C/EBPβ regulates Vegf gene expression in granulosa cells undergoing luteinization during ovulation in female rats

Masahiro Shinagawa, Isao Tamura, Ryo Maekawa, Shun Sato, Yuichiro Shirafuta, Yumiko Mihara, Maki Okada - Matsumoto, Toshiaki Taketani, Hiromi Asada, Hiroshi Tamura & Norihiro Sugino

The ovariatory LH-surge increases Vegf gene expression in granulosa cells (GCs) undergoing luteinization during ovulation. To understand the factors involved in this increase, we examined the roles of two transcription factors and epigenetic mechanisms in rat GCs. GCs were obtained from rats treated with eCG before, 4 h, 8 h, 12 h and 24 h after hCG injection. Vegf mRNA levels gradually increased after hCG injection and reached a peak at 12 h. To investigate the mechanism by which Vegf is up-regulated after hCG injection, we focused on C/EBPβ and HIF1α. Their protein expression levels were increased at 12 h. The binding activity of C/EBPβ to the Vegf promoter region increased after hCG injection whereas that of HIF1α did not at this time point. The C/EBPβ binding site had transcriptional activities whereas the HIF1α binding sites did not have transcriptional activities under cAMP stimulation. The levels of H3K9me3 and H3K27me3, which are transcriptional repression markers, decreased in the C/EBPβ binding region after hCG injection. The chromatin structure of this region becomes looser after hCG injection. These results show that C/EBPβ regulates Vegf gene expression with changes in histone modifications and chromatin structure of the promoter region in GCs undergoing luteinization during ovulation.

Angiogenesis is required for the corpus luteum (CL) formation. After the surge of luteinizing hormone (LH), endothelial cells in the theca cell layer proliferate and start to invade the granulosa cell layer during ovulation. This is the first process to form the CL. Endothelial cells rapidly form blood vessels and vascular network in the CL, which makes CL highly vascularized organs. This event is necessary for CL continuing to produce progesterone for establishing and maintaining pregnancy. In previous studies, angiogenic factors that are responsible for luteal development have been identified. Vascular endothelial growth factor (VEGF) is one of them and is highly expressed in rat and human CL. VEGF in the ovary is involved in physiologic development, maintenance and maturation of blood vessels and contributes to rapid vascularization during CL formation. Therefore, VEGF is one of important genes, which leads to CL development. The LH surge changes a number of gene expressions in granulosa cells (GCs) during ovulation. Although VEGF gene expression is increased during ovulation, the detailed molecular mechanism of this regulation has been unclear. It is well known that hypoxia inducible factor 1-alpha (HIF1α) induced by hypoxic conditions increases VEGF expression in many types of cells. However, it is unclear whether HIF1α also regulates VEGF expression in GCs undergoing luteinization.

CCAAT/enhancer-binding protein beta (C/EBPβ) is a transcription factor involved in follicle rupture and following CL formation. In C/EBPα and β knockout mice, the expressions of angiogenesis-related genes are suppressed and vascular development in CL is defective. These findings led us to hypothesize that Vegf expression is regulated by C/EBPβ in GCs during ovulation.

Not only transcription factors, but also epigenetic mechanisms regulate gene expression. Histone modification is one of them and affects the chromatin structure, which regulates the accessibility of transcription factors into gene promoter or enhancer region. In previous studies of GCs in rats undergoing luteinization, we...
reported that changes of histone modifications and chromatin remodeling are involved in the regulation of three luteinization-related genes: steroidogenic acute regulatory protein (StAR), aromatase (Cyp19a1) and cytochrome P450 cholesterol side-chain cleavage enzyme (Cyp11a1). These findings raised the possibility that Vegf expression is regulated not only by transcription factors but also by an epigenetic mechanism.

In this study, we showed that C/EBPβ, but not HIF1α, regulates Vegf expression in rat GCs undergoing luteinization.

Results

Vegf mRNA expression. Human chorionic gonadotropin (hCG) was injected to rats to induce luteinization. GCs were isolated from ovarian follicles. Vegf mRNA levels in the GCs gradually increased and reached a peak at 12 h after hCG injection (Fig. 1).

C/EBPβ and HIF1α protein expressions. We examined by Western blot analyses whether C/EBPβ and HIF1α protein expression levels were increased in rat GCs after hCG injection. C/EBPβ protein (Fig. 2A) and HIF1α protein (Fig. 2B) were expressed in rat GCs before luteinization (0 h) and were up-regulated by hCG injection (12 h).

