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Analysis of TGFB1, CD105 and FSP1 expression in human granulosa cells during a 7-day primary in vitro culture

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Abstract

The human granulosa cells (GCs) surround the oocyte and form the ovarian follicle's proper architecture. These sub-populations include mural granulosa cells, antral granulosa cells, and cumulus granulosa cells. Their main functions are to support the oocyte's growth (cumulus granulosa cells) and estradiol production (mural granulosa cells). After ovulation, the granulosa cells transform into the luteal cells of the corpus luteum and produce progesterone.

Our study investigated the expression profile of three genes: TGFB1, CD105, and FSP1 during a 7-day *in vitro* culture. The analysis was conducted using the RT-qPCR technique.

Changes in the expression of CD105 and FSP1 could be observed during the 7-day *in vitro* culture. In the case of TGFB, the expression remained at a similar level, with no statistically significant differences observed.

Running title: Expression of TGFB1, CD105 and FSP1 in granulosa cells

Keywords: granulosa cells, TGFB1, CD105, FSP1

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Introduction

The human granulosa cells (GCs) surround the oocyte and form the ovarian follicle's proper architecture. In the initial period of development, the number of GCs surrounding the oocyte is relatively low. However, with time, the intensity of their proliferation increases, which leads to the formation of a granulosa layer. The further development of this layer leads to the creation of an antrum and the development of three granulosa cell subpopulations. These sub-populations include mural granulosa cells, antral granulosa cells, and cumulus granulosa cells. Their main functions are to support the oocyte's growth (cumulus granulosa) and estradiol production (mural granulosa cells). After ovulation, the granulosa cells transform into the luteal cells of the corpus luteum and produce progesterone.

In the recent years, the ability of GCs to proliferate and differentiate in the conditions of *in vitro* culture has been demonstrated. However, there is still a large field for extensive investigation of molecular basics, as well as marker genes, responsible for these processes. Therefore, in this study, the expression of TGFB1, CD105, and FSP1 in GCs was examined.

The transforming growth factor-beta (TGFB) superfamily consists of more than 35 structurally related members [1]. It is expressed both in mammalian ovarian somatic cells and oocytes [2,3]. The most important TGFB superfamily genes include bone morphogenic proteins (BMPs) and growth differentiation factor 9 (GDF9) that are regulate critical steps of follicle growth and development. Moreover, it has also been shown that TGFB1 is involved in the regulation of follicular processes. In addition, it was postulated that TGFB might play a significant role in regulating GCs proliferation [4,5] and that TGFB1 stimulated the growth of preantral follicles isolated from adult mice ovaries and cultured *in vitro* [1].

Endoglin (CD105, ENG) is a 180 kDa homodimeric transmembrane glycoprotein. Its expression was determined in the luteinizing granulosa cells of the human ovary after culture in the presence of leukaemia inhibiting factor (LIF) [6,7]. It is also known as a mesenchymal stem cells (MSCs) marker. Its expression may indicate the multipotency of granulosa cells, which was confirmed by differentiation into neurons, chondrocytes, and osteoblasts [6].

Fibroblast-Specific Protein 1, or S100 calcium-binding protein A4 (FSP1, S100A4), is a member of the calmodulin S100 troponin C superfamily. It is a fibroblast-specific protein related to the morphology and mobility of mesenchymal cells, expressed during epithelial-mesenchymal transformations [8]. Fibrosis-related cytokines are responsible for the induction of FSP1. These include TGFB and epidermal growth factor (EGF) [8]. Additionally, factors promoting fibroblasts' growth may affect the growth, proliferation, and survival of granulosa cells [9].

Our study aimed to analyse the expression profile of these three genes during the 7-day *in vitro* culture.

Material and Methods

Granulosa cell collection

The study group consisted of 7 patients, aged 18–40 years, enrolled in *in vitro* fertilization (IVF) procedure in the Division of Infertility and Reproductive Endocrinology, Poznan University of Medical Sciences, Poland. Follicular fluid containing the GCs from patients undergoing *in vitro* fertilization (IVF) procedures was collected.

