Abstract. Previously, we demonstrated that follicle stimulating hormone (FSH) enhanced VEGF expression and facilitated ovarian cancer angiogenesis via the PI3K/AKT signaling pathway. In this study, we further investigated the involvement of microRNA-27a: ZBTB10-specificity protein pathway in the mechanism of FSH-induced VEGF, Cox2 and survivin expression. Treatment with FSH resulted in significant increase in the expression of VEGF, Cox2, survivin, Sp1 proteins and microRNA-27a in a dose-dependent manner, whereas reverse protein expression pattern was observed in ZBTB10. Downregulation of microRNA-27a using antisense microRNA-27a blocked FSH-induced VEGF, Cox2 and survivin expression. Overexpression of ZBTB10 also attenuated the FSH-induced expression of these molecules. The enhanced expression of VEGF, Cox2 and survivin was also abolished by knocking down Sp1 using small interfering RNA. Collectively, these results indicated that stimulation of ovarian cancer cell VEGF, Cox2 and survivin expression by FSH involves the microRNA-27a: ZBTB10-specificity protein pathway.

Introduction

Ovarian cancer is the most lethal gynecological malignancy in women because of occult metastases within the peritoneal cavity and the advanced stage at detection when curative therapy is ineffective. Approximately 80-90% ovarian cancer is origin from ovarian surface epithelium. The etiology of ovarian epithelial cancer (OEC) remains to be clarified, multiple factors involved in OEC development, for example, hormonal, environmental and genetic factors may play a role. Currently, the gonadotropin theory of ovarian cancer proposes that elevated serum gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), contribute significantly to the development of ovarian cancer. In previous study, Choi et al have reported that FSH enhanced ovarian cancer cells proliferation and invasion by PI3K/AKT signal pathway (1,2). Recent study in our laboratory demonstrated that FSH inhibits ovarian cancer cell apoptosis by upregulating survivin and downregulating PDCD6 and DR5 (3). These studies indicate FSH plays an important role in OEC occurrence, especially in postmenopausal women. In our previous work, it was also reported that activation of the PI3K/AKT pathway mediates FSH-stimulated VEGF expression in ovarian serous cystadenocarcinoma (4). This study also showed that survivin and HIF1α are involved in FSH-mediated VEGF expression by PI3K/AKT signal pathway. Survivin is a member of the inhibitor of apoptosis protein (IAP) family, in addition to the anti-apoptosis function, the role of survivin to regulate VEGF expression has been described in various tumor cell types (5,6). These factors contribute to tumor angiogenesis. Inflammation also facilitate tumor angiogenesis, Cox2 is an important effector molecule of inflammation and was reported to be involve in VEGF expression and tumor angiogenesis. In our recent study, it was found FSH could significantly upregulate Cox2 expression in a dose-dependent manner (unpublish data). Although these molecules contributing to tumor angiogenesis have positive response to FSH treatment, the detail mechanism and relative signal pathway is not clear.

Mertens-Talcott et al showed evidence that microRNA-27a: ZBTB10-specificity protein pathway contributed to breast cancer angiogenesis, decreased microRNA-27a resulted...
in attenuating expression of survivin and VEGF, whereas overexpression of ZBTB10 reduced the protein levels of both molecules (7). MicroRNAs are endogenous 20-25 bp small non-coding RNAs that interact with complementary binding sites in 3’-untranslated regions of target mRNA to inhibit their expression by blocking translation or enhance mRNA cleavage (8), and play essential roles in a variety of cellular processes, including cell differentiation, proliferation and fat metabolism (9-13). MicroRNA-27a also possesses these oncogenic activities. In addition, microRNA-27a was demonstrated to modulate the cardiac β-myosin heavy chain gene via thyroid hormone signaling (14). Li et al confirmed that antisense microRNA-27a and overexpression of ZBTB10 blocked estrogen-induced transactivation in breast cancer (15). These studies suggest that microRNA-27a: ZBTB10-specificity protein pathway mediates hormone-induced bio-function. However, the role of this pathway in expression of FSH-induced OEC angiogenesis related molecules has not been addressed. Therefore, in this study, we focused on the roles of microRNA-27a, ZBTB10 and Sp1 in mediating FSH-induced VEGF, Cox2 and survivin expressions.

