHR expression and meiotic resumption. Moreover, this antisense ODN against LHR could inhibit FSH-induced mitogen-activated protein kinase (MAPK) phosphorylation. This study suggested that LHR expression in cumulus cells is involved in FSH-induced oocyte meiotic resumption, which process is possibly regulated by MAPK cascade. (**Key Words :** Cumulus-enclosed Oocyte, LH Receptor, Oligodeoxynucleotide, Mouse)

INTRODUCTION

Mammalian oocytes are arrested at the diplotene stage until shortly before ovulation. Following stimulation by gonadotropin, fully grown oocytes resume meiosis and develop to the metaphase II (M II) stage (Richards et al., 1987). The mechanisms of meiosis resumption in mammalian oocytes have yet to be defined. Although both FSH and LH act through stimulatory G protein-coupled receptors (GCPRs), activation of adenylate cyclase and production of the second-messenger cyclic adenosine monophosphate (cAMP, Jonassen et al., 1982; McFarland et al., 1989), LH is believed to be the primary stimulator of meiotic maturation and ovulation in the intact animal. On the other hand, *in vitro* studies suggest that physiological concentrations of FSH are able to overcome the meiotic arrest with dibutyryl cAMP or hypoxanthine (HX) and induce the maturation of isolated cumulus-enclosed oocytes (CEOs) from medium size or large preovulatory follicles (Linder et al., 1974; Dekel et al., 1988; Downs et al., 1988; Xia et al., 1994, 2000; Tornell et al., 1995; Byskov et al., 1997; Smitz et al., 1998). On the contrary, highly purified LH or hCG (even at high concentration) has no effect at all (Andersen et al., 1999; Su et al., 1999; van Tol et al., 1996). So it is concluded that LH receptors (LHRs) are absent or expressed at very low levels on the cumulus cells surrounding the oocyte (van Tol et al., 1996; Andersen et al., 1999; Su et al., 1999).

Studies have shown tissue-specific expression of the FSH receptor (FSHR) in granulosa cells and LHR in theca cells of all growing follicles. The synergistic actions of FSH and estradiol induce LHR expression in granulosa cells of preovulatory follicles (Uilenbroek and Richards, 1979; LaBarbera and Ryan, 1981). Recent studies show that LHR is up-regulated in cumulus cells during FSH-induced meiotic maturation of CEOs (Chen et al., 1994; Shimada et al., 2003). Our previous study also demonstrated that FSH stimulation is able to increase LHR expression in cumulus cells of CEOs from no eCG-primed mice, and the induction of LHR expression precedes closely upon oocyte maturation (Fu et al., 2007). However, the functional role of synthesized LHR in cumulus cells during FSH-induced maturation of CEOs has remained unclear. Studies using

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LHR knockout (LuRKO) mice indicated that follicular development and ovulation could not be induced by high doses of FSH in the absence of LHR expression (Pakarainen et al., 2005). However, few preovulatory follicles were present in the LuRKO ovary due to lack of LH stimulation. It is therefore warranted to reassess this question in an *in vitro* model to characterize the mechanism of oocyte maturation under the influence of LH and FSH, especially, the functional role of LHR expression in cumulus cells.

In the present study, we collected CEOs from large follicle of eCG primed mice to investigate the LHR expression pattern during FSH-induced meiotic resumption of oocytes. To address the functional role of LHR expression, we used an *in vitro* antisense strategy to decrease the level of LHR protein during oocyte maturation. It has been postulated that the antisense oligodeoxynucleotide (ODN) binds specifically and efficiently to the complementary mRNA sequence and suppresses the levels of the target mRNA by triggering degradation of the RNA strand of the RNA-DNA duplex. In addition, the antisense ODNs also prevent translation of the target mRNA (Stein and Cheng, 1993; Wagner, 1994).

