FSH receptor binding inhibitor restrains ovarian cancer through down-regulating expression levels of FSHR and ERβ in mice

Zhuandi Gong¹, Suocheng Wei²*, Haoqin Liang², Luju Lai², Yingying Deng¹, Juan Yang² & Xiaoyun Shen³

¹Affiliated Hospital of Medicine College; ²College of Life Science and Engineering, Northwest Minzu University, Gansu Province, Lanzhou -730 030, People’s Republic of China
³School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang -621 010, China

Received 17 April 2018; revised 27 July 2018

Follicle stimulating hormone receptor is used as an imaging biomarker for the detection of ovarian cancer (OC). Inhibition of FSHR may attenuate the carcinogenesis particularly epithelial ovarian cancer. Here we investigated FSH receptor binding inhibitor (FRBI) effects on the follicular development, to explore their impact on expressions of FSH receptor (FSHR) and estrogen receptor β (ERβ) proteins in the ovaries. 150 female mice were assigned to FRBI+FSH (COM) group, FSH group, and control group (CG). Mice in COM-1, COM-2, and COM-3 groups were intramuscularly injected with 500, 750 and 1000 μg FRBI combined with 10 IU FSH for five consecutive days. The results showed that the numbers of secondary follicles (SF) in FSH group were increased. SF numbers of three COM groups were gradually declined as the FRBI doses rose. SF numbers of COM-2 and COM-3 groups were less than the FSH group on day 20 (P < 0.05). Ovarian cortex thicknesses (OCT) of COM-3 group was less than that FSH group (P < 0.05). Maximum longitudinal diameter (MLD) and transverse diameter (MTD) of SFs in three COM groups were dose-dependently decreased. FSHR protein levels of COM groups were significantly decreased as compared to FSH group (P < 0.05). ERβ protein levels of COM-1 and COM-2 were less than the FSH group (P < 0.05). Summarily, FRBI could reduce OCT and follicle numbers, suppress follicular development, decrease expression of ovarian ERβ and FSHR protein.

Keywords: Estrogen receptor, Follicle stimulating hormone, Follicular development, FSH receptor binding inhibitor, Mice, Ovarian cortex thicknesses, Secondary follicle

The development of ovarian follicles is precisely regulated by many factors, including follicle stimulating hormone (FSH) and other follicle-derived steroid hormones. FSH activates multiple signaling pathways to modulate genes necessary for follicular maturation. FSH acts via cognate FSH receptor (FSHR) existed on the granulosa cells of ovary follicles. FSH-FSHR interaction activates a cascade of intracellular signaling events resulting in the production of steroidogenesis. Earlier studies indicated FSHR was present at a higher level in the ovarian cancers and gynecologic malignancies. FSHR overexpression may be associated with enhanced levels of potential oncogenic pathways and increased proliferation of epithelial ovarian cancer (EOC) cells. Therefore, inhibition of FSHR overexpression may be beneficial to attenuate the carcinogenesis and progression of ovarian cancer, especially EOC.

Estrogen regulates the function of different ovarian cell types during follicular development, which is mediated by at least two Estrogen receptors (ERα and ERβ). Ovarian cancer is hormone-responsive cancer with ERs expressed in about 60-100% of ovarian cancers. Estrogen has an influence on ovarian cancer cells and it has been shown that 40-60% of ovarian cancers express estrogen receptor α (ERα). Comparing normal ovarian tissue with epithelial ovarian cancers (EOC), a loss of ERβ expression was detected. Loss of ERβ is thought to enhance ERα-mediated proliferation of hormone-dependent cancer cells. A positive correlation between ERβ expression with survival has been shown in ovarian cancer patients as well as animal models. Therefore, ERβ has been suggested to affect ovarian carcinogenesis. Currently, little documents have reported the FRBI effect on ERβ levels and E₂secretion in ovarian follicles and oophoroma.

