Vascular endothelial growth factor induces anti-Müllerian hormone receptor 2 overexpression in ovarian granulosa cells of *in vitro* fertilization/intracytoplasmic sperm injection patients

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Abstract. Misregulation of vascular endothelial growth factor A (VEGF-A) has been implicated in numerous types of ovarian disease, such as polycystic ovarian syndrome, ovarian hyperstimulation syndrome, endometriosis and ovarian cancer. VEGF regulates blood vessel permeability and angiogenesis. In our previous study, VEGF-regulated gene expression was profiled in the uterus of a transgenic mouse model with repressed VEGF expression, which indicated that VEGF is an important regulator in controlling gene expression in the uterus. The anti-Müllerian hormone (AMH) is expressed by ovarian granulosa cells (GCs) and acts through its type 2 receptor, AMH receptor 2 (AMHR2). Serum AMH levels are used to predict ovarian reserves and the small antral follicles contribute markedly to the serum AMH level. AMH recruits primordial follicles and inhibits excessive follicular development by follicular stimulating hormone (FSH). However, AMH may be influenced by suppression of gonadotrophin secretion and VEGF inhibition. In the current study, human primary ovarian GCs were isolated from ovarian follicle fluid of *in vitro* fertilization/intracytoplasmic sperm injection cycles (IVF/ICSI). It was identified that the FSH receptor was consistently expressed in the isolated cells. VEGF-A treatment stimulated AMHR2 overexpression at the gene and protein levels. In addition, VEGF induced AMHR2 expression on the surface of the isolated GCs from mature follicles. The VEGF treatment was also performed in an ovarian granulosa-like cell line, KGN. *AMH* and *AMHR2* are co-expressed in normal GCs; however, as a result of VEGF misregulation, AMHR2 overexpression increases AMH binding, which may attenuate follicular or oocyte maturation. However, the associated function and underlying mechanism requires further investigation.

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Introduction

The anti-Müllerian hormone (AMH) is produced in the ovarian granulosa cells (GCs). Through the specific receptor, AMH receptor 2 (AMHR2), AMH limits the formation of primary follicles and inhibits excessive follicular recruitment by follicle stimulating hormone (FSH) (1,2). The expression levels of AMH have been used as the indexes to predict ovarian reserves and ovarian response in assisted reproductive technology (3,4). In human ovaries, early stage antral follicles contribute markedly to the serum AMH concentration (5). AMHR2 is a single transmembrane serine/threonine kinase receptor of the transforming growth factor β (TGFβ) receptor family, which is expressed on ovarian GCs (6). The presence of AMHR2 is also observed in ovarian cancer cell lines that respond positively to treatment with recombinant AMH (7). Thus, the receptor and ligand may be important diagnostic factors or therapeutic tools.

The human ovary is a highly vascularized organ. As a key regulator of vascularization, vascular endothelial growth factor (VEGF) is crucial in regulating follicular growth, corpus luteum development and maintaining ovarian functions (8). VEGF is expressed in the ovarian granulosa, theca and granulosa lutein cells (9) and acts by binding to the tyrosine kinase receptors, VEGFR1 and 2 in the ovary. Marked production and secretion of follicular VEGF occur in response to gonadotrophin stimulation (10). Furthermore, hypersecretion of VEGF is frequently observed in patients with polycystic ovarian syndrome (PCOS) (11,12). It has been proposed that VEGF, by regulating vascular permeability, induces blood vessel leaking in ovaries exhibiting ovarian hyperstimulation syndrome (OHSS) (13). Notably, in a transgenic mouse model with engineered inducible repression of VEGF, mice were observed to become infertile upon VEGF repression (14). By digital gene expression assays, 831 uterus-specific and

2,398 VEGF-regulated differentially expressed genes were identified in the mouse uterus, which indicated that VEGF exerts a regulatory role in gene expression in the uterus.

The majority of studies regarding AMH focus on the early stage of follicle development. In a study on marmosets, AMH levels were decreased in early preantral follicles as a result of the suppression of gonadotrophin secretion and VEGF inhibition (15). The regulatory role of VEGF with regard to *AMH* and *AMHR2* signaling has remained to be elucidated in mature follicles. In the present study, VEGF, VEGFR2, FSH receptor (FSHR), AMH, AMHR2 and TGFβ expression changes were analyzed following FSH and/or VEGF treatment in human primary GCs, which were isolated from IVF/ICSI patients. Furthermore, the elevation of AMHR2 expression levels by VEGF in the ovarian granulosa-like KGN cells was identified.

