Wilms tumor 1 regulates lipid accumulation in human endometrial stromal cells during decidualization

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We previously reported that the transcription factor Wilms tumor 1 (WT1) regulates the expression of insulin-like growth factor-binding protein-1 (IGFBP-1) and prolactin (PRL) during decidualization of human endometrial stromal cells (ESCs). However, other roles of WT1 in decidualization remain to be fully clarified. Here, we investigated how WT1 regulates the physiological functions of human ESCs during decidualization. We incubated ESCs isolated from proliferative-phase endometrium with cAMP to induce decidualization, knocked down WT1 expression during decidualization, and compared the transcriptome data obtained among these three treatments. We observed that WT1 up-regulates 121 genes during decidualization, including several genes involved in lipid transport. The WT1 knockdown inhibited lipid accumulation (LA) in the cAMP-induced ESCs. To examine the mechanisms by which WT1 regulates LA, we focused on very low-density lipoprotein receptor (VLDLR), which is involved in lipoprotein uptake. We found that cAMP up-regulates VLDLR and that the WT1 knockdown inhibits it. Results of ChIP assays revealed that cAMP increases the recruitment of WT1 to the promoter region of the VLDLR gene, indicating that WT1 regulates VLDLR expression. Moreover, VLDLR knockdown inhibited cAMP-induced LA, and VLDLR overexpression reverted the suppression of LA caused by the WT1 knockdown. Taken together, our results indicate that WT1 enhances lipid storage by up-regulating VLDLR expression in human ESCs during decidualization.

Wilms tumor 1 (WT1) encodes an essential transcription factor regulating mammalian urogenital development (1). WT1-knockout mice show embryonic lethality and failure of kidney and gonad development (2). Because mutations in the WT1 gene are associated with malformation of the uterus, WT1 also has an important role in uterine development (3, 4). In addition to its expression in the embryonic uterus, WT1 is also expressed in adult human uterus (5–7), although its role in the adult endometrium is unclear. Recently, we reported that the expression level of WT1 is increased upon decidualization in human ESCs and contributes to inducing the expressions of insulin-like growth factor-binding protein-1 (IGFBP-1) and prolactin (PRL) during decidualization (7), which are well-established decidualization markers (8, 9). We also revealed that WT1 expression during decidualization is regulated by CCAAT enhancer-binding protein β (C/EBPβ), which is another important transcription factor for decidualization (7, 10).

During decidualization, a number of genes are up- or down-regulated in ESCs (11, 12). In other words, a number of cellular functions are altered in ESCs during decidualization. WT1 contributes to decidualization by up-regulating the expressions of IGFBP-1 and PRL (7). Therefore, WT1 might also be involved in the regulation of other cellular functions as a transcription factor during decidualization in human ESCs. It is interesting to note that dramatic metabolic changes occur in ESCs during decidualization (11, 13, 14). WT1 is involved in the regulation of cell metabolisms in hepatocellular carcinoma cells and osteosarcoma cells (15, 16). These facts led us to hypothesize that WT1 regulates some metabolic functions of ESCs undergoing decidualization.

Blastocyst implantation and subsequent placentation is tightly regulated by a trophoblast-endometrial microenvironment (17). Decidualized ESCs have been implicated as important sources of histotrophic support for the early conceptus (18, 19). Lipid is one essential for nutrition for the development of embryos (20). It was also reported that lipid droplet accumulation occurs in ESCs during decidualization (21). These facts suggest that decidualized ESCs work as a histotrophic storage of lipids for the early conceptus. However, it is unclear how lipid accumulation (LA) is regulated during decidualization.

VLDLR, very low-density lipoprotein receptor; GPDH, glycerol-3-phosphate dehydrogenase; EGR-1, early growth response-1; FABP, fatty acid-binding protein; FATP, fatty acid transport protein; qPCR, quantitative PCR; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; Bt,cAMP, dibutyryl cyclic AMP; IP, immunoprecipitated.
**WT1 regulates lipid accumulation during decidualization**

In this study, we performed a microarray analysis and identified a number of genes up- or down-regulated by WT1 during decidualization of human ESCs. Because several of these genes were involved in lipid metabolism, we focused on lipid metabolisms in ESCs. We found a novel function of WT1 in which it enhances LA in ESCs by up-regulating the expression of very low-density lipoprotein receptor (VLDLR) during decidualization.

