Expression and regulation of sFRP family members in human granulosa cells

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ABSTRACT: Follicular development and ovulation are major processes in the reproductive system. Understanding their complexity is important to female fertility treatments and the control of reproductive processes. Wnt signaling pathway components were shown to be involved in reproduction in animal models. The secreted frizzled-related protein-4 (sFRP4), a potential modulator of Wnt4 signaling pathway, was shown to be induced by LH in rodents and expressed in the corpus lutea, but the pattern of its expression in human ovaries remains unknown. We evaluated the expression pattern of sFRP4 and other sFRP family members in human mural and cumulus granulosa cells (GCs), as well as their regulation by LH/hCG. GCs were obtained from follicles aspirated during in vitro maturation and IVF procedures. GCs were also plated and grown in culture. We showed that the human sFRP4 expression decreases as follicles grows to the preovulatory stage and its expression was higher in cumulus GCs than in mural GCs. Interestingly, LH/hCG stimulation of GCs in vivo and in culture resulted in decreased expression of sFRP4. Of the other sFRP family members, sFRP5 expression was found in mural and cumulus GCs in vivo and was shown to be induced by LH/hCG in vitro and in vivo. In summary, sFRP4 is expressed in human GCs and its expression declines during late antral follicular growth. sFRP4 expression is also inhibited by LH/hCG, unlike its rodent homolog. In human GC, sFRP5 may substitute the role of sFRP4 in mouse GC.

Key words: granulosa cells / IVF / IVM / LH/hCG / sFRP4/sFRP5/sFRP2

Introduction

Follicular development and ovulation are complex processes that are central to the female reproductive system. Several signaling pathways were shown to be involved in follicular development and ovulation, among them the transforming growth factor family (Elvin et al., 1999), the insulin-like growth factor pathway (Adashi et al., 1985), the protein kinase C pathway (Varnold and Smith, 1990), the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase pathway (Fan et al., 2009) and the Wnt pathway (Cadigan and Nusse, 1997). The Wnt pathway is named after its ligand, the Wnt family of secreted glycoprotein ligands. Wnts act by binding frizzled (FZD) receptors, which are members of a specific class of seven pass transmembrane receptors (Bhanot et al., 1996). The Wnt pathway is implicated in many developmental processes, such as tissue differentiation, proliferation and apoptosis (Nusse and Varmus, 1992). It is modulated by endogenous secreted substances, including the members of the Dickkopf (Dkk) and secreted FZD-related protein (sFRP) families. sFRPs comprise a family of secreted glycoproteins that bind to either Wnts or FZD receptors. FZD-related proteins contain an N-terminal cysteine-rich domain of ~110 residues that is homologous to the putative ligand-binding domain of FZD proteins (Rattner et al., 1997). The exact function of sFRP4 is still unknown, although sFRPs have generally been described as Wnt antagonists.

Several components of the Wnt pathway were implicated in follicular development and ovulation (Hsieh et al., 2002). The ligand Wnt-4 has been identified as playing a key role in the development of the female reproductive system (Vainio et al., 1999). The receptors, Fzd4 and Fzd1 are expressed and induced by the LH surge (Hsieh et al., 2003), and the modulator, sFRP4, was detected in the ovary of mice (Hsieh et al., 2003) and rats (Hsieh et al., 2003), and in human serous ovarian tumors (Drake et al., 2009). In the rodent model, sFRP4 was shown

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to be induced by LH, independent of the progesterone receptor (Hsieh et al., 2003; Fan et al., 2009, 2010). It was also found to be expressed in granulosa cells (GCs) of preovulatory follicles and in corpus luteal cells (Hsieh et al., 2003). While the role of sFRP4 in ovulation is unclear, mice null for frizzled4 (Fzd4−/−) are infertile and exhibit impaired corpora lutea that suggest a putative role for sFRP4 in corpora luteal formation and function. In addition, the Wnt signaling pathway has been associated with apoptosis (Marti et al., 1999); however, Drake et al.’s (2003) examination of a possible correlation between sFRP4 expression and apoptosis in rat ovary yielded no significant results.

The aim of the study was to investigate the human mural and cumulus GC expression of sFRP4 in the developing and preovulatory follicles using in vivo GCs obtained from in vitro maturation (IVM) and IVF procedures. We also determine sFRP4 hormonal regulation using an in vitro GC model.