The IVF procedure was based on a controlled ovarian hyperstimulation protocol adapted to the patient's initial cause of infertility, as well as predicted and current ovarian response. Stimulation with human recombinant FSH (Gonal-F; Merck, Darmstadt, Germany) and highly purified human menopausal gonadotropin (hMG-HP; Menopur; Ferring) has been performed according to protocol. Gonadotropin-releasing hormone (GnRH) antagonist-cetrorelix acetate (Cetrotide; Merck, Darmstadt, Germany), injection has been given at the appropriate dose to suppress the function of the pituitary gland. Induction of ovulation was based on the subcutaneous injection of 6.500 h of human chorionic gonadotropin (hCG; Ovitrelle; Merck, Darmstadt, Germany). The doses of gonadotropins and GnRH antagonist have been precisely controlled and recorded for every patient. The follicular fluid has been collected during transvaginal ultrasound-guided oocyte pick-up, 36 h after administration of human chorionic gonadotropin. GCs have been taken from follicles with a diameter of over 16 mm. Directly after ovarian puncture, the complete content of the ovarian follicle (follicular fluid containing GCs and oocytes) was passed on to a qualified embryologist that extracted all of contained oocytes that were subsequently used in further stages of the IVF procedure (conducted at Division of Infertility and Reproductive Endocrinology, Department of Gynecology, Obstetrics and Gynecological Oncology, Poznan University of Medical Sciences, Poznan, Poland). Meanwhile, the remaining granulosa cell containing follicular fluid, usually discarded after this step, was passed on to the employees of Department of Anatomy, Poznan University of Medical Sciences, in which the further research was conducted.

Primary cell culture

The procedure of GCs isolation was performed as described previously [10,11]. Briefly, the follicular fluid, were washed twice by centrifugation at 200 × g for 10 min at RT. Medium consisted of Dulbecco's Modified Eagle's Medium (DMEM; Merck, Darmstadt, Germany), 2% Fetal Bovine Serum FBS (FBS; Merck, Darmstadt, Germany), 4 mM L-glutamine (Invitrogen; Thermo Fisher Scientific, Inc.), 10 mg/ml gentamycin (Invitrogen; Thermo Fisher Scientific, Inc.), 10,000 U/ml penicillin, and 10,000 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were cultivated at 37°C under aerobic conditions (5% CO_2). The medium was changed every 48 hours of culture.

Total RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR) analysis

RNA was isolated at 7 time periods, after each day of cultivation. Total RNA was isolated using the Chomczyński-Sacchi method [12]. The GCs were suspended in 1 ml mixture of guanidine thiocyanate and phenol in monophase solution (TRI Reagent; Merck, Darmstadt, Germany). In the next step, the chloroform solution (Merck, Darmstadt, Germany) was added and centrifuged to separate 3 phases. RNA has been located in an aqueous phase. In the last step, the RNA has been precipitated with 2-propanol (Merck, Darmstadt, Germany), in amount accurate per 1 ml of TRI-reagent and has been washed with 75% ethanol. Resulting RNA has been used for further analysis. The total RNA was determined from the optical density at 260 nm and the RNA purity was estimated using the 260/280 nm absorption ratio (NanoDrop spectrophotometer, Thermo Fisher Scientific, Inc).

The obtained cDNA was used to perform the RT-qPCR reaction. The RT-qPCR analysis was performed using LightCycler real-time PCR detection system (Roche Diagnostics, Mannheim, Germany), with SYBR® Green I (Qiagen, Hilden, Germany) serving as a detection dye. The relative abundance of TGFB1, CD105 and FSP1 was standardized to the internal standards ACTB. For amplification, 2 µl of cDNA solution was added to 18 µl of QuantiTect® SYBR® Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) and primers (**Tab. 1**). One RNA sample of each preparation was processed without the RT-reaction to provide a negative control for subsequent PCR.

Statistical analysis

The data obtained from the RT-qPCR analysis were calculated using the $2^{-\Delta\Delta Ct}$ method [13]. The change in expression of the target genes was normalized to ACTB. In the next step, normalized values were compared to the control. The time point for which the normalized Ct value was the highest was selected as a control. After the results were obtained, statistical analysis was performed using Python 3.8 and the SciPy 1.5.4 library. To check that the data was drawn from a normal distribution, the Shapiro-Wilk test was used [14,15]. To determine the statistical significance between the time intervals of the analysed genes, Student's T-test was used.