Materials and methods

Chemicals, antibodies, plasmids and reagents. Lipofectamine 2000, DMEM/F12 medium and fetal bovine serum were purchased from Invitrogen. Human follicle stimulating hormone was obtained from Sigma-Aldrich. ZBTB10, SP1, Cox2, survivin, VEGF, GAPDH and β-actin antibodies were purchased from Abcam (Cambridge, UK). MicroRNA mirvRNA extraction kits, the reverse transcription and real-time PCR amplification kits were obtained from Applied Biosciences. As-microRNA-27a (as-miR-27a) was purchased from Applied Biosciences. ZBTB10 expression plasmid and empty plasmid (pCMV6-XL4) were get from Origene. Sp1 siRNA was obtained from Dharmacon.

Cell lines and cell culture. Human ovarian cancer cell lines, A2780, OVCAR-3, ES-2, HO8910PM, Hey and HO8910, were obtained from the American Type Culture Collection (Manassas, VA) and cultured in 1:1 DMEM/F12. These cell lines were cultured in medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum. Cultures were maintained at 37°C in a humidified incubator containing 95% room air and 5% CO2 atmosphere. In addition, the Moody cell line was kindly provided by Dr W. Zheng (Arizona University, Tucson, AZ, USA), it is a normal ovarian epithelial cell line that was transfected with hTERT. The cells were maintained in MCDB109/M199 medium supplemented with 15% FBS.

Hormone treatment. Ovarian cancer Hey and HO8910 cells were plated in 6-well plates with a cell density of 1x10^4 cells/ml, respectively. At 60% confluence, the media were changed to Opti-MEM without serum, starvation for 24 h, the cells were treated with different doses of FSH (0, 25, 50 and 100 mIU/ml) for 24 h, the cells were harvested and used for determining the effect of FSH on microRNA-27a expression by real-time PCR. Similarly, both cell lines were treated with different doses of FSH for 48 h, following the cells were collected for western blot analysis to examine the expression patterns of Cox2, survivin and VEGF protein. To investigate the effects of as-microRNA-27a on FSH-induced VEGF, Cox2 and survivin, prior to 50 mIU/ml FSH treatment, Hey and HO8910 cells were treated with as-microRNA-27a, the cells were harvested and the microRNA27a expression was examined by real-time PCR, checked the proteins expression by western blot analysis. In addition, in order to investigate the roles of ZBTB10 and Sp1 on FSH-induced VEGF, Cox2 and survivin expressions, after overexpression of ZBTB10 plasmid or knockdown Sp1, 50 mIU/ml FSH was used to treat the cells for another 48 h, the different expression profiles of VEGF, Cox2 and survivin were analyzed by western blot analysis.

Western blot analysis. The western blot analysis was performed as previously reported. Briefly, after lysis, 60 µg proteins were loaded on 10% SDS-PAGE gels, transferred to polyvinylidene fluoride (PVDF) membranes, and incubated with specific primary antibodies at 4°C overnight, followed by 1-h incubation with the appropriate secondary antibody at room temperature, then the bands were visualized with the ECL Plus system (Amersham, GE Healthcare). GAPDH or β-actin served as a loading control.

Transfection with as-miR-27a and real-time PCR. Transfection with as-miR-27a was done using siPORT™ NeoFX™ Transfection Agent (Invitrogen) according the manufacturer's instruction. After transfection, the treated cells were harvested and the microRNA was extracted using Taqman microRNA RT kit, followed by amplification using Taqman universal PCR Master mix.

Sp1 siRNA and ZBTB10 plasmid transfections. siRNA transfections were performed as previously reported, briefly, 1x10^6 cells were seeded in 6-cm dishes and incubated for 24 h, the culture mediums were changed with opti-MEM without serum prior to transfection with 50 nM Sp1 siRNA. After 12 h, the cells were treated with 50 mIU/ml FSH or PBS for another 48 h.