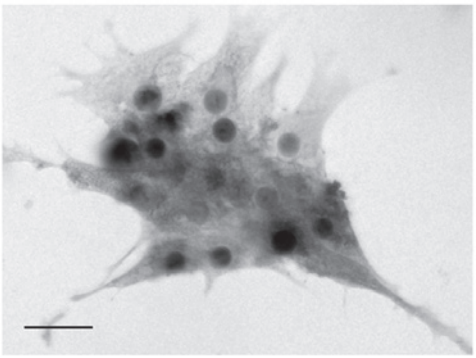

Figure 1. FSHR was expressed on isolated human luteinized granulosa cells. Representative immunocytochemistry of FSHR (n=3). Scale bar, 20 μm. FSHR, follicle-stimulating hormone receptor.

Materials and methods

The current study was performed on cultured GCs, which were derived from human ovarian follicular fluid. The female participants provided written informed consent, and the protocol of the study was approved by the ethical committee of Jilin Province People's Hospital (Changchun, China).

Primary ovarian GCs. GCs were isolated at the time of oocyte pick-up from 18 IVF/ICSI patients with male factors only (age, 31.3±4.5 years) between April 2013 and December 2014. Eighteen follicular fluid samples were collected and the follicular aspirates obtained from individual patients were centrifuged at 900 x g at room temperature. Blood contaminants were removed from GCs by Histopaque® 1077 (Sigma-Aldrich, St. Louis, MO, USA) gradient centrifugation at 800 x g for 20 min at room temperature. The GC pellet was resuspended in a 20-ml volume of medium [Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Beyotime Institute of Biotechnology, Inc., Haimen, China)] and washed by a further 10-min centrifugation at 500 x g. The GC pellet was resuspended in 1 ml GC preparation medium containing 0.02% (w/v) EDTA and gentle repeated pipetting was performed to break up any cellular clumps. Following further washing, the cell stock was resuspended in the GC culture medium. The cells were seeded in six-well cell culture plates at a density of 100,000 cells/well in medium. Following an overnight incubation (37˚C, 5% CO_2), cells were treated with 30 ng/ml human recombinant FSH (450 IU; GONAL-F; Merck Biopharma China, Beijing, China) and/or 100 ng/ml VEGF-A (CB055-0231; ExCell Biotech, Shanghai, China) for 24 or 48 h. Phosphate-buffered saline (PBS) was added to the control group as a vehicle.

Cell line. The human granulosa tumor-derived cell line, KGN was obtained from Riken BioResource Center (Riken Cell Bank, Tsukuba, Japan). The KGN cells used in these experiments were passages 8-12. The cell line was validated by short tandem repeat polymorphism analysis, which was performed by Riken Biosource Center.

Gene expression analysis. Total RNA was isolated from primarily cultured cells according to previous methods (16). First-strand cDNA synthesis was performed using 1 μg total RNA in a 20-μl volume. Random primers and 200 units Moloney murine leukemia virus reverse transcriptase (Takara Biotechnology Co., Ltd., Dalian, China) were used for reverse transcription (RT). The SYBR Green PCR master mix (Roche Diagnostics, Shanghai, China) was used as a double-stranded DNA-specific fluorescent dye. Gene expression was assessed by RT-quantitative polymerase chain reaction (qPCR) using the Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.). PCR conditions were as follows: 2 min at 50˚C and 10 min at 95˚C, followed by 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec. The specificity of amplification was confirmed by melting curve analysis and gel electrophoresis. All results were normalized to the levels of 18S ribosomal (r)RNA, and relative quantification was calculated using relative quantification (ΔΔCq) values for each biological replicate (16,17). Values are expressed as the mean ± standard error of the mean (SEM; triplicate samples and three repeats). The following primer sequences (Genewiz, Suzhou, China) were used for RT-PCR: Forward, CGAAGTGGTGAAGTT CATGG and reverse, GTACTCGATCTCATCAGGGT for VEGF; forward, CACTGGCTTCTACAGCTGCA and reverse, CGAAAGGTCTACCTGTATC for VEGFR1; forward, CGG TCAACAAAGTCGGGAGA and reverse, CAGTGCACCACA AAGACACG for VEGFR2; forward, GGAACATCATAG TGCTAGTG and reverse, CCAGTCAATGGCATAGTTGT for FSHR; forward, GTCCTACACCTGGAGGAAGT and reverse, AGCCCTCGTCACAGTGACCT for AMH; forward, GATTTGAGGCCTGACAGCAG and reverse, GCCAGGTGG ATGGGATGTAG for AMHR2; forward, ACTACTACGCCA AGGAGGTC and reverse, CGGAGCTCTGATGTGTTGAA for TGFβ; and forward, CATTCGAACGTCTGCCCTAT and reverse, GATGTGGTAGCCGTTTCTCA for 18S rRNA.