MATERIALS AND METHODS

Isolation and culture of cumulus-enclosed oocytes

All animal procedures were approved by the Animal Care Committee of China Agricultural University. Mice were housed under controlled temperature and light conditions and fed *ad libitum*. Immature, 22 to 24 days old Kunming White mice (outbreed strain) were injected with 5 IU of eCG (Hua Fu Medical Co., Tian Jin, China) and were killed by cervical dislocation 44 h later. The ovaries were immediately removed and placed in M199 (GIBCO-invitrogen, Carlsbad, CA, USA) medium supplemented with 4 mM hypoxanthine (HX) to maintain the oocytes in a germinal vesicle stage. CEOs with intact cumulus cell connection were isolated under a stereomicroscope by manual rupture of large follicles using a pair of 26 gauge needles, washed three times with maturation medium (M199 medium containing 4 mM HX, 3 mg/ml bovine serum albumin, 0.23 mM pyruvate, 2 mM glutamine, all from Sigma, St. Louis, MO, USA). Pools of 50-60 CEOs were plated in 0.5 ml culture medium in 24-well plates (NUNC, Roskilde, Denmark) at 37°C in a 5% CO_2 in air atmosphere.

ODNs and transfection

We designed a 20-mer antisense (5'-GCCGGGACC CGCCGCCCCAT-3') ODN from mouse LH/hCG receptor cDNA sequence (NM_013582) covering the ATG translation initiation codon. A 20-mer sense (5'-ATGGGGCGGC GGGTCCCGGC-3') ODN was used as control. To avoid degradation by exonucleases, ODNs were protected by incorporating nuclease resistant phosphorothioate bonds in the last nucleotides at the 5'- and 3'-ends, respectively. All ODNs were synthesized by Augt inc (Beijing, China), then resuspended (400 μM) in sterile water and stored at -80°C in small aliquots until used.

Transient transfections were carried out according to the manufacturer's instruction for TRANSfection Reagent (Tiangen Biotech Co., Ltd., USA). Briefly, CEOs were plated in 24-well cell culture plates and then cultured in 0.4 ml serum-free, penicillin/streptomycin-free culture medium. Preliminary experiments were performed in order to maximize transfection efficiency and minimize cytotoxicity using TRANSfection Reagent according to the manufacturer's instructions. The following parameters were optimized: ratio of ODN to TRANSfection Reagent, length of exposure of cells to complexes, and ODN concentration. To optimize the amount of ODN, various concentrations (0, 2, 4, 8 μM) of antisense and sense ODNs were added and cells were cultured for 8-16 hours. With ODN concentration and exposure time held constant, the amount of TRANSfection Reagent was varied to determine the optimal concentration. Western immunoblotting analysis was performed to determine the LHR protein level in cumulus cells transfected with either sense or antisense ODNs. Optimal condition was achieved as the following procedure. The lipid: ODN mixture was added drop-wise to a final volume of 100 μl. For each well, 5 μl ODN (at a final concentration of 4 μM) and 5 μl TRANSfection Reagent were first diluted separately in 45 μl culture medium. The 50 μl of diluted TRANSfection Reagent was incubated for 5 min at room temperature. The 50 μl of diluted ODN was incubated for 10 min at room temperature. The ODN-TRANSfection Reagent complexes were mixed and incubated at room temperature for 20 min before they were added to the wells. Control CEOs were treated as described above with the ODNs omitted. Plates were incubated in a humidified 5% CO_2 atmosphere at 37°C. At 2 h post-transfection, FSH from porcine pituitary (pFSH, Sigma, F-2293) or recombinant human FSH (rhFSH, Serono, Switzerland) were added at a final concentration of 50 U/L. Cells were harvested at different times for analysis.