ZBTB10 plasmid transfection was carried out using Lipofectamine 2000 reagent following the manufacturer's protocol. Briefly, after serum starvation for 24 h, 4 µg plasmid or empty plasmid were mixed with serum-free DMEM/F12 and Lipofectamine 2000 reagent, respectively, following incubated in room temperature for 25 min, then added to the ovarian cancer cells.

Data analysis. Data are presented as the mean ± standard deviation (SD). The statistical significance of the results was assessed by the Student's t-test or a one way ANOVA using SPSS 11.5 software with p<0.05 being considered significant.

Results

The expression patterns of microRNA-27a and Sp1 in normal ovarian epithelial cells and ovarian cancer cells. To examine the possible contribution of microRNA-27a:ZBTB10-specificity protein pathway on ovarian cancer development, we evaluated the expression profiles of microRNA-27a and Sp1 proteins. As illustrated in Fig. 1A, microRNA-27a was overexpressed in
ovarian cancer cells compared with that of Moody cells, which is a normal ovarian epithelial cell line, transfected with hTERT, suggesting that microRNA-27a may play an important role in ovarian cancer development. Similar expression pattern was observed in Sp1, the Sp1 protein levels were higher in ovarian cancer cells than that in Moody cells (Fig. 1B).

**FSH activates microRNA-27a: ZBTB10-specificity protein pathway and induces tumor angiogenesis factor expression.**

In a previous study, we demonstrated that FSH enhanced VEGF expression and promoted angiogenesis (4), however, the detail signal pathway involved in FSH regulation of VEGF and angiogenesis remains to be clarified. Considering that microRNA-27a and Sp1 were overexpressed in ovarian cancer, we further determined whether FSH activated the microRNA-27a: ZBTB10-specificity protein pathway. As showed in Fig. 2A, FSH potently enhanced microRNA-27a expression in both Hey and HO8910 cell lines in a dose-dependent manner, the maximal peak of increase of microRNA-27a was observed when the cells were treated with 50 mIU/ml FSH for 24 h. Moreover, western blot analysis demonstrated that FSH treatment resulted in significantly increased expression of survivin, Cox2, VEGF and Sp1 (Fig. 2B and C), on the contrary, it showed a reverse expression pattern in ZBTB10 protein, decreasing ZBTB10 protein was observed accompanying the increasing dose of FSH (Fig. 2C).

**Inhibition of microRNA-27a blocks FSH-activating microRNA-27a: ZBTB10-specificity protein pathway and abolishes FSH-induced tumor angiogenesis factor expression.** To investigate the role of microRNA-27a on FSH-induced tumor angiogenesis factor expression, inhibition of microRNA-27a was performed using as-miR-27a. As showed in Fig. 3A, transfection of as-miR-27a obviously decreased micro-27a expression in both Hey and HO8910 cell lines. As illustrated in Fig. 3B, transfection of as-miR-27a induced approximately a 2-fold (Hey) or 1.5-fold (HO8910) increase of ZBTB10, the effect was abolished by 50 mIU/ml FSH treatment for 48 h. However, transfection of as-miR-27a resulted in a 10-16-fold decrease of Sp1, 7-9-fold decrease of Cox2, 2-3-fold decrease of survivin, 2-3-fold decrease of VEGF. Moreover, the FSH-induced Sp1, Cox2, survivin and VEGF expression were blocked by transfection of as-miR-27a (Fig. 3B and C).

**Overexpression of ZBTB10 blocks microRNA-27a: ZBTB10-specificity protein pathway and FSH induces tumor angiogenesis factor expression.** To analyse the role of ZBTB10 on FSH-induced tumor angiogenesis factor expression, overexpression of ZBTB10 was performed. As showed in Fig. 4A, transfection with ZBTB10 significantly enhanced ZBTB10 protein expression, whereas FSH treatment attenuated this effect. As expected, overexpression of ZBTB10 potently inhibited Sp1, survivin, Cox2 and VEGF expression in both Hey and HO8910 cell lines, moreover, the increased expression induced by FSH also was attenuated (Fig. 4A and B).