Materials and Methods

IVF protocol

GCs from large follicles were obtained at the time of oocyte retrieval for IVF patients. Briefly, normo-ovulatory patients (<37 years old) undergoing IVF due to male or tubal factors were selected. Treatment protocols included the ‘long protocol’ with the mid-luteal GnRH agonist (Decapeptyl 3.75 mg, Ferring), or the ‘antagonist protocol’ with the GnRH antagonist (Cetrorelix; Merck Serono or Orgalutran; Schering Plough). Ovarian stimulation by means of a daily subcutaneous (Fadini et al., 2009) dose of hMG (Menogon, Ferring) or recombinant FSH (Gonal-F, Merck-Serono or Puregon Pen, Schering-Plough) was started on the third day of the menstrual cycle. The initial dose depended on the age, BMI and treatment history. When the leading follicles reached 18 mm in diameter, the patients received hCG (Ovitrelle 250 mcg, Merck Serono, Geneva, Switzerland). Oocyte retrieval was performed by transvaginal ultrasound-guided needle aspiration.

IVM protocols

In the hormonal-stimulated protocol, normo-ovulatory patients underwent an IVM cycle according to accepted IVM protocols (Fadini et al., 2009). Briefly, a baseline evaluation that included a hormonal profile and an ultrasound scan was performed on Day 2–3 of the menstrual cycle. On day three, 150 IU/day recombinant FSH was added for 3 days. A second evaluation was performed on Day 6 of the menstrual cycle. An injection of 10 000 IU hCG (Pregnyl; Organon, Oss, Holland) was administered s.c. when the endometrial thickness was ≥5 mm and the leading follicle was at least 12 mm. Transvaginal oocyte retrieval was scheduled 36–38 h after hCG injection. The follicular fluid was collected and the follicular fluids were separated into two groups of small (4–8 mm) and large (10–14 mm) follicles.

For the non-stimulated protocol, normo-ovulatory patients who were unable to receive hormonal treatment underwent an IVM cycle without any hormonal supplementation (Fadini et al., 2009). They also underwent a baseline evaluation that included a hormonal profile and an ultrasound scan that was performed on Day 2–3 of the menstrual cycle. A second evaluation was performed on Day 6 of the menstrual cycle. Transvaginal oocyte retrieval was scheduled when the endometrial thickness was ≥5 mm and the leading follicle was at least 12 mm. The follicular fluid collection was the same as for the stimulated protocol.

The study was approved by the local Institutional Review Board committee, and written informed consent was obtained from each patient.

Mural and cumulus GC extraction and purification

Mural GCs were collected from follicular fluid under a microscope, carefully avoiding blood clots and re-suspended in IVF medium or phosphate-buffered solution (PBS). After allowing the cells to settle by gravity for few minutes, the top medium was aspirated, and this step was repeated 2–3 times until the medium became clear and the cells’ pellet was white. The cells were centrifuged at 200g for 5 min at room temperature, and the resulting pellets were re-suspended in red blood cell lysing buffer (Sigma-Aldrich, St Louis, MO). After a 1 min incubation, the mix was diluted with 12 ml PBS and the cells were centrifuged at 200g for 5 min at room temperature, after which the resulting pellets were subjected to RNA purification.

Cumulus GCs were obtained during oocyte denudation for the ICSI procedure. Briefly, after oocyte retrieval, the cumulus cells were removed from the cumulus—oocyte complex by means of hyaluronidase. The cells were centrifuged at 200g for 5 min at room temperature and the resulting pellets were subjected to RNA purification. Denuded oocytes were then injected with one sperm each. Fertilization was confirmed by the appearance of two pronuclei 24 h later. White blood cells (WBCs) of the same patients were purified from whole blood by Leucosep (Greiner Bio-One) by fractionation as described in the manufacturer’s manual.

GCs in culture

Preovulatory GCs that had been obtained from follicles during the IVF procedure as described earlier were plated into culture at a density of 50 × 10³ cells/well of 24-well plates in basic medium [10% fetal calf serum; Invitrogen, Carlsbad, CA] in Dulbecco’s modified Eagle’s medium (DMEM) with 1% penicillin/streptomycin. After 48 h, the medium was replaced with fresh basic medium with or without 1 U/ml hCG (Ovitrelle, Merck Serono). The medium was collected for hormonal measurements at different times, and the cells were collected and subjected to total RNA purification, RT and quantitative real-time PCR (qRT-PCR). Progesterone levels were measured by chemiluminescent enzyme immunoassay (Immulite 2000 Siemens Medical Solutions Diagnostics, Los Angeles, CA).