Western immunoblotting

Eight hours after hormone treatment, cumulus cells were separated from 120 CEOs and washed three times in PBS. Proteins were extracted using 2×SDS sample buffer and heated to 100°C for 4 min. After cooling on ice and centrifuging at 12,000 g for 5 min at 4°C, total proteins were separated by discontinuous 10% SDS-PAGE under reducing conditions. The proteins were electro-blotted to

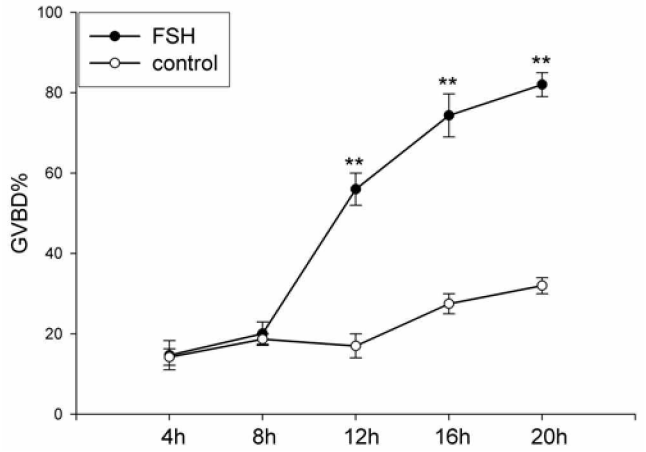

Figure 1. Time course of FSH-induced meiotic resumption *in vitro*. CEOs were incubated in maturation medium with or without pFSH (50 U/L). At the indicated times, oocytes were isolated from the CEOs and scored for GVBD. Each point is the mean±SEM of three experiments using 50 to 60 CEOs per time point. ** $p<0.01$.

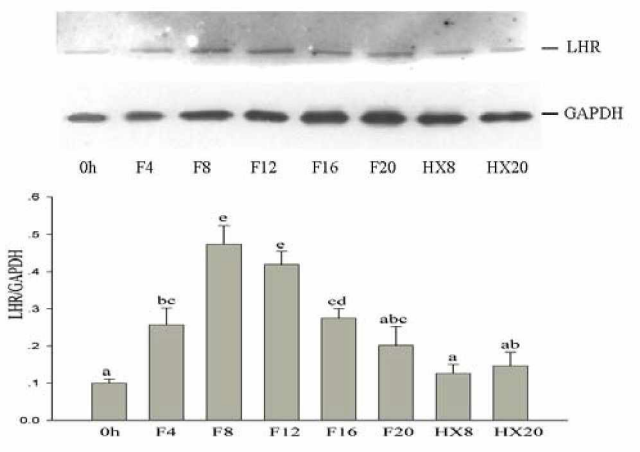

Figure 2. *In vitro* regulation of LHR expression by FSH. Western blot analysis of LHR protein expression in cumulus cells of cultured CEOs at different times after FSH stimulation *in vitro*. The ratio between the intensity of the bands is reported as the mean±SEM of the three experiments performed. Columns labeled with different letters exhibit significant difference ($p<0.05$).

nitrocellulose membranes and detected by using a 1:500 dilution of LH/hCG receptor antibody (sc-26343, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then a 1:5,000 dilution of horseradish peroxidase (HRP)-labeled second antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were also blotted with a 1:4,000 dilution of monoclonal mouse anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Standard molecular weight marker proteins were run in an adjacent lane to determine the molecular size of the proteins. Bands were visualized using chemi-luminescence (ECL, Amersham Pharmacia Biotech, Sweden) and quantified by image analysis with AlphaEaseFC Software.

Proteins of cumulus cells from 60 CEOs were treated as above for MAPK activity analysis. The phosphorylated forms of MAPK were detected using a monoclonal mouse anti-MAPK, activated ERK1/2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The second antibody used for detection of MAPK primary antibody complex was HRP-conjugated rabbit anti-mouse IgG. After the initial analysis, the membranes were washed in a stripping buffer (100 mM β-mercaptoethanol, 20% SDS and 62.5 mM Tris, pH 6.7) to remove bound antibodies and were re-probed with polyclonal rabbit anti-ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect the total amount of MAPK (phosphorylated and unphosphorylated forms). The second antibody used for the re-probing was HRP-conjugated goat anti-rabbit IgG diluted 1:5,000, and final processing was as described above. All experiments were repeated at least three times.

Statistical analysis

All the experiments were performed in triplicate and repeated at least three times on different occasions. The data presented are the means and their SEM for all values. One-way analysis of variance and Duncan's multiple range tests were used for statistical analysis of the data. All frequencies were subject to arcsine transformation before analysis. Differences were considered significant at $p<0.05$.