Immunocytochemistry. Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 30 min. Cells were blocked with 5% bovine serum albumin (Sigma-Aldrich) in PBS for 1 h at room temperature and incubated overnight with rabbit anti-human FSHR antibody (cat. no. BA2317; Beijing Bioss Biosynthesis Biotechnology Co., Ltd., Beijing, China) or rabbit anti-human MIS antibody (cat. no. sc-6886; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) primary antibodies (dilution, 1:500) at 4˚C and horseradish peroxidase (HRP)-conjugated goat

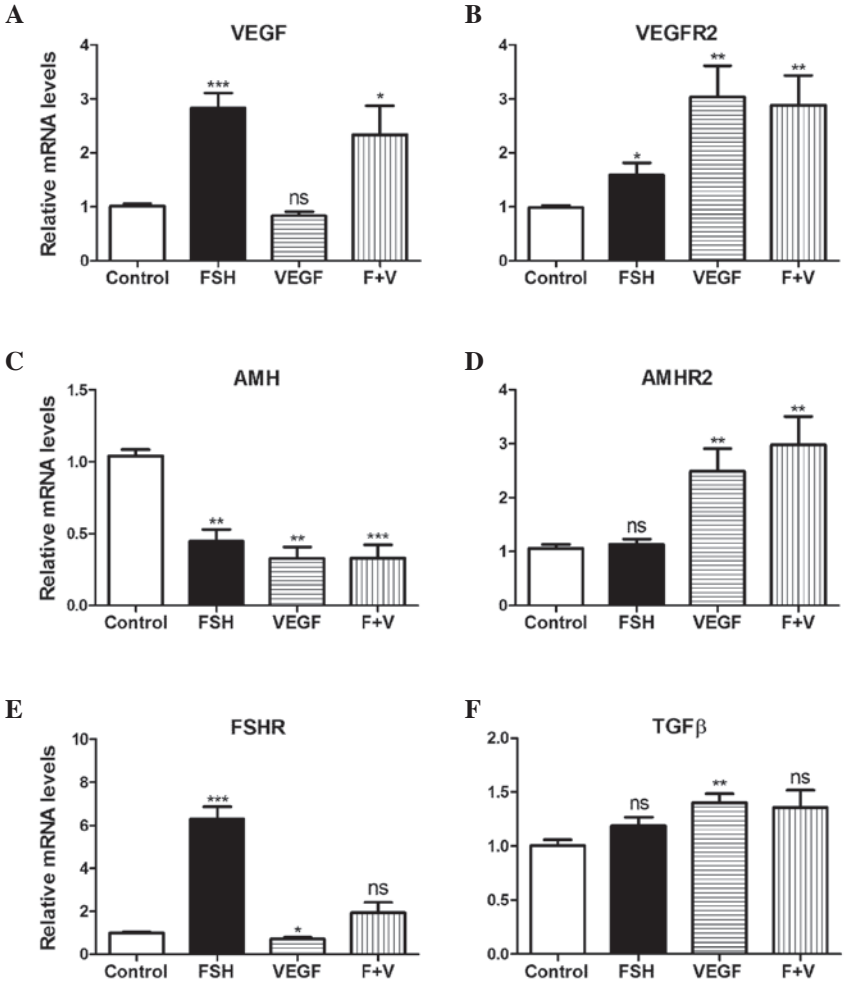

Figure 2. VEGF treatment induced changes at the mRNA level of primary granulosa cells. rhFSH (30 ng/ml), rhVEGF (100 ng/ml), and rhFSH (30 ng/ml) and rhVEGF (100 ng/ml; F + V) were used to treat the primary granulosa cells. Gene expression levels of (A) *VEGF*, (B) *VEGFR2*, (C) *AMH*, (D) *AMHR2*, (E) *FSHR* and (F) *TGFβ* were analyzed by reverse transcription quantitative polymerase chain reaction (n=6). Data are presented as means ± standard error of the mean. *P<0.05, **P<0.01, ***P<0.001 compared with the vehicle control. ns, no significance; VEGF, vascular endothelial growth factor; rh, recombinant human; AMH, anti-Müllerian hormone; FSHR, follicle-stimulating hormone receptor; TGFβ, transforming growth factor β.