One non-steroidal low molecular weight factor, FSH receptor binding inhibitor (FRBI), was identified from the sheep and human follicular fluid. This
FRBI, as an FSH antagonist, suppressed the binding of FSH to rat granulosa cells. FRBI blocked the binding of FSH to FSHR and altered FSH action at the receptor level\(^6\,\text{and}^22\). \textit{In vivo} administration of FRBI resulted in the suppression of ovulation and caused follicular atresia of mice\(^{21}\), further impaired the fertility in marmosets at doses of 100 μg/day and 300 μg/day\(^{22}\). Early studies reported that the progesterone (P) secretion was inhibited after rat granulosa cells were treated with FRBI in the presence or absence of FSH, thereby regulating steroidogenesis\(^{23}\). Up to date, it remains unknown whether FRBI treatment of COCs influences the levels of FSH receptor (FSHR) and estrogen receptors ER\(^\alpha\)\(^{24,25}\). Currently, little information has been recorded about FRBI effects on follicular development and ovarian tumorigenesis in human and animals\(^{5,26}\).

Based on the previous studies, we hypothesized that the FSH receptor binding inhibitor (FRBI) can impact FSH effects on the ovarian and follicular development by modulating hormone receptor expression related to ovarian cancer. The present study was undertaken to investigate FRBI effects on the follicular development and expressions of FSHR and ER\(\beta\) proteins in the ovaries, further to explore the FRBI inhibiting cancer efficacy through altering levels of FSHR and ER\(\beta\) in the ovarian follicles. We expected to find a novel anti-cancer therapeutics.

**Materials and Methods**

**Preparation of FSH receptor binding inhibitor (FRBI)**

The FSH receptor binding inhibitor (FRBI) peptide of 99.9 % purity was synthesized by Nanjing Peptide Biotech Co. Ltd., Nanjing, China (CAS: 163973-98-6) and was characterized before using it for experimental work. The homogeneity of FRBI peptide was checked by analytical RP-HPLC. The amino acid composition and the peptide content were evaluated by amino acid hydrolysis of FRBI peptide. FRBI activity was determined by the radioreceptor assay as reported earlier\(^{23}\). To prepare FRBI solution, 100 mg FRBI was dissolved in 10% dimethyl sulfoxide (DMSO), the sterile normal saline was added into the above solution to a total volume of 100 mL, the concentration of FRBI was 1000 μg/mL. The FRBI solution was kept at \(-20°C\).

**Animals and ethics statement**

One hundred and fifty Kunming female mice (\textit{Mus musculus}), 21 days old and body weight of 18.00 ± 1.23 g, were purchased from Experiment Animal Center, Lanzhou University [License No. SCXK (Gansu) 2005-0007]. All mice were randomly allocated to FRBI combining with FSH (COM), FSH and control groups (CG) (n =30). COM group was again divided into COM-1, COM-2 and COM-3 group. The mice in COM-1, COM-2, and COM-3 were intramuscularly injected with 500 μg FRBI+10 IU FHS, 750 μg FRBI+10 IU FHS, and 1000 μg FRBI+10 IU FHS once a day for five consecutive days. Mice in FSH group were intramuscularly injected with 10 IU FSH once a day for five consecutive days, which was taken as a positive group. Mice in CG were injected intramuscularly with 0.2 mL saline for five days, once a day. Injections were made in the morning (from 8 to 9 AM) each day. All mice were accurately weighed each day using an electronic balance, and raised in the group and kept in mice cages equipped with automatic water dispensers under the same conditions in the room maintained at 22-24°C and 30% to 50% relative humidity. The light cycle in the room provided 12 h light/day. Mice received a commercial diet (Lanzhou Taihua Feed Co. Ltd., China). Water was provided \textit{ad libitum}. The experiment was launched following a 7 day adjustment period of mice. All procedures referring to animal treatment were approved by the Experiment Animal Care and Use Committee of Gansu province, the People’s Republic of China.