RESULTS

Time course of FSH-induced meiotic resumption *in vitro*

To determine the time course during FSH-induced meiotic resumption, CEOs were cultured in maturation medium with or without pFSH (50 U/L). GVBD were scored at 4, 8, 12, 16 or 20 h of culture. Compared with the control treatment, pFSH had not obvious effect on GVBD before 8 h, but significantly induced meiotic resumption after treatment for 12 h (56% vs. 17%, Figure 1), 16 h (74.4% vs. 29.5%) and 20 h (82% vs. 32%).

Time course of FSH action on LHR protein levels

Western blot analysis indicated LHR expression was induced by FSH in a time-dependent manner. While the CEOs cultured in maturation medium exhibited little LHR expression, LHR proteins obviously increased in FSH treatment as early as 4 h, peaked at 8 h, decreased at 12 h and then returned towards baseline by 20 h (Figure 2).

Antisense ODNs suppress FSH-induced LHR expression in cumulus cells

To block the expression of LHR on cumulus cells, we used phosphorothioate antisense ODNs directed against the LHR mRNA and the complementary sense ODNs as control ODNs. We found that a dose of 4 μM ODN was optimal for

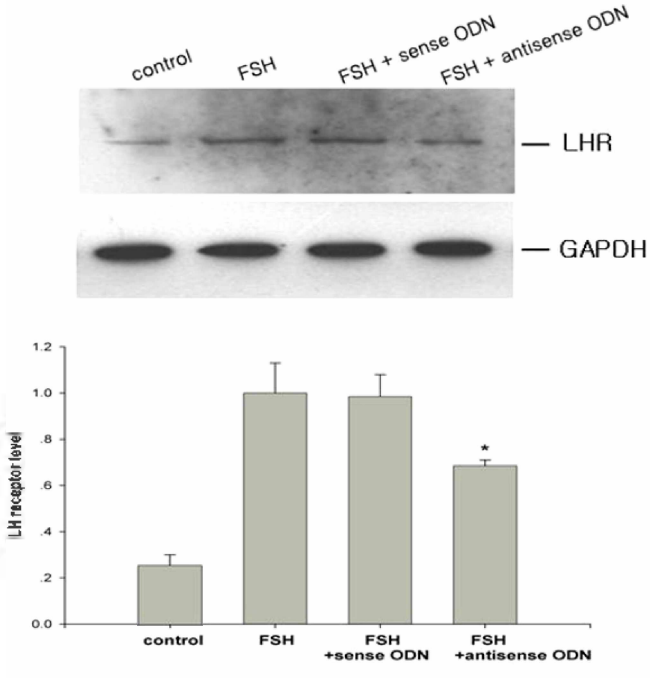

Figure 3. Effects of the antisense ODNs on LHR protein abundance. Cultured CEOs were transfected with 4 μM sense or antisense ODNs, then cultured with FSH (50 IU/L) 2 h later. Cumulus Cells were harvested 8 h after the addition of FSH. LHR expression was estimated by densitometry analysis and expressed as relative intensity (%) compared with untreated control cells. Statistical summary of the results of three individual experiments are shown, with a representative western blot using GAPDH as an internal standard. * $p<0.05$ vs. control.

suppression of LHR in cultured CEOs (as described in Methods, data not shown). CEOs isolated from eCG primed immature mice were cultured with 4 μM LHR antisense ODNs or the corresponding sense ODNs, followed by the addition of FSH (50 U/L).Twelve or sixteen hours after this procedure, the cumulus cells were collected, and whole proteins were extracted for Western blot analysis.

In the experiment described in Figure 3, the CEOs that were treated with 4 μM antisense ODNs (lane 4) exhibited significantly reduced LHR protein compared with that of FSH or the sense ODNs treatment (lane 2 and lane3, $p<0.05$, respectively). Hybridization of the blot with GAPDH displayed signals of comparable intensities in both lanes, indicating that GAPDH expression was not significantly affected by either sense or antisense LHR ODNs. These results demonstrate that pretreatment with antisense ODN results in suppression of FSH-induced LHR expression in the cumulus cells of cultured CEOs.