RNA extraction from GCs

Total RNA was extracted from GCs or WBCs by a Mini RNA Isolation I Kit (Zymo Research Corp, Irvine, CA), according to the manufacturer’s instructions. For RT, total RNA (100 ng) from each sample was used for cDNA synthesis using Verso™ RT–PCR Kit (Abgene Limited, Epsom, UK) according to the manufacturer’s instructions: 42°C for 30 min followed by 95°C for 2 min with random hexamer primers (25 ng/µl final) in 20 µl total volume reaction. For PCR, aliquots of the cDNA (10%), ReadyMix RedTaq PCR reactive mix (Sigma-Aldrich) and specific primers were mixed to amplify sFRP4, and β-actin served as an internal control. Amplification was performed using DNA Engine (Bio-Rad, Hercules, CA) with 50°C as annealing temperature and 25 cycles for all primer pairs (Table I).

Carry out qRT-PCR, the RT was performed separately from the PCR. An aliquot (10%) of cDNA was then subjected to 40 amplification cycles of PCR (Applied Biosystems Prism 7000 sequence detection system) using Platinum SYBR Green qPCR SuperMix-UDG w/ROX (Invitrogen). Specific primers were used to amplify sFRP4. β-Actin was included in each experiment as loading control. Cycling parameters were 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 s and at 60°C for 1 min. A melting curve analysis was performed at the end of each run to ensure a single amplicon. For primers’ sequence, see Table II. sFRP4 levels were calculated relative to the β-actin level in the same sample.
**Table I** PCR primers sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers sequence</th>
<th>Product length</th>
<th>Acc no.</th>
</tr>
</thead>
</table>
| sFRP4 | Sense 5′-GAGAGCAAGGTGAGACAGATTCC  
Antisense, 5′-CCTTTTTGGGAGAATGAATCTTT | 342            | NM_003014.3 |
| β-Actin | Sense, 5′-AGAAATCTGGGACACCCACACC  
Antisense, 5′-CCTCTTAATGTACGCAGCA | 395            | NM_001101.3 |

**Table II** Real-time PCR primers sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers sequence</th>
<th>Product length</th>
<th>Acc no.</th>
</tr>
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</table>
| sFRP4 | Sense, 5′-TGGCAACGTATCTCAAGCACA  
Antisense, 5′-GGATGGTGATGAGGACTTTG | 154            | NM_003014.3 |
| sFRP5 | Sense, 5′-ACCAAGATCTGCGCCAGGTTG  
Antisense, 5′-TCAGCTTGGGTTCCTCCACCTCT | 130            | NM_003015.3 |
| sFRP2 | Sense, 5′-AGGACAACGACCTTTGCATC  
Antisense, 5′-TTTCAGCCAGCTTCACATTC | 89             | NM_003013.2 |
| β-Actin | Sense, 5′-CAGGGAGCTTCAGCAAGAGA  
Antisense, 5′-CAGCGGAACCGCTTTCGAGCA | 117            | NM_001101.3 |

**Statistical analysis**

Paired comparisons were performed using the un-paired, two-sided Student’s t-test or analysis of variance single factor. P < 0.05 was considered statistically significant.

**Results**

To investigate the expression of sFRP4 in human GCs and its expression pattern during late follicular maturation, we analyzed sFRP4 expression in mural luteinized GCs obtained from the follicular fluid of small follicles (4–8 mm) during the IVM procedure. Figure 1A shows the expression of sFRP4 in mural GCs of small IVM follicles. Since some WBCs might be present during the isolation of GCs, we included WBC samples from the same patients to ensure that sFRP4 was not expressed in the WBCs. We also examined sFRP4 expression in three different follicle size groups: small (4–8 mm), large (10–14 mm) luteinized follicles from IVM procedures and large preovulatory (>17 mm) luteinized follicles from IVF procedures. The results (Fig. 1B) showed that the expression of sFRP4 was inversely correlated to follicular size: specifically, that mural GCs obtained from small luteinized IVM follicles expressed higher levels of sFRP4 than mural GCs obtained from large luteinized IVM follicles, and that sFRP4 expression further decreased in mural GCs obtained from preovulatory luteinized follicles.