Binding activity of C/EBPβ and HIF1α to the Vegf promoter. The JASPAR database lists consenus binding sequences for several transcription factors in the Vegf promoter, including C/EBPβ (−1115 bp to −1106 bp) and HIF1α (−913 bp to −906, −714 bp to −707 bp, −434 bp to −423 bp) (Fig. 3A). Because C/EBPβ and HIF1α protein levels were increased after hCG injection in rat GCs (Fig. 2), we hypothesized that C/EBPβ and HIF1α bind to the Vegf promoter region and regulate Vegf expression. Therefore, we designed chromatin immunoprecipitation (ChIP) primers surrounding these sites, −1148 bp to −1024 bp (C/EBPβ binding region), −976 bp to −857 bp (HIF1α binding region−1), −724 bp to −645 bp (HIF1α binding region−2) and −470 bp to −369 bp (HIF1α binding region−3), respectively (Fig. 3A). The binding activity of C/EBPβ to the Vegf promoter region was significantly increased in GCs 12h after hCG injection (Fig. 3B). The binding activities of HIF1α were observed in all HIF1α binding regions before luteinization (Fig. 3C, 0h). However, they were not increased 12h after hCG injection (Fig. 3C, 12h). These results indicate that C/EBPβ, but not HIF1α, regulates Vegf gene expression by binding to the promoter region in rat GCs undergoing luteinization.

Effect of C/EBPβ knockdown on VEGF mRNA expression. To elucidate the involvement of C/EBPβ in Vegf mRNA expression, we examined the effect of C/EBPβ knockdown on Vegf mRNA expression. Because the efficiency of knockdown in primary GCs is quite low, we used KGN cells (human granulosa tumor cells) that are widely used for experiments on luteinization22. Because KGN cells do not respond to hCG stimulation, cAMP, a second messenger of hCG, was used to induce luteinization in KGN cells as reported previously22,23. First, we confirmed that VEGF mRNA was increased by cAMP stimulation in KGN cells (Fig. 4A). Knockdown of C/EBPβ knockdown on VEGF mRNA expression. To elucidate the involvement of C/EBPβ in VEGF mRNA expression, we examined the effect of C/EBPβ knockdown on VEGF mRNA expression. Because the efficiency of knockdown in primary GCs is quite low, we used KGN cells (human granulosa tumor cells) that are widely used for experiments on luteinization22. Because KGN cells do not respond to hCG stimulation, cAMP, a second messenger of hCG, was used to induce luteinization in KGN cells as reported previously22,23. First, we confirmed that VEGF mRNA was increased by cAMP stimulation in KGN cells (Fig. 4A). Knockdown of C/EBPβ significantly decreased the VEGF mRNA expression level (Fig. 4B). These results indicate that C/EBPβ is involved in the regulation of Vegf gene expression in rat GCs undergoing luteinization.

Figure 1. Vegf mRNA expression in rat GCs undergoing luteinization. The relative mRNA levels of Vegf in GCs before (0 h) and 4, 8, 12, 24 h after hCG injection were assessed by qPCR. Gapdh was used as an internal control. Data were shown as a ratio of those of 0 h. Each value represents the mean ± SEM of 6 animals. a, P < 0.05 vs. 0 h. mRNA expression was also assessed by RT-PCR. The images of representative gels are shown.
We examined whether hCG stimulation changes the binding of EZH2, which induces H3K27me3, to the C/EBPβ binding region, as measured by ChIP assay, (Fig. 5A). When cells were transfected with the promoter construct (−1171 bp to +115 bp), CAMP significantly increased the luciferase activities. Deletion of the C/EBPβ binding site completely blocked it (the construct of −976 bp to +115 bp) and the CAMP-induced luciferase activities were also decreased to the basal level (without CAMP stimulation). Mutation of the HIF1α binding sites (the mutated construct of −1171 bp to +115 bp) did not affect either CAMP-increased or basal luciferase activities. We further examined the luciferase activities of the rat VEGF promoter region under the condition of C/EBPβ knockdown. Both the construct of −1171 bp to +115 bp and siRNA were transfected to KGN cells and then cells were treated with CAMP. As shown in Fig. 5B, the increase in luciferase activities by the stimulation of CAMP was completely blocked by the knockdown of C/EBPβ. We also examined the effect of CoCl2, which induces HIF1α protein expression, on the luciferase activities. CoCl2 increased the luciferase activities of the VEGF promoter region, and mutation of HIF1α binding sites blocked it (Supplementary Figure 1). These findings show that HIF1α binding sites in the rat VEGF promoter region have the transcriptional activities under CoCl2 stimulation, but not under CAMP stimulation.