**Measurements and sample collections**

Five mice from each group were sacrificed by cervical dislocation on days 0, 7, 10, 15, 20 and 30, respectively, after they were anesthetized by injecting intramuscularly 0.1 mg/kg xylazine. Every ovary was aseptically harvested. Ovarian weight was weighed immediately on an electronic scale. The numbers of secondary follicles (SF, 2-5 mm in diameter) were counted in each ovary under the optical microscope (Leica, Japan). The average value was calculated from the left and right ovaries. Blood samples were also collected on days 0, 7, 10, 15, 20 and 30, respectively. Serum was separated and stored at \(-20°C\).

**Histological observations and image measurement of ovaries**

Ovarian samples fixed in 10% formaldehyde were embedded with paraffin wax, sliced (5 μm), and stained with hematoxylin and eosin (H & E). The sections were observed under the light microscope (Leica, Japan). Secondary follicles were found and counted. Microscopic images of the ovaries were photographed. A total of 150 sites were measured for
each group (6 sites in each section and 5 sections in every mouse). The ovarian cortex thickness, maximum longitudinal diameter (MLD) and transverse diameter (MTD) of each secondary follicle were measured, respectively, using Images Advanced 3.2 and Image Pro Plus 2.0 (MOTIC Company, Hong Kong, China).

**Western blots of ERβ and FSHR proteins in mouse ovaries.**

To evaluate the expression levels of ERβ and FSHR proteins in ovarian tissues, Western blot was implemented referred to in our previous report. Rabbit anti-sheep FSHR and ERβ polyclonal antibodies (Sigma, 1:200) (BOSTER Biological Technology Co. Ltd, Wuhan, China) and β-actin polyclonal antibody (1:1000) were diluted and incubated at 4°C overnight, followed by 1 h incubation with the appropriate secondary antibody (1:2000). The blots were further developed using a chemiluminescence reagent (Super Signal West Pico, Rockford, IL, USA). The integral optical density (IOD) of the scanned band images was achieved by using Quantity One software (Bio-Rad Company, Hercules, CA, USA). The relative concentrations of ERβ and FSHR proteins were presented as the ratio between gray values of ERβ and FSHR divided by that of β-actin. Assays were executed in triplicate.

**Measurement of serum concentrations of E2 and FSH**

In order to access the FRBI impacts on the secretory functions in mice, the serum concentrations of estradiol (E2) and FSH were measured with especial E2 and FSH kit for mice (ELISA) according to the manufacturer’s instructions (Cusabio Biotech Co., Ltd. Wuhan, People’s Republic of China), respectively. Analytical sensitivities were 0.01 ng/mL for FSH, 0.02 pg/mL for E2. All samples were tested in duplicate in the same assay. The detailed operation steps were presented in our initial research.

**Pearson correlation analysis**

Pearson correlation analysis was used to determine relationships between FRBI doses and other indexes in CG and three COM groups on day 20.

**Data statistical analyses**

Statistical analysis was done using SPSS v. 21.0 (SPSS Inc. Chicago, IL, USA). For each group, all the parameters described above were calculated on the basis of the data of 5 mice in each subgroup, respectively. Data are presented as means ± SD. After a square root transformation of the data, all variables of the three groups complied with the assumptions for a one-way ANOVA. When significant differences were identified, supplementary Turkey’s post-hoc tests were conducted to investigate pair wise differences. *P* <0.05 was considered significant.

**Results**

**Ovarian cortex thickness (OCT)**

As shown in (Fig. 1), the OCT of FSH-treated group was increased as compared to CG. The OCTs of COM groups were reduced in comparison with CG and FHS group. OCT of the COM-3 group was less than that FSH group on days 20 and 30 (*P* <0.05). The results demonstrated that FRBI could block FSH action on and suppress on ovarian development of mice.