**sFRP4 expression in cumulus GCs correlated to oocyte maturation**

To further study sFRP4 gene expression and its role in the human ova
tory cascade, we compared its expression in cumulus and mural GCs of the same patients. The sFRP4 transcript levels were significantly higher in cumulus GCs than in mural GCs (Fig. 2A). These results are in agreement with recently published microarray data (Koks et al., 2010). We also analyze the expression of other members of the sFRP family, sFRP2 and sFRP5 that were previously shown to be expressed in GCs (Hernandez-Gonzalez et al., 2006, Koks et al., 2010). Unlike sFRP4 that is expressed predominantly in cumulus GCs, sFRP5 expressed at similar levels in mural and cumulus GCs (Fig. 2A). Therefore, in cumulus GCs the sFRP4 and sFRP5 expression levels are comparable but in mural GCs, sFRP5 is the more abundant sFRP member (Fig. 2A) as was previously indicated in human beings (Koks et al., 2010). Although sFRP2 was shown to be expressed in the cumulus and induced by LH/hCG in the mouse (Hernandez-Gonzalez et al., 2006), we found that its expression in human GCs in vivo was too low to be accurately quantified.

To test whether the expression of sFRP4 in the cumulus GCs correlated to oocyte maturation, we collected cumulus GCs according to oocyte maturation as follows: germinal vesicle (GV), metaphase 1 (MI) and metaphase 2 (MII) oocytes. The levels of sFRP4 expression (Fig. 2B) were higher in the cumulus of GV oocytes than that of MI oocytes and were even lower in the cumulus of MII oocytes.

**LH/hCG-dependent expression of sFRP family members in vivo and in vitro**

To study the LH/hCG effect on sFRP family members, the in vitro murine GC culture model was used. Mural GCs obtained from IVF follicles grown in culture and then stimulated with hCG (1 U/ml) for different times. The results showed that while progesterone secretion (Fig. 3A) was induced by LH/hCG action, sFRP4 expression was decreased significantly at 8 h after stimulation and then decreased further compared with untreated cells (Fig. 3B). Similarly, the results show that sFRP2 is repressed by LH/hCG (Fig. 3C) in vitro. Interestingly, sFRP5 is induced by LH/hCG (Fig. 3D) but with different kinetics. While the effect of LH/hCG on sFRP4 and sFRP2 (data not shown) can be seen as early as 4 h, the effect on sFRP5 was shown...
only after <8 h. The slower response may suggest an indirect effect. These in vitro results imply that human sFRP4 and sFRP2 are down-regulated and sFRP5 is up-regulated by LH/hCG. Finally, to confirm the LH/hCG effect on sFRP genes, we assessed the expression of the human sFRP genes in mural GCs obtained from similar size follicles of two different IVM protocols, i.e. non-luteinized mural GCs that were obtained from IVM procedures without hCG priming, and luteinized mural GCs that were obtained from hCG-primed IVM cycles. The sFRP4 levels in the non-luteinized GCs were significantly higher than its expression in the luteinized sample. These results are expressed as mean ± SEM of five women in each group. Values with different superscripts are significantly different (P < 0.05).

Discussion

The results of this study are the first to demonstrate the expression of sFRP family members in human GCs. We showed that sFRP4 was expressed in higher levels in the earlier stages of antral follicular development and that its expression decreased as the follicles grow to the preovulatory stage. We were also able to show that this gene is expressed mainly in the cumulus in preovulatory GCs and in inverse correlation to oocyte maturity, i.e. its expression was lowest in the cumulus from MII oocytes. Interestingly, sFRP4 expression was found to be down-regulated by LH/hCG.