Histone modifications, chromatin structure, and binding activities of enhancer of zeste homolog 2 (EZH2). Because not only transcription factors but also epigenetic mechanisms regulate gene expression,14–16 we examined in rat GCs whether hCG stimulation changes histone modification levels of the C/EBPβ binding region in the VEGF promoter. The levels of H3K9me3 and H3K27me3, which are correlated with the suppression of transcription, significantly decreased at 12 h after hCG injection. The level of H3K4me3, which is correlated with the activation of transcription, was not affected by hCG injection (Fig. 6A).

The chromatin remodeling of the C/EBPβ binding region after hCG injection were examined by FAIRE (formaldehyde-assisted isolation of regulatory elements)-qPCR. The relative FAIRE enrichment ratio in this region increased by hCG injection (Fig. 6B), indicating that hCG stimulation loosens the chromatin structure of the C/EBPβ binding region.

We examined whether hCG stimulation changes the binding of EZH2, which induces H3K27me3, to the C/EBPβ binding region. The EZH2 binding activity to the C/EBPβ binding region, as measured by ChiP assay, significantly decreased after hCG injection (Fig. 6C).

Discussion

The present study demonstrated that C/EBPβ is an important transcription factor regulating VEGF expression in rat GCs undergoing luteinization during ovulation. C/EBPβ is well known to be transcription factor necessary for ovulation,12, and belongs to the ERK-1/2 signaling pathway, which activates in GCs undergoing luteinization. We reported that C/EBPβ up-regulates the expression of STAR and Cyp11a1 and promotes the production of progesterone, which play central roles in follicle rupture and subsequent CL formation.13,20,21 Our results show a new finding that VEGF is one of downstream target genes of C/EBPβ in rat GCs after the LH surge, suggesting that C/EBPβ contributes to angiogenesis for CL formation by up-regulating VEGF expression.
This is the first report to show the involvement of C/EBPβ in the regulation of Vegf gene expression in rat GCs. The promoter region of the rat Vegf gene has the consensus binding sequence of C/EBPβ, which is highly conserved among species. However, this site has not been examined so far. C/EBPβ clearly binds to this site in rat GCs undergoing luteinization. In addition, by knocking down C/EBPβ protein in KGN cells, we demonstrated that C/EBPβ regulates the basal and cAMP-increased Vegf mRNA expressions. Taken together, these findings show that C/EBPβ up-regulates Vegf gene expression through the binding to the novel regulatory region in rat Vegf promoter region in GCs undergoing luteinization.

HIF1α has been reported to regulate Vegf gene expression by binding to the promoter region in many types of cells. HIF1α protein is expressed in granulosa cells before luteinization and is up-regulated by hCG stimulation, which is consistent with our findings. Because both VEGF and HIF1α expression show the same trend toward luteinization, HIF1α was thought to be involved in the increase of Vegf expression by luteinization. However, the direct evidences showing that the increase in Vegf expression by luteinization is mediated by HIF1α have not been reported so far. We proved by ChIP assay that the binding activities of HIF1α were observed in all HIF1α binding regions in rat Vegf promoter region before luteinization. However, these binding activities were not increased after hCG injection, which suggests that HIF1α does not contribute to the up-regulation of Vegf expression by hCG stimulation. In addition, we showed by luciferase assay that hypoxia response elements (HREs) in rat Vegf promoter region are not involved in the regulation of Vegf expression by cAMP. In our study, we did not show the involvement of HIF1α in the regulation of Vegf in our experimental condition including the use of KGN cells. However, we do not exclude the role of HIF1α in Vegf induction during luteinization, because HIF1α may regulate Vegf expression at different time points of luteinization process, or bind to an unknown promoter or enhancer region of Vegf gene undergoing luteinization in GCs. On the other hand, Rico et al. showed that HRE deficient mice responded to LH with an increase in Vegf expression in granulosa cells in vivo, suggesting that Vegf expression by LH is not regulated by HIF1α in mouse granulosa cells, which supports our findings. Kim et al. used Echinomycin, which inhibits the binding ability of HIF1α to HREs, and found that Echinomycin inhibits hCG-increased Vegf mRNA expression in mice granulosa cells. Although they concluded that hCG up-regulates Vegf expression through HIF1α, it should be noted that...
Echinomycin directly affects many gene expressions. Taken together, we concluded that C/EBPβ is an important factor to regulate Vegf gene expression in rat GCs undergoing luteinization after the LH surge.