Antisense ODNs attenuates FSH-induced oocyte maturation *in vitro*

As indicated in Figure 4A, where porcine FSH (pFSH) was added, the percentage of oocytes remaining in the GV stage was significantly higher ($p<0.05$) in the antisense

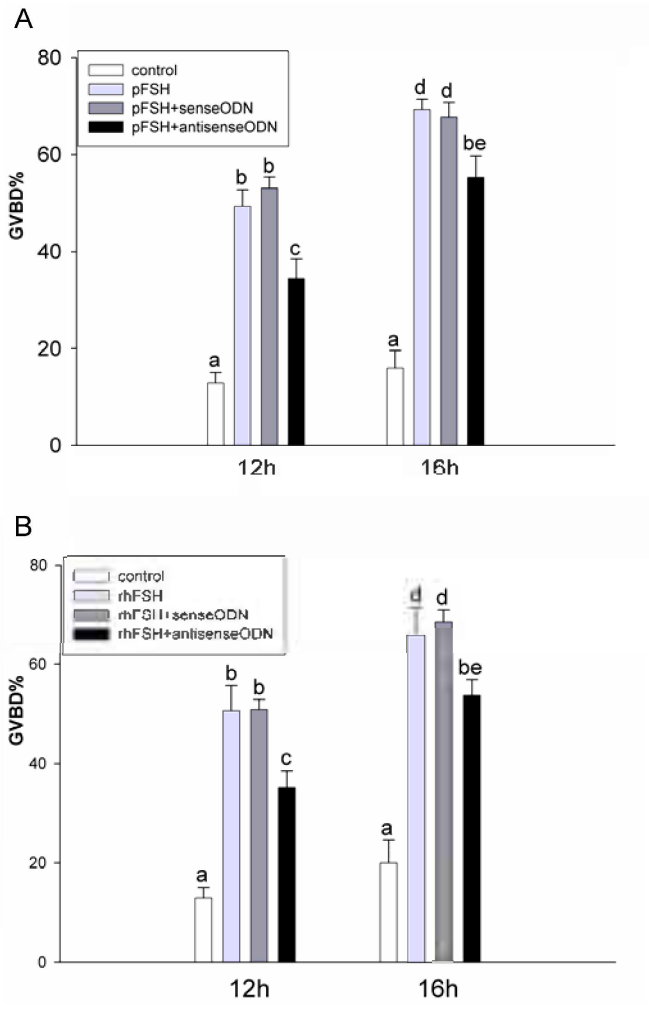

Figure 4. Effects of antisense and sense ODNs on FSH-induced oocyte maturation. CEOs were transfected with sense ODN and antisense ODN followed by culturing with 50 U/ml pFSH (A) or rhFSH (B) as described in *Material and Methods*. Columns labeled with different letters exhibit significant difference ($p<0.05$).

ODNs group (65.54%) than in control or sense ODNs groups (50.67% and 46.89%, respectively) at 12 h of culture. After 16 h of culture, the progression of meiosis to MII stage was retarded ($p<0.05$) in the antisense ODNs group (55.27%) compared with the control (69.33%), whereas there was no significant difference between the sense ODNs (67.7%) and control treatments. Similar results were obtained when porcine FSH was replaced with recombinant human FSH (rhFSH) (Figure 4B).

Antisense ODNs suppress FSH-induced MAPK phosphorylation in cumulus cells

Previous studies have showed that MAPK activity in cumulus was elevated before GVBD during *in vitro* gonadotropin-induced meiotic maturation (Su et al. 2002; Su et al., 2003, Chul-Wook et al., 2004). Interestingly, in