Ethical approval

The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved with resolution 558/17 by Poznan University of Medical Sciences Bioethical Committee.

Informed consent

Informed consent has been obtained from all individuals included in this study.

Results

Changes in the expression of CD105 and FSP1 could be observed during the 7-day *in vitro* culture. In the case of TGFB, the expression level remained unchanged, and no statistically significant differences in its expression level were observed (**Fig. 1**).

In the case of CD105, a low level of expression was observed on the first day of *in vitro* culture, followed by an increase in CD105 expression until day 5. Thereafter, CD105 expression decreased and remained similar on days 6 and day 7.

With FSP1, there was an irregular expression pattern. The highest level of expression was noticed on day 3. On days 2, 4, and 6, FSP1 expression was lower than on days 3, 5, and 7. However, the expression level on all days was higher than at the start of *in vitro* culture.

Discussion

The conducted research shows that follicular fluid is rich in proteins and functional cells. In addition to oocyte cells, the follicular fluid contains granulosa, theca, and ovarian surface epithelial cells [16]. To confirm the absence of epithelial cells in the culture, we performed an analysis of keratin 14 (KRT14) expression. The identified value of KRT14 expression was below the detection level (data not presented).

TABLE 1 Oligonucleotide sequences of primers used for RT-qPCR analysis

GENE	GENE ACCESSION NUMBER	PRIMER SEQUENCE (5'-3')	PRODUCT SIZE (BP)
TGFB1	NM_000660	GGCCTTTCCTGCTTCTCATG CCTTGCGGAAGTCAATGTACA	150
CD105 (ENG)	BC014271	CACTAGCCAGGTCTCGAAGG CTGAGGACCAGAAGCACCTC	165
FSP1 (S100A4)	CR450345	TCTTGGGGAAAAGGACAGATGA CCCAACCACATCAGAGGAGT	195
ACTB	NM_001101	AAAGACCTGTACGCCAACAC CTCAGGAGGAGCAATGATCTTG	132