Although there are several reports showing that HIF1α is associated with Vegf mRNA expression in GCs, most of them are based on the data from in vitro experiments done under the conditions of hypoxia or CoCl2 stimulation. Martinez-Chequer et al. reported that CoCl2 increased VEGF expression in monkey GCs. Alam et al. reported that CoCl2 induced HIF1α protein expression in rat GCs. Kim et al. showed that HIF1α is involved in VEGF expression induced by CoCl2 in mouse GCs. Yalu et al. showed the involvement of HIF1α in the CoCl2-induced VEGF expression by knocking down HIF1α in human GCs. Our results also showed that HIF1α contributes to VEGF expression under CoCl2 stimulation, but not under cAMP stimulation (Supplementary Figure 1). From these results, it is likely that HIF1α is involved in the regulation of Vegf expression in GCs under CoCl2 stimulation in vitro.

Our data also showed that epigenetic changes such as histone modifications and chromatin remodeling occurred in the Vegf promoter region in luteinizing rat GCs. These changes included decreases of H3K9me3 and H3K27me3 levels along with a decrease of EZH2 binding to the C/EBPβ binding region in the Vegf promoter all of which are associated with the activation of transcription by loosening the chromatin. This result is consistent with our previous findings that stimulating rats with hCG caused decreases in H3K9me3, H3K27me3 and EZH2 binding of the Cyp11a1 promoter region in GCs and increases in their mRNA expressions. Our results showed...
that hCG loosened the chromatin structure of the Vegf promoter region, allowing C/EBPβ to access its response element of the Vegf promoter region. These findings suggest that these epigenetic changes are closely associated with the up-regulation of Vegf expression in GCs undergoing luteinization. H3K4me3 is a histone modification related to active transcription. In our study, the levels of H3K4me3 were not changed by hCG stimulation. This is not surprising, because the changes of H3K4me3 levels are not always correlated with those of other histone modifications, and because gene expression is regulated by not only one histone modification, but also the combination of various histone modifications.

In summary, the present study shows a molecular mechanism by which Vegf is up-regulated in rat GCs undergoing luteinization after the LH surge. We found that C/EBPβ, but not HIF1α, regulates Vegf gene expression by binding to the novel binding site in the rat Vegf promoter region. In addition to transcription factors, histone modifications and chromatin structure of the Vegf promoter region are involved in the regulation of Vegf expression. Because Vegf plays a key role in angiogenesis, our results should help to better understand the regulation of angiogenesis in GCs undergoing luteinization during ovulation.

Methods
This study was reviewed and approved by The Committee for the Ethics on Animal Experiment in Yamaguchi University Graduate School of Medicine. All experiments were performed in accordance with relevant guidelines and regulations.

Animal models. Female Sprague Dawley rats (21- to 24-day old) were purchased from Japan SLC (Hamamatsu, Japan). They were injected subcutaneously with 15 IU of equine chorionic gonadotropin (eCG) (Sigma, St. Louis, MO, USA) to promote follicular growth followed by 15 IU of hCG (Sigma) injection to induce ovulation and luteinization. The ovaries were obtained before hCG (0), and 4, 8, 12, and 24 hours (h) after hCG injection. The follicles were punctured to isolate GCs. Cells were centrifuged and washed in PBS and provided for the experiments.

Cell culture. KGN cells (Nikon, Tokyo, Japan), which were derived from a human granulosa cell tumor, were cultured in DMEM/Ham's F-12 medium.