![Fig. 1 — Ovarian cortex thickness (OCT) of mice (×100). Note: Administration of 0 μg/mL FRBI was used as the blank control group (CG). 10 IU/mL FSH treatment was taken as a positive control (FSH group). *P* <0.05 as compared to FSH group](image-url)
Histology structures of follicles

To determine the effects of FRBI regulating FHS function on follicular development, the ovarian structure was observed under a microscope (×100). Histological changes on day 20 were described in all groups as followings.

Control group (CG)
The primordial follicles (POF) and primary follicles (PF) were small. A few of the secondary follicles (SF) and mature follicles (MF) existed. The structures of the ovaries and follicles were complete. The zona pellucida (ZP) was clear. (Fig. 2A)

FSH group
The rich SFs and MFs were distributed. POF and PF became larger, and SF numbers were obviously increased in comparison with CG (Fig. 2B). The follicular antrum was formed in many MFs. Dense granular layers distributed over SF. Numbers of POF and PF were reduced in comparison with CG. Follicles developed fully.

The structures of the ovaries and follicles were complete. Fewer numbers of SF and MF were distributed on the ovaries as compared to FSH group. But the numbers of SF in the COM-1 group were more plentiful in comparison with CG (Fig. 2C). Follicles developed poorly in the COM-3 group (Fig. 2D & E).

These results showed FSH treatment could enhance follicle development, increase the numbers of SF and MF. FRBI administration in the presence of FSH down-regulated the FSH promoting effect on ovarian follicles.

Numbers of secondary follicles of mouse ovaries
Data in (Table 1) showed the numbers of secondary follicles (SF) in FSH group was increased when

Table 1 — Numbers of secondary follicles in different times

<table>
<thead>
<tr>
<th>Group</th>
<th>0 Days</th>
<th>7 Days</th>
<th>10 Days</th>
<th>15 Days</th>
<th>20 Days</th>
<th>30 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>3.83±0.38</td>
<td>4.50±0.42</td>
<td>4.96±0.43</td>
<td>5.21±0.49</td>
<td>5.65±0.51</td>
<td>6.50±0.59</td>
</tr>
<tr>
<td>FSH</td>
<td>3.84±0.33</td>
<td>5.64±0.56</td>
<td>6.27±0.59</td>
<td>7.01±0.63*</td>
<td>9.50±0.52**</td>
<td>7.63±0.62</td>
</tr>
<tr>
<td>COM-1</td>
<td>3.81±0.35</td>
<td>4.62±0.40</td>
<td>5.32±0.57</td>
<td>6.03±0.64</td>
<td>7.02±0.77#</td>
<td>6.86±0.71</td>
</tr>
<tr>
<td>COM-2</td>
<td>3.82±0.35</td>
<td>4.35±0.41</td>
<td>4.97±0.46</td>
<td>5.68±0.61*</td>
<td>6.29±0.69#</td>
<td>6.03±0.68</td>
</tr>
<tr>
<td>COM-3</td>
<td>3.81±0.32</td>
<td>4.06±0.37</td>
<td>4.30±0.42</td>
<td>4.97±0.51*</td>
<td>5.11±0.56**</td>
<td>5.97±0.40</td>
</tr>
</tbody>
</table>

Note: *P <0.05 when compared to control group (CG). **P <0.05 when compared to FSH group.
#P <0.05 when compared to FSH group.
compared to CG, reaching a maximum value on day 20 ($P < 0.01$). In COM groups, SF numbers of COM-1 and COM-2 groups were slightly greater than that of CG during the experiment. However, SF numbers of three COM groups were gradually declined as the FRBI doses rose in comparison to FSH group. SF numbers of COM-3 groups were significantly less than that of the FSH group after 15 days of the first FRBI injection ($P < 0.05$ or $P < 0.01$). The results indicated that FRBI could attenuate FSH promoting effect on the follicular development.