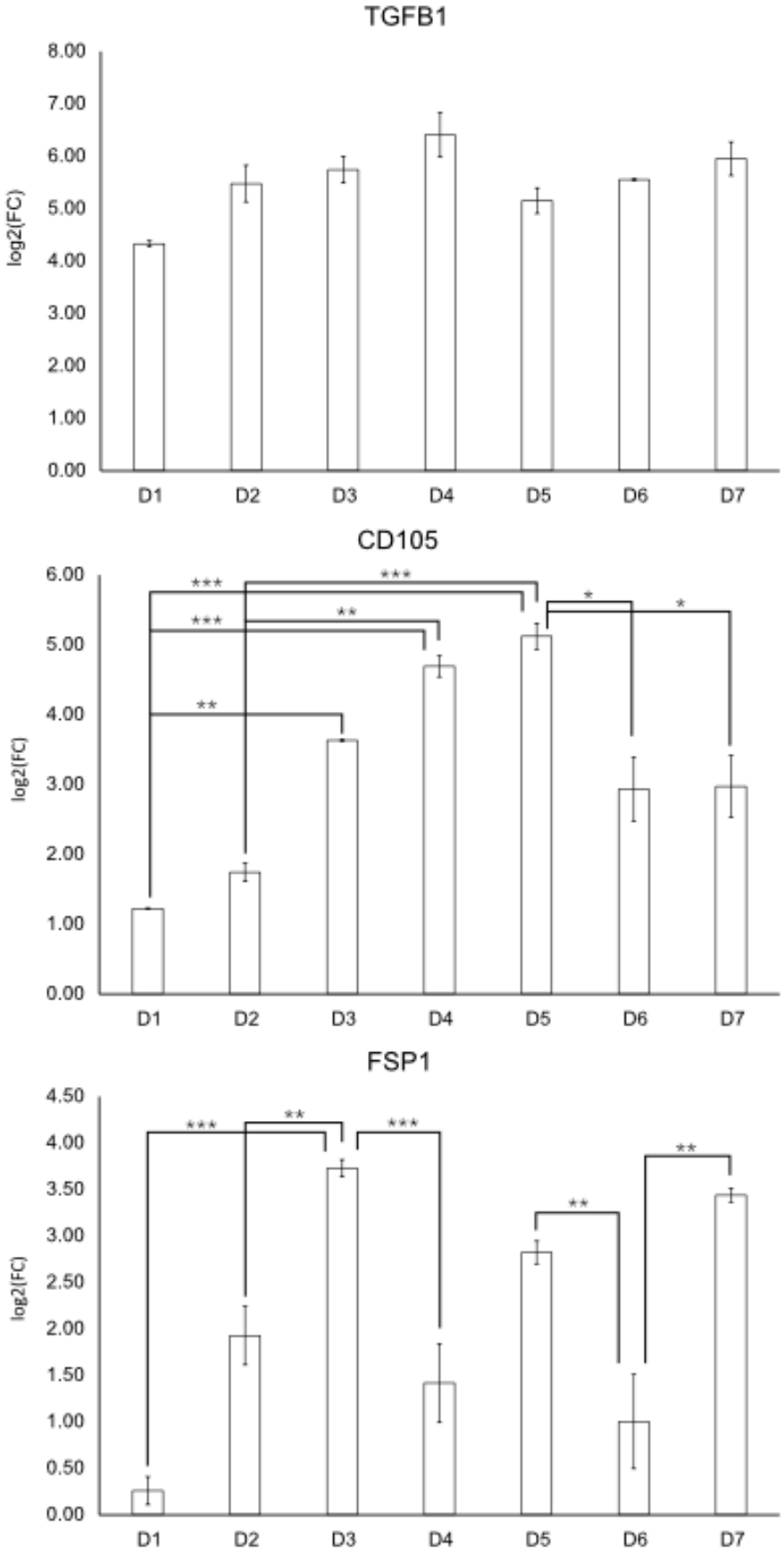

FIGURE 1 TGFB1, CD105 and FSP1 expression in granulosa cells during 7-day *in vitro* culture. Data was presented like logarithm of fold change. Statistical analysis of the data was performed using the T-test and the differences were considered to be significant at the level of p<0.001 (***), p<0.01 (**), *p*<0.05 (*)

TGFB proteins affect many different cells in the body and perform various functions. Early studies showed that products of the TGFB might have a capacity for autocrine or paracrine modulation of granulosa cell differentiation [17]. On the other hand, TGFB may exhibit tumour-promoting activity through the ability to repress apoptosis in the juvenile form of granulosa cell tumours [18]. It was also showed that abnormal TGFB signalling in granulosa cells is related to the pathological conditions of polycystic ovary syndrome (PCOS) [19].

In our research, due to the regulatory role in the proliferation and differentiation of GCs, TGFB1 expression was maintained at a similar level during the 7 days of *in vitro* culture.

The second analysed molecule, endoglin (CD105), is associated with the TGFB superfamily. CD105 is a cell-surface co-receptor for TGFB1 and TGFB3 isoforms [7,20]. It is a glycoprotein that is expressed in various cell types, with high expression identified in endothelial cells [21]. CD105 is also expressed in female gonadal cells [7], as well as is one of the mesenchymal stem cells (MSCs) markers. Since GCs originate from the mesoderm, they should show a mesenchymal cell expression pattern. Kossowska-Tomaszczuk K. et al. showed that the freshly collected GCs were positive for CD29, CD44, CD90, CD105, CD166, and negative for CD73 [6]. Using immunohistochemistry, the expression of CD105 was demonstrated in the cytoplasmic area of the oocyte and in all groups of granulosa cells. However, CD105 was more concentrated in oocytes than granulosa cells [7]. Our research showed that the expression of CD105 on the first day after seeding was on a low level, raising in the following days. The highest level of CD105 expression was confirmed on day 5. It needs to be noted that GCs may lose their properties during multiple passages, which may also affect the process of cell immortalization. Furthermore, the expression of CD105 in immortalized granulosa cells was not confirmed by Ai et al. [16].

It is known that CD105 plays a role in vasculature development, and it was confirmed that follicle development is dependent on the establishment and continual remodelling of a complex vascular system. It was suggested that GCs might behave as endothelial cells and contribute to follicular angiogenesis [22].