Figure 5. Transcriptional activities of the C/EBPβ binding site in rat Vegf promoter region. (A) The reporter constructs, −1171 bp to +115 bp, −976 bp to +115 bp (ΔC/EBPβ) and −1171 bp to +115 bp (mutation of HREs) were transfected into KGN cells. After 24 h of transfection, cells were treated with and without cAMP for 24 h. The firefly luciferase activity was normalized according to Renilla luciferase activities. Values of the luciferase activities were expressed as a ratio of control treatment with −1171 bp to +115 bp. Each value represents the mean ± SEM of 3 independent incubations. a, P < 0.05 vs. control treatment of the construct of −1171 bp to +115 bp. b, P < 0.05 vs. control treatment of the construct of −1171 bp to +115 bp. c, P < 0.05 vs. cAMP treatment of the construct of −1171 bp to +115 bp. d, P < 0.05 vs. control treatment of the construct of −1171 bp to +115 bp (mutation of HREs). (B) The reporter construct of −1171 bp to +115 bp and siRNA (C/EBPβ siRNA or non-targeting siRNA) were transfected into KGN cells. After 24 h of transfection, cells were treated with and without cAMP for 24 h. The firefly luciferase activity was normalized according to Renilla luciferase activities. Values of the luciferase activities were expressed as a ratio of control treatment with −1171 bp to +115 bp. Each value represents the mean ± SEM of 3 independent incubations. a, P < 0.05 vs. control treatment. b, P < 0.05 vs. cAMP treatment in the control siRNA.
Quantitative real-time PCR. RNA was extracted with RNeasy Mini (QIAGEN, Chatsworth, CA, USA) and was converted to cDNA with reverse transcriptase (Invitrogen, Waltham, MA, USA) as reported previously. Quantitative real-time PCR reaction was carried out as described previously. Primer sequences are listed in Table 1.

Western blotting. Total protein were extracted and subjected to SDS-PAGE and then transferred to membrane as described previously. The membranes were incubated for overnight with the following antibodies: C/EBPβ (Santa Cruz Biotechnology), HIF1α (Novus Biologicals, USA) and Histone H3 (Cell Signaling Technology, Tokyo, Japan). Amersham ECL Western Blotting Detection Reagent and hyperfilm-ECL (GE health care UK Ltd., Buckinghamshire, UK) were used to detect immunoblot bands.

ChIP assay. The histone modification levels and recruitments of transcription factors were examined by ChIP assays as described previously with some modifications. The following antibodies were used in this assay: H3K4me3 (Upstate Biotechnology, Lake placid, NY, USA), H3K9me3 (Abcam, Cambridge, UK, USA), H3K27me3 (generous gift from Dr. Kimura), EZH2 (Cell Signaling Technology), C/EBPβ, HIF1α and non-specific normal IgG (Cell Signaling Technology). Real-time qPCR was performed with primers listed in Table 1 to calculate the relative enrichment.
Transfection of siRNA. Transfection of siRNAs was performed as reported previously\(^{46}\). After 24 h of transfection, KGN cells were incubated with or without dibutyryl-cAMP (cAMP, 0.5 mM) (Sigma) for 24 h.

Luciferase assay. Three 5′-flanking regions of the rat VEGF gene; 1) the construct including both C/EBPβ binding sites and HIF1α binding sites (−1171 bp to +115 bp), 2) the construct lacking the C/EBPβ binding site (−976 bp to +115 bp) and 3) the construct that four HIF1α binding sites are mutated (−1171 bp to +115 bp), were amplified using the primer sets in Table 1. These constructs were inserted to pGL3 basic vector (Promega, Tokyo, Japan). Transfection of the constructs were performed as described\(^{20}\) and then cells were incubated with or without CAMP (0.5 mM) or CoCl\(_2\) (0.5 mM) for 24 h. Luciferase activities were examined as described\(^{29}\). Luciferase assays combined with C/EBPβ knockdown were performed as reported previously\(^{23}\). The reporter construct (the construct of −1171 bp to 115 bp) and siRNA (C/EBPβ) or non-targeting siRNA) were transfected simultaneously. Then, KGN cells were incubated with or without cAMP and luciferase activities were examined. Luciferase assays were done in triplicate and repeated three times.

FAIRE-qPCR. To examine the chromatin accessibility of C/EBPβ binding region in the VEGF promoter, FAIRE-qPCR was performed as described\(^{47,48}\) with some modifications. Real-time qPCR was performed to determine and calculate the FAIRE enrichment with same primers used in ChIP assay (Table 1).

Statistical analyses. Unpaired student t-test was used to compare the mean values in two groups. To analyze differences between groups, one-way ANOVA followed by Tukey-Kramer test was used. P < 0.05 was considered significant.