**Longitudinal diameter (MLD) and transverse diameter (MTD) of secondary follicles**

The maximum longitudinal diameter (MLD) and transverse diameter (MTD) of secondary follicles were measured under the microscope (×100). During the whole experiment, MLD and MTD of FSH-treated groups were slightly increased as compared to CG. MLD and MTD of three COM groups were dose-dependently decreased in comparison with the FSH group (Fig. 3). The MTD of COM-3 group was significantly less than that of the FSH group on day 30. The results demonstrated that a high dose of FRBI (1000 μg) treatment blocked the follicle development of mice and down-regulate FSH action on follicular development.

**Expression levels of ERβ and FSHR proteins in ovaries**

In comparison with CG, ERβ and FSHR protein levels were increased in the FSH group from day 7 afterward of the first FSH administration (Fig. 4). ERβ and FSHR protein levels of three COM groups were gradually reduced with a maximum reduction of COM-3. ERβ protein levels of COM-1 and COM-2 were less than the FSH group ($P < 0.05$). FSHR protein levels of COM groups were significantly decreased as compared to FSH group ($P < 0.05$). In addition, the FSHR level of the COM-3 group was lower than CG from day 15 later ($P < 0.05$). The results exhibited that administration of FRBI suppressed the expression of ERβ and FSHR protein in the ovaries of mice.

**Serum concentrations of FSH and estradiol (E₂)**

Serum FSH concentrations of all mice rose gradually (Fig. 5). Serum FSH concentration of FSH-treated mice was higher than that of CG ($P < 0.05$) on days 20 and 30. FSH contents of three COM groups were less than that of FSH group.

E₂ concentrations of three COM groups were lower than that of FSH group (Data omitted). There was a significant reduction of E₂ concentrations of COM-2 and COM-3 on days 20

---

**Fig. 3** — Maximum transverse diameter (MTD) of secondary follicles (×100). [MTD of three COM groups was decreased. On the contrary, the MTD of FSH group was increased in comparison to CG]

**Fig. 4** — Levels of ERβ and FSHR protein of mouse ovaries. In comparison with CG, ERβ and FSHR protein levels were increased in FSH mice. ERβ protein levels of COM-1 and COM-2 were less than the FSH group on days 20 and 30. FSHR protein levels of COM groups were significantly decreased as compared to FSH group. FSHR protein level of the COM-3 group was lower than CG on day 15 later. *$P < 0.05$ as compared to control group (CG); **$P < 0.05$ as compared to FSH group
and 30 (Fig. 5). The findings indicated that 1000 μg of FRBI administration could decrease the promoting secretory effects of FSH on FSH and E₂ in mice.

**Pearson correlation analysis**

As listed in (Table 2), the Pearson correlation analyses in CG and three COM groups showed that FRBI administration doses had significant negative correlations to OCT, MLD and MTD (P <0.05). MLD had positive correlations with MTD (P <0.05). The findings indicated administration of FRBI had obvious effects on ovarian and follicular development.

**Discussion**

The development and growth of ovarian follicles are precisely regulated by many hormones, such as FSH¹². FSH induces oocyte maturation via binding its cognate FSHR that is mainly expressed by granulosa cells in follicle⁶.

FSH-receptor binding inhibitor (FRBI) influences the efficacy of FSH²² by blocking the binding of FSH to FSHR in the presence of FSH²⁴. The interaction blockage of FSH-FSHR resulted in the decline of FSH action². Administration of FRBI resulted in the suppression of ovulation of mice²¹,²⁸ and further impaired the proliferation of granulosa cells. Currently, little information has been documented regarding FRBI influences on follicular development and reproduction functions in human and animals⁵,²⁶.