anti-rabbit immunoglobulin G (IgG) secondary antibody (cat. no. SA1028; Beijing Bioss Biosynthesis Biotechnology Co., Ltd.) (dilution, 1:1,000) for 2 h at room temperature, followed by 3,3'-diaminobenzidine and hematoxylin staining (Beijing Chemical Co., Beijing, China). Washes with PBS (three times for 3 min each) were performed between incubations. A microscope (CX31; Olympus Corp., Tokyo, Japan) and Image J software (version 1.46r; National Institutes of Health, Bethesda, MD, USA) were used to perform analyses.

Protein analysis. For western blot analysis, cells were lysed in radioimmunoprecipitation assay buffer [0.5% Nonidet P-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5); Beijing Chemical Co.]. The lysates were resolved by 10% SDS-PAGE (Pierce Biotechnology, Inc., Rockford, IL, USA), transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) at 10 V over 2 h and probed with rabbit anti-human FSHR, rabbit anti-human MIS and mouse anti-human GAPDH antibody (cat. no. sc-20357; Santa Cruz Biotechnology, Inc.) at 1:500 dilution at 4˚C overnight. The secondary antibody conjugated to HRP (goat anti-rabbit IgG-HRP; cat. no. sc-2030 or goat anti-mouse IgG-HRP; cat. no. sc-2031; Santa Cruz Biotechnology, Inc.) was applied successively at 1:1,000 dilution followed by incubation at room temperature for 2 h. Blots were developed using the Chemiluminescence WB solution ABC kit (DW101-01; Beijing TransGen Biotech, Beijing, China).

Statistical analysis. Values are expressed as the mean ± SEM. Statistical analysis was performed using an unpaired *t*-test with GraphPad Prism software (version 5.1; GraphPad Inc., La Jolla, CA, USA). Grouped data were analyzed by one-way analysis of variance and P<0.05 was considered to indicate a statistically significant difference.

Results

AMHR2 expression in primary GCs. Human luteinized GCs were isolated from the follicle fluid of IVF/ICSI patients. FSHR expression is considered to be the biological marker of ovarian GCs, thus, the isolated cells were identified by immunocytochemistry staining of FSHR (Fig. 1). The negative controls were established by incubation with an equal quantity of non-immunized rabbit IgG.

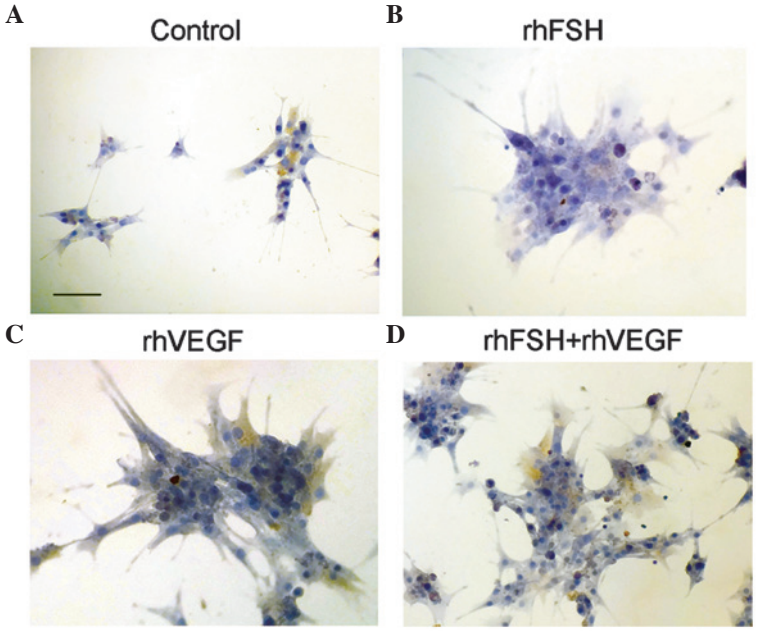

Figure 3. AMHR2 expression in primary human luteinized granulosa cells. (A) Control (treated with vehicle). Primary cells were treated with (B) rhFSH (30 ng/ml); (C) rhVEGF (100 ng/ml); and (D) rhFSH (30 ng/ml) and rhVEGF (100 ng/ml; rhFSH +rhVEGF). Representative immunocytochemistry with anti-AMHR2 was conducted (n=3). Scale bar, 40 μm. rh, recombinant human; FSH, follicle-stimulating hormone; VEGF, vascular endothelial growth factor; AMHR2, anti-Müllerian hormone receptor 2.