References
1. Fraser, H. M. & Duncan, W. C. SRB Reproduction, Fertility and Development Award Lecture 2008. Regulation and manipulation of angiogenesis in the ovary and endometrium. Reproduction, Fertility and Development 21, 377–392 (2009).
2. Sugino, N., Matsuoka, A., Taniguchi, K. & Tamura, H. Angiogenesis in the human corpus luteum. Reproductive Medicine and Biology 9, 91–103 (2008).
3. Kizuka, F. et al. Involvement of bone marrow-derived vascular progenitor cells in neovascularization during formation of the corpus luteum in mice. Biol Reprod 87, 55 (2012).
4. Ferrara, N. et al. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nature medicine 4, 336–340 (1998).
5. Phillips, H. S., Hains, J., Leung, D. W. & Ferrara, N. Vascular endothelial growth factor is expressed in rat corpus luteum. Endocrinology 127, 965–967 (1990).
6. Ferrara, N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. American journal of physiology. Cell physiology 280, C1358–1366 (2001).

Table 1. Primers Used in This Study.

| Gene                                    | Primer (5′ to 3′)                                      | Amplification Size (bp) |
|-----------------------------------------|------------------------------------------------------|------------------------|
| RT-PCR                                  |                                                      |                        |
| VEGF (rat)                              | For, CACCTGGCAACCTGGCTTACT Rev, GAGCTCCTAATGACCGCTCA | 111                    |
| HIF1α (rat)                             | For, TGTTGCTAAACAGATGATGGGA Rev, CATGTGACATTGACGTTA  | 123                    |
| Gapdh (rat)                             | For, CTCAAGCAGCAATCCAAGCC Rev, TTGATCCGTGAGTTGGTTA   | 155                    |
| VEGF (human)                            | For, CCATGCTGCTTCACCTGCAC Rev, GCAGTCTGCGCAGTATGTA  | 119                    |
| GAPDH (human)                           | For, AGGTGAAGGTGCCGAGAC Rev, GTCTTCAATGACGCAAGAC   | 99                     |
| ChIP-qPCR                               |                                                      |                        |
| VEGF promoter of C/EBPβ binding region | For, ATGCCTACCGAGGACCGCTCCTCTTCCTCCCA Rev, ACTAAAGCACGGTGGGCAAAT | 125                    |
| VEGF promoter of HIF1α binding region 1 | For, GAAGAGCAGGCTCTCCTCTGG Rev, GGAAAGCGAGCAAGTGTCAA | 120                    |
| VEGF promoter of HIF1α binding region 2 | For, TACTTTCCCTTCCTCAGGCC Rev, CCCACAGTGTGCTGTTGTA | 80                     |
| VEGF promoter of HIF1α binding region 3 | For, GAGCTCAGTGAGGAAGAGAC Rev, CAGTGTCTCTCCTCCATCTC | 102                    |
| Luciferase assay                        |                                                      |                        |
| −1171/+115 (ΔC/EBPβ)                    | For, GGTACCCGAGGAGCCATGCTCCTGGCAAAAT Rev, AAGCTTTGCGATGATGCCTGAAT | 1286                   |
| −976/+115 (ΔC/EBPβ)                     | For, GGTACCCGAGGAGCCATGCTCCTGGCAAAAT Rev, AAGCTTTGCGATGATGCCTGAAT | 1091                   |
| −1171/+115 (mutation of HREs)-1         | For, GCACTACTCTGCTCCTCCCAAGCTGAGTCTTCTCCCTCG Rev, GAAGCCGAGACTGCTGCTGACGAGAG | 1286                   |
| −1171/+115 (mutation of HREs)-2         | For, GGCTTCCAGTGGCCCCCGCCGATA Rev, GGCTTCCAGTGGCCCCCGCCGATA | 1286                   |
| −1171/+115 (mutation of HREs)-3,4       | For, GTGCGTCTCTCTCATGCTGAGGTGTGTTGATGATGTTG Rev, CATGAGAGAGACACTACAGTCCAGTGCAGTCCCTC | 1286                   |

Table of Primers Used in This Study.
7. Berisha, B., Schams, D., Rodler, D. & Pfäffli, M. W. Angiogenesis in The Ovary - The Most Important Regulatory Event for Follicle and Corpus Luteum Development and Function in Cow - An Overview. Anat Histol Embryol 45, 124–130 (2016).

8. Wissing, M. L. et al. Identification of new ovulation-related genes in humans by comparing the transcriptome of granulosa cells before and after ovulation triggering in the same controlled ovarian stimulation cycle. Human Reproduction 29, 997–1010 (2014).

9. Watkins, W. M. et al. Hypoxia-induced expression of VEGF splice variants and protein in four retinal cell types. Exp Eye Res 116, 240–246 (2013).

10. Kim, J., Bagchi, I. C. & Bagchi, M. K. Signaling by hypoxia-inducible factors is critical for ovulation in mice. Endocrinology 150, 3392–3400 (2009).