**Knockdown of Sp1 abolishes microRNA-27a: ZBTB10-specificity protein pathway and FSH induces tumor angiogenesis factor expression.** To test whether Sp1 is involved in FSH-induced tumor angiogenesis factor expression, knockdown of Sp1 was performed by siRNA. Transient transfection of Sp1 siRNA obviously blocked Sp1 protein expression in both Hey and HO8910 cell lines (Fig. 5A). In addition, it resulted in decreasing of Cox2, survivin and VEGF levels. Moreover, FSH-induced Cox2, survivin and VEGF expressions were abolished by knocking down siSp1 (Fig. 5B).

**Discussion**

Based on the gonadotrophin theory, the hormone environment is an important factor for ovarian cancer occurrence, especially in postmenopausal women due to increased FSH and LH levels resulting from loss of feedback of estrogen. Currently, aberrant FSH level is considered as a high risk factor for OEC development. Except for enhancing OEC cell proliferation, migration and invasion, and blocking of apoptosis, increasing evidence indicates that FSH induces angiogenesis which is a pivotal step in ovarian cancer development, growth, and invasion beyond the regional border (4,16,17). VEGF is a glycoprotein...
Figure 2. FSH activates miRNA-27a: ZBTB10-specificity protein pathway and upregulates VEGF, Cox2 and survivin. (A) FSH induced microRNA-27a expression in a dose-dependent manner, and was determined by real-time PCR in both Hey and HO8910 cells. (B) The protein levels of VEGF, Cox2 and survivin were measured by western blot analysis after treatment with various doses of FSH. (C) The expression patterns of ZBTB10 and Sp1 were detected by western blot analysis after treatment with various doses of FSH.
Figure 3. Involvement of microRNA-27a (miR-27a) in FSH-induced VEGF, Cox2 and survivin expression via ZBTB10 and Sp1. (A) Inhibition of transfection of as-miR-27a-induced microRNA-27a expression was measured by real-time PCR assay. The effects of the decrease of microRNA-27a plus 50 mIU/ml FSH treatment on (B) ZBTB10 and Sp1, (C) VEGF, Cox2 and survivin were determined by western blot analysis. Each experiment was repeated three times. \( p < 0.05 \) compared with control group (no as-miR-27a and no FSH treatment); \( p < 0.05 \) compared with FSH-treated group. The left column presents the results of Hey cell line, the right presents the results of HO8910 cells.
which is associated with tumor angiogenesis, higher levels of VEGF are consistent with ovarian cancer poor prognosis and tumor progression (18). In our previous study, it was found that FSH enhanced VEGF expression mediated by survivin

Figure 4. Overexpression of ZBTB10 blocks FSH-induced VEGF, Cox2 and survivin expression. Hey (left column) and HO8910 (right column) cells were transfected with ZBTB10 expression plasmid and empty vector using Lipofectamine 2000, respectively. After transfection for 12 h, the cells were treated with PBS or 50 mIU/ml FSH for another 48 h, western blot analysis was performed to measure (A) ZBTB10, Sp1 and (B) VEGF, Cox2 and survivin expression. *p<0.05 compared with control group (no ZBTB10 expression plasmid transfection and no FSH treatment); †p<0.05 compared with ZBTB10-overexpression cells.
and HIF1α via PI3K/AKT signal pathway (4). In this study, we showed the evidence that FSH upregulated not only VEGF, but also Cox2 and survivin in a dose-dependent manner. Cox2 plays an important role in regulating VEGF expression, several studies demonstrated that Cox2-mediated VEGF expression might contribute to tumor metastasis via lymphangiogenesis or angiogenesis pathways (19-23). These results imply that overexpression of Cox2, survivin and VEGF facilitates tumor angiogenesis. Although there are positive response between

Figure 5. Involvement of Sp1 in FSH-induced VEGF, Cox2 and survivin expression. Hey (left column) and HO8910 (right column) cells were transfected with siSp1 and siCon, respectively, after transfection for 12 h, the cells were treated with PBS or 50 mIU/ml FSH for another 48 h, western blot analysis was performed to measure (A) Sp1 and (B) VEGF, Cox2 and survivin expression. *p<0.05 compared with siCon group; *p<0.05 compared with the group treated with siCon plus 50 mIU/ml FSH.