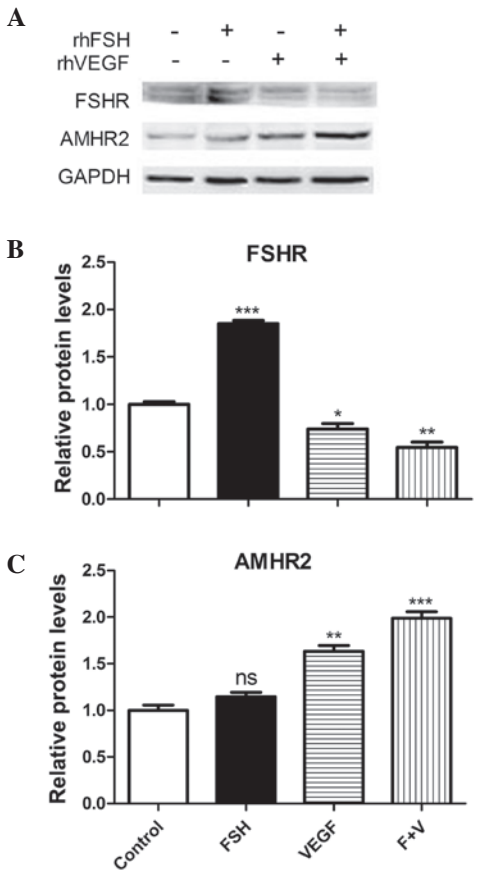

Figure 4. VEGF treatment induces changes in mRNA expression levels in primary granulosa cells. Primary cells were treated with rhFSH (30 ng/ml), rhVEGF (100 ng/ml), and rhFSH (30 ng/ml) and VEGF (100 ng/ml; F + V). (A) Representative western blot analysis and the quantification of (B) FSHR and (C) AMHR2 expression levels (n=3). Values are expressed as the mean ± standard error of the mean. *P<0.05, **P<0.01, ***P<0.001 vs. control. rh, recombinant human; FSH, follicle-stimulating hormone; VEGF, vascular endothelial growth factor; AMHR2, anti-Müllerian hormone receptor 2, ns, no significance.

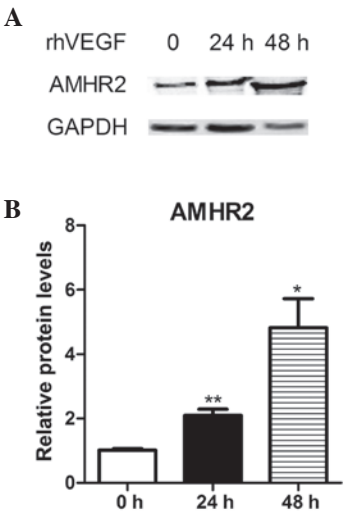

Figure 5. VEGF induced representative AMHR2 protein expression in the KGN cells. KGN cells were treated with rhVEGF (100 ng/ml) for 0, 24 and 48 h. (A) Representative western blot analysis and (B) quantification of AMHR2 protein expression (n=3). Data are presented as means ± standard error of the mean. *P<0.05, **P<0.01 vs. 0 h. rh, recombinant human; AMHR2, anti-Müllerian hormone receptor 2; VEGF, vascular endothelial growth factor.

FSH- and VEGF-induced gene expression varies in primary GCs. Gonadotrophin contributes to GC growth and proliferation (15). To investigate the gonadotrophin- and VEGF-induced gene expression changes, RT-qPCR was performed on the primary GCs, and human recombinant FSH and VEGF were used to treat the cells. The mRNA expression of *VEGF* was significantly elevated as a result of treatment with FSH, and FSH + VEGF by 2.8- (P<0.05) and 2.3-fold (P<0.01), respectively; however, no significant difference was noted in the VEGF only group (Fig. 2A). *VEGFR2* mRNA expression levels

were significantly increased in the VEGF and VEGF plus FSH groups by ~3-fold (P<0.01) and in the FSH group by ~1.5-fold (P<0.05) (Fig. 2B), whereas *AMH* mRNA expression levels were significantly decreased in all groups (P<0.01) (Fig. 2C). Compared with the control, the *AMHR2* mRNA expression levels increased as a result of VEGF, and FSH + VEGF treatment by 2.4- and 3.2-fold, respectively (P<0.01); however, the increase observed in the FSH only treatment group was not significant (Fig. 2D). *FSHR* mRNA expression levels increased due to FSH treatment, by 6.2-fold (P<0.001) and significantly decreased following VEGF treatment, by 0.7-fold (P<0.05) (Fig. 2E). *TGFβ* mRNA expression levels increased significantly as a result of VEGF treatment (P<0.01; Fig. 2F).