11. Loureiro, R. M. & D’Amore, P. A. Transcriptional regulation of vascular endothelial growth factor in cancer. Cytokine Growth Factor Rev 16, 77–89 (2005).

12. Fan, H. Y., Liu, Z., Johnson, P. F. & Richards, J. S. CCAAT/enhancer-binding proteins (C/EBP)-alpha and -beta are essential for ovulation, luteinization, and the expression of key target genes. Mol Endocrinol 25, 253–268 (2011).

13. Lee, L. et al. Changes in histone modification and DNA methylation of the SSR and Cyp19a1 promoter regions in granulosa cells undergoing luteinization during ovulation in rats. Endocrinology 154, 458–470 (2013).

14. Maekawa, R. et al. Genome-wide DNA methylation analysis reveals a potential mechanism for the pathogenesis and development of uterine leiomyomas. PLoS One 8, e66632 (2013).

15. Sato, S. et al. Potential mechanisms of aberrant DNA hypomethylation on the x chromosome in uterine leiomyomas. The Journal of reproduction and development 60, 47–54 (2014).

16. Tamura, I. et al. Genome-wide analysis of histone modifications in human endometrial stromal cells. Mol Endocrinol 28, 1656–1669 (2014).

17. Dogan, N. et al. Occupancy by key transcription factors is a more accurate predictor of enhancer activity than histone modifications or chromatin accessibility. Epigenetics Chromatin 8, 16 (2015).

18. Tamura, I. et al. Importance of C/EBPbeta binding and histone acetylation status in the promoter regions for induction of IGFBP-1, PRL, and Mn-SOD by cAMP in human endometrial stromal cells. Endocrinology 155, 275–286 (2014).

19. Li, B., Carey, M. J. & Workman, J. L. The role of chromatin during transcription. Cell 128, 707–719 (2007).

20. Okada, M. et al. Epigenetic Changes of the Cyp11a1 Promoter Region in Granulosa Cells Undergoing Luteinization During Ovulation in Female Rats. Endocrinology 157, 3344–3354 (2016).

21. Maekawa, R. et al. Changes in gene expression of histone modification enzymes in rat granulosa cells undergoing luteinization during ovulation. J Ovarian Res 9, 15 (2016).

22. Nishi, Y. et al. Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. Endocrinology 142, 437–445 (2001).

23. Mizutani, T., Takashita, T. et al. C/EBPbeta (CCAT/enhancer-binding protein beta) mediates progestosterone production through transcriptional regulation in co-operation with SF-1 (steroidogenic factor-1). The Biochemical journal 460, 459–471 (2014).

24. Yalu, R., Oyesiji, A. E., Eisenberg, I., Imbar, T. & Meidan, R. HIF1A-dependent increase in endothelin 2 levels in granulosa cells: role of hypoxia, LH/cAMP, and reactive oxygen species. Reproduction (Cambridge, England) 149, 11–20 (2015).

25. Cao, R. et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science (New York, N.Y.) 298, 1039–1043 (2002).

26. Czermak, B. et al. Drosophilia enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 111, 185–196 (2002).

27. Rosenbloom, K. R. et al. The UCSC Genome Browser database: 2015 update. Nucleic Acids Res 43, D670–681 (2015).

28. Goldberg, M. A. & Schneider, T. J. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. The Journal of biological chemistry 269, 4355–4359 (1994).

29. Forsythe, J. A. et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16, 4604–4613 (1996).

30. Boonyaprapob, U., Gadsby, J. E., Hedgpeth, V., Routh, P. A. & Almond, G. W. Expression and localization of hypoxia inducible factor-alpha mRNA in the porcine ovary. Canadian Journal of veterinary research - Revue canadienne de recherche vétérinaire 69, 215–222 (2005).

31. Nishimura, R. & Okuda, K. Hypoxia is important for establishing vascularization during corpus luteum formation in cattle. The Journal of reproduction and development 56, 110–116 (2010).

32. van den Driesche, S., Myers, M., Gay, E., Thong, K. J. & Duncan, W. C. HCG up-regulates hypoxia inducible factor-1 alpha in luteinized granulosa cells: implications for the hormonal regulation of vascular endothelial growth factor A in the human corpus luteum. Mol Hum Reprod 14, 455–464 (2008).

33. Tam, K. K. et al. Hormonally regulated follicle differentiation and luteinization in the mouse is associated with hypoxia inducible factor activity. Mol Cell Endocrinol 327, 47–55 (2010).