Figure 6. Proposed signal pathways involved in FSH-induced angiogenesis. FSH may enhance VEGF, Cox2 and survivin expression through activation of the microRNA-27a: ZBTB10-Sp1 signal pathway, further induces ovarian tumor angiogenesis.
these three molecule expressions and FSH treatment, the detail mechanism and signal pathway are not clear.

In the current study, it was found that microRNA-27a is required for FSH-induced VEGF, Cox2 and survivin expression. MicroRNA-27a possesses oncogenic activity, it is involved in cancer development in various tumor types, such as breast and ovarian cancer (7,24). It was found that microRNA-27a overexpressed in several ovarian cancer cell lines, compared with the Moody cell line, a normal ovarian epithelial cell line, which suggests that microRNA-27a is functional in OEC occurrence. In addition, obvious increase in microRNA-27a expression pattern was identified in the present study together with increased dose of FSH. We found that transfection of as-microRNA-27a reduced Cox2, survivin and VEGF expressions, moreover, the induced expressions of the three molecules by FSH were decreased by microRNA-27a, which indicates microRNA-27a contributes to FSH-induced ovarian cancer angiogenesis.

Under rich FSH conditions, reduced ZBTB10 expression was observed. This putative zinc finger protein suppresses specificity protein (Sp) transcription factors and Sp-dependent gene expression (7,25). It was reported that microRNA-27a targets ZBTB10 gene in breast cancer and regulates cell proliferation (7). Our data clearly showed that transfection of as-microRNA-27a induced ZBTB10 expression, the enhanced effect attenuated inhibition of FSH-induced ZBTB10 expression. This is consistent with the results of Mertens-Talcott et al (7). To investigate the role of ZBTB10 on FSH-induced angiogenesis, transfection of ZBTB10 plasmid was performed. Our present result showed that overexpression of ZBTB10 protein inhibited the expressions of VEGF, Cox2 and survivin, and it abolished FSH-induced expression. These data imply that ZBTB10 plays an opposite role in mediating FSH-induced angiogenesis.

Sp1 is a critical molecule in microRNA-27a: ZBTB10-specificity protein pathway and is a target gene of ZBTB10, which belongs to the Sp/krüppel-like factor family of transcription factors (26-28). Previous reports stated that Sp1 was overexpressed in several type of cancer tissues (29-31), moreover, it is a significant predictor of survival in human gastric cancer (30-32). In the current study, overexpression of Sp1 was observed in ovarian cancer cell lines, which indicates that Sp1 may be involved in OEC occurrence. Moreover, the Sp1 protein level was elevated with increased dose of FSH, whereas, transfection of as-microRNA-27a or overexpression of ZBTB10 inhibited Sp1 expression, the inhibition effect abolished FSH-induced Sp1 expression. Present data also clearly showed that RNA interference directed against Sp1 blocked the induction of VEGF, Cox2 and survivin by FSH, suggesting that Sp1 is a critical molecule in mediating FSH-induced angiogenesis. These results are consistent with previous studies of Sp1 involved in regulation of VEGF expression (33-38).

In conclusion, this study shows that FSH stimulates the microRNA-27a: ZBTB10-specificity protein pathway to induce VEGF, Cox2 and survivin expression (Fig. 6), which is the first evidence of direct linkage of FSH to microRNA activation and VEGF, Cox2 and survivin expression. Our research may help to understand the molecular mechanism of FSH-induced angiogenesis.

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