FSH- and VEGF-induced protein expression varies in primary GCs. AMHR2 expression was observed in the primary ovarian GCs by immunocytochemical staining (Fig. 3). Expression of the FSHR and AMHR2 protein was analyzed by western blotting. FSHR protein expression was significantly increased by FSH stimulation (P<0.001), and significantly decreased by VEGF and VEGF + FSH treatment (Fig. 4A and B). Conversely, AMHR2 protein expression was significantly increased as a result of VEGF and VEGF + FSH treatment (P<0.01). Although the AMHR2 expression was increased as a result of FSH treatment, the difference was not significant (Fig. 4C).

VEGF stimulated AMHR2 overexpression in KGN cells. The human granulosa-like cell line, KGN is an ovarian cancer cell line that expresses FSHR (18). The KGN GC tumor cells were derived from a patient who presented with a recurrent, metastasized granulosa cell tumor in the pelvic region. The cell line maintains ovarian GC features, such as expression of the FSH receptor and production of estrogen in response to FSH (7). In the present study, the expression levels on primary cells were varied in specific individuals. To demonstrate the induction of AMHR2, KGN cells were treated with VEGF. The cells were treated with 100 ng/ml VEGF for 24 and 48 h, and the representative AMHR2 protein levels increased by 2- and 5-fold, respectively (Fig. 5A and B).

Discussion

VEGF is important in maintaining and regulating the functions of the female reproductive system. White adipose tissue, which is the major organ of VEGF secretion, surrounds the human ovaries and uterus and assists with maintaining their functions. VEGF has been detected in the follicular fluid of PCOS females undergoing controlled ovarian hyperstimulation, which modulates the effects of gonadotrophins in GCs (8,10). AMH is exclusively produced by ovarian GCs of the developing preantral and antral follicles (19,20). Ovarian function and reserve can be assessed by serum AMH levels to evaluate infertility. Age-specific serum AMH levels and the antral follicle count (AFC) baseline are evaluated to predict ovarian reserve, particularly in females with PCOS (21-23).

AMH acts via its type II receptor, AMHR2, which has recently been increasingly investigated. In a previous study, AMHR2 expression was evaluated in the mouse uterus (13). Genetic variants of *AMHR2* appear to be associated with unexplained infertility and PCOS risk (24). A primary study demonstrated that antral follicles (size, 5-8 mm) contribute markedly to serum AMH levels, and the *AMHR2* gene is co-expressed with *AMH* in the GCs of small antral follicles; however, the correlation between *AMHR2* gene expression level and follicle fluid AMH concentration was not identified to be significant (5). In the present study, FSHR and AMHR2 were detectable in the ovarian follicular fluid-derived GCs of the patients. *AMH* mRNA levels were decreased in response to FSH and/or VEGF treatment. Furthermore, TGFβ is important in controlling cell proliferation and exerts a regulatory role in ovarian angiogenesis (25). TGFβ expression levels were increased in response to VEGF treatment in the current study. Thus, VEGF may have prompted the maturation of GCs and the reduction in AMH expression.

In the present study, AMHR2 expression levels were increased when the AMH expression level was repressed following VEGF exposure. The *in vitro* study of KGN cells indicated that the induction of AMHR2 by VEGF was time-dependent. VEGF and its receptor, VEGFR2 are highly expressed in ovarian GC tumors. In the present study, AMHR2 protein expression was increased in response to VEGF stimulation in the KGN cells. In a previous study, high levels of circulating VEGF were observed in the serum of patients with primary GC tumors (26). Thus, elevated VEGF levels increase AMHR2 levels in carcinoma tissues, which may contribute to the malignancy of the tumor.

In conclusion, AMH exerts paracrine and hormonal actions, and expression levels of the specific receptor, AMHR2 also varies in different cells or tissues. VEGF misregulation increases AMHR2 expression, which may result in binding of AMH from the circulation, leading to paracrine AMH binding and attenuation of the maturation of follicles in individuals using assisted reproductive technology. AMH may exert other actions in reproduction-associated organs; however, this requires further investigation.

Acknowledgements

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