In contrast to our findings in the human ovary, sFRP4 had been shown by others to be up-regulated by LH in the mouse and rat ovary (Hsieh et al., 2003). Different expression patterns of genes between species were previously described for other ovarian genes [e.g. oocyte-specific homeobox gene NOBOX (Rajkovic et al., 2004; Qin et al., 2007) and activin (Richards et al., 1999; Martins da Silva et al., 2004)]. Different mRNA regulation may be due to a difference in the transcription levels (promoter) or post-transcription level (stability). To assess the possibility that the difference in sFRP4 regulation is related to the transcription level, we examined the promoter region of the human sFRP4 gene and compared it with the mouse and rat genes. We found one and two nucleotides’ substitutions in the main regulatory binding sites of the mouse and rat sFRP4 promoter, i.e., STAT3 and cAMP response element-binding protein (Wong et al., 2003; Yam et al., 2003) respectively, but the functionality of these substitutions remains to be determined. Another explanation
for our observation of sFRP4 regulation being different from that of other studies might be related to differences in the time course analysis of sFRP4 expression following LH/hCG administration. Careful examination of the previously reported LH effects over time in mice (Hsieh et al., 2003) demonstrated a complex pattern. Specifically, after a sharp induction lasting up to 4 h, sFRP4 expression declines over a period of up to 24 h and is then again induced over the ensuing 48 h. This means that sFRP4 expression is decreased for 20 of the 48 h after LH/hCG had been administered. Because of the obvious ethical limitation of obtaining human follicles, we could not perform such a systematic analysis on human samples. Therefore, although we observed only decreased expressions of sFRP4 by LH/hCG in all conditions tested, we cannot rule out the possibility of a temporary increase in sFRP4 expression. Our in vitro culture studies, however, do not support the likelihood of such an increase.

The down-regulation of sFRP4 expression in the human ovary during the late antral follicular development and the down-regulation by LH/hCG strongly suggest a role for that gene in human reproduction and ovulation. Drake et al. (2009) showed an association between sFRP4 expression and apoptosis markers at the time of ovulation (12 h post-LH/hCG) in rats. It was also suggested that sFRP4 might be involved in apoptotic processes of the corpus luteum (Drake et al., 2003). The possibility that sFRP4 participates in apoptosis was also suggested in other tissues (Marti et al., 1999). In our study, the expression pattern of elevated sFRP4 expression in human GCs of healthy antral follicles and the decrease towards the preovulatory stage suggest a different role for sRFP4 in human ovarian physiology. Interestingly, the expression in healthy follicles and the non-apoptotic-related functions were also proposed by Hsieh et al. (2003) in their study on rodents. Other functions suggested for sFRP4 was to antagonize the β-catenine suppression of LH/hCG pathway (Fan et al., 2010). Our data showing the SFRP4’s down-regulation by LH does not seem to support this mechanism. However, sFRP5, which was found to be induced by LH/hCG, may carry out this function.

Wnt signaling proteins are involved in tissue patterning (Marlow et al., 2002). At the preovulatory stages, we observed that sFRP4 was more

**Figure 3** Effect of LH/hCG on sFRP genes expression in vitro. (A–D) Mural GCs of IVF procedures were plated (50 000 cells per well of 24-well plates) in 10% FCS in DMEM with 1% penicillin/streptavidin. After 3 days, the medium was replaced with fresh medium with or without 1 U/ml hCG. At indicated times, the medium was collected and subjected to (A) progesterone measurement, and the cells were harvested and subjected to total RNA purification followed by RT and qRT-PCR. Primers used were sFRP4 (B), sFRP2 (C), sFRP5 (D) and β-actin (loading control). sFRP4 expression was calculated relative to the β-actin level in the same sample. The level in time 0 was set to 1 and the other time points were calculated relative to time 0. The graph expressed as mean ± SEM of a total of five independent experiments (*P < 0.05).
abundant in the cumulus GCs than in their mural GCs counterparts. The strong effect of LH/hCG on sFRP4 regulation and the high expression of sFRP4 in preovulatory cumulus cells might suggest a role for sFRP4 in cumulus expansion. sFRP4 was shown to be induced equally by LH/hCG in wild-type and progesterone receptor knockout mice (Hsieh et al., 2003). Therefore, sFRP4 is not a progesterone-receptor-regulated gene, and it might be regulated through the cyclooxygenase-2 (COX-2) pathway, which is essential for cumulus matrix formation (Edson et al., 2009). Finally, it was previously reported that sFRP4 expression was increased after FSH stimulation in hypophysectomized rats (Hsieh et al., 2009). Therefore, the results presented in this study document the sFRP4, sFRP5 and sFRP2 expression pattern in human GCs. They also showed that human sFRP genes are LH-dependent genes and that they might have a role in follicular development and ovulation. The implication of these findings on the Wnt signaling pathway in the ovarian tissue and the expression of sFRP family genes in the human corpus lutea still remain to be determined.

Authors’ roles

All authors made substantial contributions to conception and design, revising the article critically for important intellectual content and final approval of the version to be published.

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