34. Duncan, W. C., van den Driesche, S. & Fraser, H. M. Inhibition of vascular endothelial growth factor in the primate ovary up-regulates hypoxia-inducible factor-1alpha in the follicle and corpus luteum. Endocrinology 149, 3313–3320 (2008).

35. Rico, C. et al. HIF1 activity in granulosa cells is required for FSH-regulated Vegfa expression and follicle survival in mice. Biol Reprod 90, 135 (2014).

36. Martinez-Chequer, J. C., Stouffer, R. L., Hazzard, T. M., Patton, P. E. & Molskness, T. A. Insulin-like growth factors-1 and -2, but not hypoxia, synergize with gonadotropin hormones to promote vascular endothelial growth factor-A secretion by monkey granulosa cells from preovulatory follicles. Biol Reprod 68, 1112–1118 (2003).

37. Alam, H. et al. Role of the phosphatidylinositol-3-kinase and extracellular regulated kinase pathways in the induction of hypoxia-inducible factor (HIF)-1 activity and the HIF-1 target vascular endothelial growth factor in ovarian granulosa cells in response to follicle-stimulating hormone. Endocrinology 150, 915–928 (2009).

38. Tamura, I. et al. Differential effects of progesterone on COX-2 and Mn-SOD expressions are associated with histone acetylation status of the promoter region in human endometrial stromal cells. J Clin Endocrinol Metab 96, E1073–1082 (2011).

39. Rada-Iglesias, A. et al. A unique chromatin signature uncovers early developmental enhancers in humans. Nature 470, 279–283 (2011).

40. Barski, A. et al. High-resolution profiling of histone methylations in the human genome. Cell 129, 823–837 (2007).

41. Tamura, I. et al. Induction of IGFBP-1 expression by CAMP is associated with histone acetylation status of the promoter region in human endometrial stromal cells. Endocrinology 153, 5612–5621 (2012).

42. Matsuoka, A. et al. Progesterone increases manganese superoxide dismutase expression via a CAMP-dependent signaling mediated by noncanonical Wnt5a pathway in human endometrial stromal cells. J Clin Endocrinol Metab 95, E291–299 (2010).

43. Tamura, I. et al. Novel Function of a Transcription Factor WT1 in Regulating Decidualization in Human Endometrial Stromal Cells and Its Molecular Mechanism. Endocrinology 158, 3696–3707 (2017).

44. Nishimoto, Y. et al. Decreased carbonyl reductase 1 expression promotes tumor growth via epithelial mesenchymal transition in uterine cervical squamous cell carcinomas. Reprod Med Biol 17, 173–181 (2018).

45. Maekawa, R. et al. Tissue-Specific Expression of Estrogen Receptor 1 Is Regulated by DNA Methylation in a T-DMR. Mol Endocrinol 30, 335–347 (2016).

46. Tamura, I. et al. The distal upstream region of insulin-like growth factor-binding protein 1 enhances its expression in endometrial stromal cells during decidualization. The Journal of biological chemistry 293, 5270–5280 (2018).
47. Simon, J. M., Giresi, P. G., Davis, I. J. & Lieb, J. D. Using formaldehyde-assisted isolation of regulatory elements (FAIRE) to isolate active regulatory DNA. *Nat Protoc* **7**, 256–267 (2012).

48. Arase, M. *et al.* Dynamics of chromatin accessibility during TGF-beta-induced EMT of Ras-transformed mammary gland epithelial cells. *Sci Rep* **7**, 1166 (2017).

**Acknowledgements**

This work was supported in part by JSPS KAKENHI (Grant Number 15K10720, 16K11091, 16K11142, 16K20191, 17K11239, 17K11240 and 18K16772). We thank Dr. Hiroshi Kimura (Tokyo Kogyo University, Tokyo, Japan) for the gift of anti-H3K27me3 antibody.

**Author Contributions**

M.S., I.T. and N.S. designed the study. M.S., I.T., S.S, Y.S., Y.M. and M.O.-M. performed the assays. S.S., R.M., T.T., H.A. and H.T. analyzed the data. M.S. and I.T. drafted the first manuscript. N.S. directed the research and drafted the final manuscript. All authors approved the final manuscript.

**Additional Information**

**Supplementary information** accompanies this paper at https://doi.org/10.1038/s41598-018-36566-y.

**Competing Interests:** The authors declare no competing interests.

**Publisher's note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019