Fibroblast-Specific Protein 1 (FSP1) is a member of the calmodulin S100 troponin C superfamily [23]. The FSP1 gene encodes a filament-binding S100 protein with paired EF-hands, specifically expressed in fibroblasts [24]. FSP1 was indicated as an improved marker for lung fibroblasts that could be useful for investigating pulmonary fibrosis's pathogenesis [23]. Studies have shown that FSP1 protein is present in the cytoplasm of fibroblasts, but not the epithelium. However, in kidney fibrosis occurring due to persistent inflammation, many fibroblasts could be identified in interstitial sites. This may be related to the local transition from epithelium to fibroblasts, with focal FSP1 overexpression observed [25]. In the case of granulosa cells, the process of fibrosis may affect their proliferation and development [9]. In our studies, FSP1 expression showed significant changes on the individual days of *in vitro* culture, which may indicate the dynamics of their internal changes.

Conclusions

Our study showed that the expression of TGFB1 was high relative to CD105 and FSP1 and remained at a similar level during 7-day *in vitro* culture. This may highlight the important role of TGFB1 in the growth and differentiation of GCs. In contrast to TGFB1, FSP1 expression showed a heterogeneous pattern, indicating dynamic changes during culture. In the case of CD105, an increase in expression up to day 5 and subsequent stabilization of the expression level was observed. This may indicate a high proliferation potential on day 5.

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Conflict of interest statement

The authors declare they have no conflict of interest.

References

1. Xu Y, Niu J, Xi G, Niu X, Wang Y, Guo M, Yangzong Q, Yao Y, Sizhu SL, Tian J. TGF-β1 resulting in differential microRNA expression in bovine granulosa cells. Gene. 2018;663:88–100; DOI:10.1016/j.gene.2018.04.036.
2. Corduk N, Abban G, Yildirim B, Sarioglu-Buke A. The effect of vitamin D on expression of TGF β1 in ovary. Exp Clin Endocrinol Diabetes. 2012;120:490–3; DOI:10.1055/s-0032-1314858.
3. Nagashima T, Kim J, Li Q, Lydon JP, DeMayo FJ, Lyons KM, Matzuk MM. Connective tissue growth factor is required for normal follicle development and ovulation. Mol Endocrinol. 2011;25:1740–59; DOI:2019041122541184000.
4. Piotrowska H, Kempisty B, Sosinska P, Ciesiolka S, Bukowska D, Antosik P, Rybska M, Brüssow KP, Nowicki M, Zabel M. The role of TGF superfamily gene expression in the regulation of folliculogenesis and oogenesis in mammals: a review. Vet Med. 2013;58:505–15; DOI:10.17221/7082-VETMED.
5. Kranc W, Budna J, Kahan R, Chachuła A, Bryja A, Ciesiółka S, Borys S, Antosik P, Bukowska D, Brüssow KP, Bruska M, Nowicki M, Zabel M, Kempisty B. Molecular basis of growth, proliferation, and differentiation of mammalian follicular granulosa cells. J Biol Regul Homeost Agents. 2017;31:1–8.
6. Kossowska-Tomaszczuk K, De Geyter C, De Geyter M, Martin I, Holzgreve W, Scherberich A, Zhang H. The multipotency of luteinizing granulosa cells collected from mature ovarian follicles. Stem Cells. 2009;27:210–9; DOI:10.1634/stemcells.2008-0233.
7. Tepekoy F, Ozturk S, Sozen B, Ozay RS, Akkoyunlu G, Demir N. CD90 and CD105 expression in the mouse ovary and testis at different stages of postnatal development. Reprod Biol. 2015;15:195–204; DOI:10.1016/j.repbio.2015.10.004.
8. Okada H, Danoff TM, Kalluri R, Neilson EG. Early role of Fsp1 in epithelial-mesenchymal transformation. Am J Physiol. 1997;273:F563-574; DOI:10.1152/ajprenal.1997.273.4.F563.