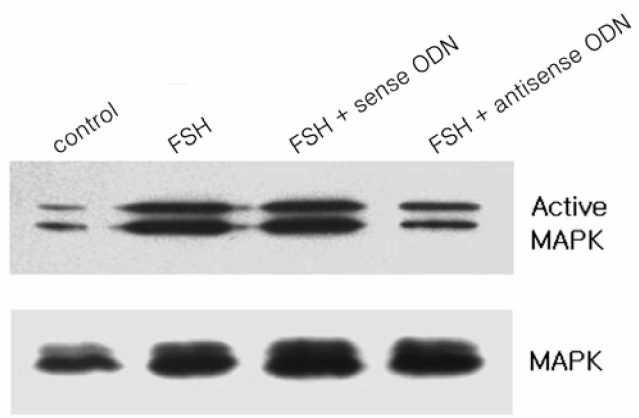

Figure 5. Effects of the antisense ODNs on MAPK activity. Cultured CEOs were transfected with 4 μM sense or antisense ODNs, preincubated for 2 h and then cultured with FSH (50 U/L) for an additional 8 h. At the end of culture, cumulus cells were harvested and homogenized for MAPK activity analysis. MAPK activity was obviously lower in the antisense ODNs group than that in FSH and sense ODNs groups. The experiment was repeated three times.

cumulus cells isolated from the CEOs treated with antisense ODNs, full phosphorylation of ERK1/2 was also obviously inhibited as observed by Western blotting (Figure 5).

DISCUSSION

Here, we reported an adoption of the inhibitory AS-ODN strategy as an alternative approach to establish a more conclusive functional study for the LHRs during meiotic maturation of oocytes, since there are no selective LHR antagonists available. The LHR protein and MAPK activity increased significantly just before FSH-induced mouse oocyte meiotic resumption, which was significantly inhibited by phosphorothioate antisense LH/hCG receptor ODN. Concomitant with this inhibition, oocyte meiotic resumption response to FSH were retarded. These results suggested that FSH-induced LHR protein expression in cumulus cells, possibly via MAPK, was involved in FSH-induced mouse meiotic resumption.

We collected CEOs from eCG-primed mice to investigate the LHR expression pattern during FSH-induced meiotic resumption of oocytes. The results showed that there was a trend towards increased LHR abundance coincident with advancing oocyte maturation, consistent with previous studies (Chen et al., 1994; Shimada et al., 2003). Using RT-PCR and *in situ* hybridization, our recent results also showed that the induction of LHR expression precedes closely upon the oocyte maturation of non-eCG primed mice (Fu et al., 2007). These results indicated that LHR expression is necessary for FSH-induced maturation of mouse oocytes.

The use of ODNs to modify gene expression is limited by their low cellular uptake and their rapid degradation by extracellular nucleases(Lezoualc'h et al., 1995). Therefore, according to previous experience, ODNs were complexed with cationic liposome carrier which can protect them from being degraded and enhance their intracellular uptake (Kerkela et al., 2002). Moreover, ODNs were rendered resistant to nucleases by introducing phosphorothioate linkages. A number of recent reviews have suggested guidelines in interpreting antisense studies (Opalinska and Gewirtz, 2002; Aboul-Fadl 2005). Using these methods, we measured LH receptor protein expression changes in response to pFSH by Western blot. In our experiments, phosphorothioate antisense, but not sense, LH/hCG receptor ODN, significantly inhibited LHR synthesis in cumulus cells. Concomitant with this inhibition, meiotic resumption response of oocytes to FSH was retarded. In order to eliminate the possibility of pFSH contamination with LH (Chen et al., 1994), rhFSH, which is devoid of any bioactivities of LH, was used and the same results were obtained. All these results suggest that LHR expressed in cumulus cells, but not LH, plays a functional role in mediating FSH-induced meiotic resumption by oocytes. A limitation of this study is the moderate effects of LHR antisense ODN on LHR protein level. Only 20-30% inhibition in this model is somewhat difficult to rationalize but it may be related to the lower cell density or low cell uptake efficiency of phosphorothioate ODN. Another reason may be that the expression level of LHR protein was constitutively elevated because of the previous administration of FSH.