In the present study, varying doses of FRBI were administrated in female mice, so as to assess FRBI effects on ovarian and follicular development. OCT of the FSH-treated group was increased as compared to CG. The OCTs of COM groups were reduced in comparison with CG and FHS group. The numbers of secondary follicles (SF) in FSH group were increased when compared to CG, reaching a maximum value on day 20 (P <0.01). However, SF numbers of three COM groups were gradually declined as the FRBI doses rose in comparison to FSH group. The results indicated that FSH increased transverse and longitudinal diameters of follicles (MLD and MTD), and enhanced follicular development. As compared to CG and FSH group, a higher dose of FRBI (1000 μg) reduced ovarian cortex thickness (OCT), numbers of ovarian secondary and maturation follicles (SF and MF), decreased dose-dependently MLD and MTD in the presence of FSH, resulting in the poor development of follicles. Additionally, the Pearson correlation analyses showed that FRBI administration doses had significant negative correlations to OCT, MLD, and MTD. Therefore, FRBI down-regulated the promoting effect of FSH on ovarian follicle development. These findings were in agreement with earlier reports²⁴,²⁸. Our findings have to be further investigated in the future. The mechanism still needs a thorough exploration.

A previous study indicated that significantly lower ERβ1 expression was detected in high-grade cancers. Higher nuclear (n) ERβ5 and lower cytoplasmic (c) ERβ5 expressions were associated with ovarian clear
cell subtypes and overall survival. Overexpression of ERβ inhibited growth and motility of ovarian cancer cells and induced apoptosis since ERβ overexpression enhanced ovarian cancer cell migration, invasion, and proliferation via FAK/c-Src activation. The nERβ was a potential prognostic marker and therapeutic target in ovarian cancer. However, little is known about the molecular mechanisms of ERβ function in ovaries and ovarian cancers. The role of ERβ as a tumor suppressor and the expression decrease in ovarian cancer cells raises the question, whether ERβ expression in ovarian cancer cells might be high enough to make this receptor a potential target in ovarian cancer therapy.

Estrogen exerts its function via binding to at least two cognate estrogen receptors (ERα and ERβ) which depended on their main mode of signaling. Estrogen receptor β (ERβ) is up-regulated only in a few cell types during the late follicular phase. Estradiol (E2) activates the growing large ovarian follicles and promotes follicular growth and differentiation. E2 promotes cell cycle progression of healthy follicles. Treatment of granulosa cells with FRBI inhibited FSH action and progesterone secretion, then hampered the growth and differentiation of granulosa cells. Up to date, little information has been documented about FRBI effect on ERβ levels and E2 secretion in ovarian follicles.

ERβ, being the predominant type of ER in normal ovary tissue, has not only been linked with the pathogenesis of ovarian cancer but also with a response to treatment. Unlike ERα, which is primarily linked with cell growth, ERβ presence is prominent in signaling pathways, cell cycle regulation and apoptosis. ERβ and its sub-cellular localization seem to play a crucial role in the pathogenesis of ovarian cancer. Studies on the exact roles of ERβ in altering effectiveness and toxicity of ovarian cancer treatment regimens are lack.

In the present work, ERβ and FSHR protein levels of COM groups were gradually reduced after the administration of FSH combined with FRBI at different doses. The maximum reduction was found in the COM-3 group. The actual effects have to be testified in the other animals. The action mechanism needs to be thoroughly elucidated.

E2 concentrations were generally increased in ovarian cancer patients. Estrogens levels are at least 100- to 1000-fold higher than normal circulating levels. 17β-estradiol promotes ovarian cancer cell migration.

Our results also showed that E2 concentrations of COM-2 and COM-3 were significantly reduced on days 20 and 30 in comparison to FSH group. FRBI administration could decrease secretion of FSH and E2 of mice. FRBI could decline FSH regulatory action on the secretion of reproductive hormones. These findings were consistent with the previous document.

Conclusion

Our results revealed that FRBI administration in the presence of FSH could reduce ovarian cortex thickness and follicle numbers, suppress follicular development, decrease expression of ERβ and FSHR protein in the ovaries, additionally decline secretion of FSH and E2 of mice. FRBI down-regulate FSH action on follicular development. Taken together, our study provides a scientific basis for thoroughly elucidating the mechanism of FRBI.

These findings, for the first time, contribute to regulating ovarian and follicular functions, and further to treat diseases of the ovaries as well as to promote fertility of human and animals.

References