9. Amin Marashi F, Torabi A, Beaudry F. Granulosa cells exposed to fibroblast growth factor 8 and 18 reveal early onset of cell growth and survival. Int J Reprod Biomed. 2019;17:435–42; DOI:10.18502/ijrm.v17i6.4815.
10. Brązert M, Kranc W, Celichowski P, Ożegowska K, Budna-Tukan J, Jeseta M, Pawelczyk L, Bruska M, Zabel M, Nowicki M, Kempisty B. Novel markers of human ovarian granulosa cell differentiation toward osteoblast lineage: A microarray approach. Mol Med Report. 2019; DOI:10.3892/mmr.2019.10709.
11. Kranc W, Brązert M, Budna J, Celichowski P, Bryja A, Nawrocki MJ, Ożegowska K, Jankowski M, Chermuła B, Dyszkiewicz-Konwińska M, Jeseta M, Pawelczyk L, Bręborowicz A, Rachoń D, Bruska M, Nowicki M, Zabel M, Kempisty B. Genes responsible for proliferation, differentiation, and junction adhesion are significantly up-regulated in human ovarian granulosa cells during a long-term primary in vitro culture. Histochem Cell Biol. 2019;151:125–43; DOI:10.1007/s00418-018-1750-1.
12. Chomczyński P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987;162:156–9; DOI:10.1016/0003-2697(87)90021-2.
13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2(−delta delta Ct) method. Methods. 2001;25:402–8; DOI:10.1006/meth.2001.1262.
14. Razali NM, Wah YB. Power comparisons of Shapiro-Wilk, Kolmogorov-Smirnov, Lilliefors and Anderson-Darling tests. J Stat Mod Anal. 2011;2:13.
15. Shapiro SS, Wilk MB. An Analysis of Variance Test for Normality (Complete Samples). Biometrika. 1965;52:591–611; DOI:10.2307/2333709.
16. Ai A, Tang Z, Liu Y, Yu S, Li B, Huang H, Wang X, Cao Y, Zhang W. Characterization and identification of human immortalized granulosa cells derived from ovarian follicular fluid. Exp Ther Med. 2019;18:2167–77; DOI:10.3892/etm.2019.7802.
17. Dodson WC, Schomberg DW. The effect of transforming growth factor-beta on follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. Endocrinology. 1987;120:512–6; DOI:2016092613452600865.
18. Mansouri-Attia N, Tripurani SK, Gokul N, Piard H, Anderson ML, Eldin K, Pangas SA. TGFβ signaling promotes juvenile granulosa cell tumorigenesis by suppressing apoptosis. Mol Endocrinol. 2014;28:1887–98; DOI:2020071613391521600.
19. Li Y, Xiang Y, Song Y, Wan L, Yu G, Tan L. Dysregulated miR-142, -33b and -423 in granulosa cells target TGFBR1 and SMAD7: a possible role in polycystic ovary syndrome. Mol Hum Reprod. 2019;25:638–46; DOI:10.1093/molehr/gaz014.
20. Nakerakanti S, Trojanowska M. The role of TGF-β receptors in fibrosis. Open Rheum J. 2012;6; DOI:10.2174/1874312901206010156.
21. Ríus C, Smith JD, Almendro N, Langa C, Botella LM, Marchuk DA, Vary CP, Bernabéu C. Cloning of the promoter region of human endoglin, the target gene for hereditary hemorrhagic telangiectasia type 1. Blood. 1998;92:4677–90.
22. Basini G, Falasconi I, Bussolati S, Grolli S, Di Lecce R, Grasselli F. Swine Granulosa Cells Show Typical Endothelial Cell Characteristics. Reprod Sci. 2016;23:630–7; DOI:10.1177/1933719115612130.
23. Lawson WE, Polosukhin VV, Zoia O, Stathopoulos GT, Han W, Plieth D, Loyd JE, Neilson EG, Blackwell TS. Characterization of fibroblast-specific protein 1 in pulmonary fibrosis. Am J Respir Crit Care Med. 2005;171:899–907; DOI:10.1164/rccm.200311-1535OC.
24. Okada H, Danoff TM, Fischer A, Lopez-Guisa JM, Strutz F, Neilson EG. Identification of a novel cis-acting element for fibroblast-specific transcription of the FSP1 gene. Am J Physiol. 1998;275:F306-314; DOI:10.1152/ajprenal.1998.275.2.F306.
25. Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, Neilson EG. Identification and characterization of a fibroblast marker: FSP1. J Cell Biol. 1995;130:393–405; DOI:10.1083/jcb.130.2.393.