Earlier studies using purified FSH preparations or recombinant FSH have suggested that FSH alone is capable of inducing oocyte maturation and ovulation in rats (Harrington et al., 1970; Nuti et al., 1974; Tsafriri et al., 1976; Galway et al., 1990) and mice (Wang and Greenwald, 1993). Recent studies using LHR-knockout mice indicate that follicular development and ovulation could not be induced by high doses of FSH in the absence of LHR expression, even if the LuRKO mice were primed with E_2 in advance (Pakarainen et al., 2005), demonstrating that functional LHR expression is essential for the follicular maturation preceding ovulation. We also showed that blocking LHR expression by antisense ODN inhibited FSH-induced maturation of mouse CEOs.

In vitro, gonadotropin-induced GVBD requires the participation of MAPK activity in the cumulus cells (Su et al., 2002). Inhibition of MAPK activation prevents gonadotropin-stimulated resumption of meiosis as well as cumulus expansion (Su et al., 2003). When activated by FSH, MAPK may stimulate the secretion of an uncharacterized factor(s) of somatic cell origin (Su et al., 1999), for example follicular fluid meiosis-activating sterol (FF-MAS), which could serve as a signal for the resumption

of meiosis (Byskov et al., 1997; Lu et al., 2000; Xie et al., 2004). In our study, FSH-induced ERK1/2 phosphorylation was obviously inhibited by LHR antisense ODN in cumulus cells. These data suggest that the inhibitory effect of ODN on FSH-induced meiotic resumption might be via the MAPK pathway.

It is puzzling that newly synthesized LHR was involved in FSH-induced meiotic maturation in the absence of any LH activity. Since the LH/CG receptor lacks binding affinity with FSH (Thomas and Segaloff, 1994), one can supposes that LH/CG receptors may be coupled to other proteins co-expressed in cumulus cells which are essential to meiotic resumption, such as FSH receptor, and these assemblies may have potential regulatory influences on the receptor function. A recent report described that hFSHR and hLHR (exon 9) can form receptor complexes and reduce the protein levels of hFSHR at the cell membrane surface (Yamashita et al., 2005), expanding the possibility of receptor heterodimerization being an important factor for gonadotropin receptors. Furthermore, the discovery of heterodimerization of GPCRs complicates the understanding of the general mechanism of GPCR modulation and function. For example, obligate heterodimerization is required for signaling via GABAB receptors (Jones et al., 1998; White et al., 1998). Another example of cross-talk between signaling pathways involving heterodimeization is provided by the observation that AT1 and AT2 angiotensin II receptor association negatively regulates signaling via AT1 receptors (AbdAlla et al., 2001). In the case of β-adrenergic receptor (βAR), the heterodimerization of β1AR and β2AR inhibits β2AR-dependent activation of the ERK1/2 MAPK signaling pathway (Lavoie et al., 2002). Although FSH and LH have divergent effects *in vivo*, they share a high degree of amino acid sequence homology between the two hormones (Pierce and Parsons, 1979; Dias et al., 2002; Fan and Hendrickson, 2005) and between their two receptors (Simoni et al., 1997; Ascoli et al., 2002; Dias et al., 2002; Vassart et al. 2004; Fan and Hendrickson, 2005). Moreover both the LH/LHR and the FSH/FSHR complexes use Gs/adenylyl cyclase/cAMP as their main signaling pathway (Simoni et al., 1997; Ascoli et al., 2002; Dias et al., 2002; Vassart et al., 2004). Based on our present results and previous results mentioned above, we propose a possible functional mechanism of LHR in meiotic regulation: FSH induced LHR expression increases and the newly synthesized LHRs forms heterodimers with other G proteins (such as FSHRs) coexpressed in cumulus cells, resulting in the activation of the ERK1/2 MAPK signaling pathway, and then the oocyte resumes meiosis.

In summary, our results suggest that depletion of LHR using antisense ODNs attenuates FSH-induced LHR protein expression and oocyte maturation and decreases MAPK phosphorylation, providing an initial indication that LHR expression in cumulus cells is required for FSH-induced maturation of the mouse oocyte. It is an important new step towards understanding the physiological actions of gonadotropins during oocyte maturation. Further work should yield useful insights into the molecular mechanisms by which LHR functions in oocyte maturation.

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