**Results**

**WT1-regulated genes in ESCs undergoing decidualization**

First, we investigated the genes regulated by WT in human ESCs during decidualization. For this purpose, ESCs were transfected with WT1 siRNA and then treated with or without dibutyryl cAMP, a decidualization stimulus, for 4 days to induce decidualization (22). Total RNAs were extracted from the three groups (no treatment with control siRNA, cAMP treatment with control siRNA, and cAMP treatment with WT1 siRNA) and subjected to transcriptome analysis to identify differentially expressed genes among these groups. cAMP up-regulated 631 genes, 121 of which were suppressed by WT1 knockdown. The 121 genes were designated as WT1 up-regulated genes during decidualization. cAMP also down-regulated 1251 genes, 149 of which were suppressed by WT1 knockdown. The 149 genes were designated as WT1 down-regulated genes during decidualization. To identify potential roles of WT1 in decidualization, the up- and down-regulated genes were subjected to gene ontology and pathway analyses. In the WT1 up-regulated genes, some well-known terms associated with decidualization, such as “response to hypoxia,” “embryo implantation,” “female pregnancy,” “angiogenesis,” and “PI3K-Akt signaling pathway” were identified as enriched (Table S1). In addition, terms associated with cellular transport (“lipid transport,” “glycoprotein transport,” “transmembrane transport,” and “ABC transporters”) were identified. Because these terms included several genes involved in the transport of lipids, we hypothesized that WT1 plays a key role in the cellular transport of lipid during decidualization. Therefore, we focused on the lipid metabolism in ESCs during decidualization. Some of the terms associated with the WT1 down-regulated genes included cell cycle and cell proliferation (Table S2). These terms included genes that positively regulate cell proliferation, such as the cyclin family genes (CDK, CCND, and CCNE) (23).

**Involvement of WT1 in LA in ESCs during decidualization**

We examined changes in the accumulation of lipid in ESCs during decidualization. ESCs were treated with or without cAMP to induce decidualization. We confirmed that cAMP treatment significantly increased intracellular cAMP levels (Fig. S1). Lipid droplets were stained with a cell-permeable lipophilic fluorescent dye, BODIPY 493/503. Lipid droplet accumulation was significantly increased by cAMP (Fig. 1A). We also examined changes in the intracellular triglyceride level during decidualization. It was also significantly increased by cAMP (Fig. 1A, lower panel). Because activation of lipogenesis or suppression of lipolysis increase the lipid accumulation, we examined whether the abilities of lipogenesis and lipolysis are altered by cAMP. The increase of glycerol-3-phosphate dehydrogenase (GPDH) activity in the cells indicates the activation of lipogenesis (24), and the increase of glycerol concentration in the culture medium indicates the activation of lipolysis (25). However, these parameters were not altered by cAMP treatment (Fig. 1B). These results suggest that the increase of lipid uptake into ESCs is responsible for LA during decidualization.

To examine whether the LA during decidualization of cAMP are mediated by WT1, ESCs were transfected with WT1 siRNA. cAMP increased WT1 protein expression and this increase was suppressed by siRNA treatment (Fig. 1C, upper panel). The expression levels of IGFBP-1 and PRL, which are specific markers of decidualization, were increased by cAMP, and these increases were inhibited by WT1 knockdown (Fig. 1C, lower panel). Lipid droplet accumulation and intracellular triglyceride level were increased by cAMP, and these increases were abolished by WT1 knockdown (Fig. 1D). These results showed that WT1 not only regulates marker gene expressions, but also contributes to LA in ESCs during decidualization.

**Regulation of VLDLR expression by WT1 during decidualization**

Our microarray analysis showed that VLDLR, which belongs to gene ontology term “lipid transport” (Table S1), was one of the genes up-regulated by WT1 during decidualization. Because VLDLR mediates uptake of triglyceride-rich lipoproteins and contributes to LA in cells (26, 27), we hypothesized that VLDLR is involved in the LA mediated by WT1 during decidualization. First, mRNA expression of VLDLR was examined at days 2 and 4 after cAMP stimulation. cAMP increased VLDLR mRNA expression in a time-dependent manner, and significantly increased it at day 4 (Fig. 2A). This increase was inhibited by WT1 knockdown, in agreement with the microarray result (Fig. 2B). This was confirmed at the protein level by Western blotting (Fig. 2C). To show that VLDLR expression actually increases by decidualization in vivo, we immunohistochemically examined VLDLR expression in human endometrium. The VLDLR expression level was low in stromal cells obtained from the proliferative phase endometrium (Fig. 2D). Stronger expression of VLDLR was observed in the stromal cells in the late secretory phase endometrium, which were morphologically identified as predecidual cells (Fig. 2D). These results show that VLDLR expression increases during decidualization and the increase is regulated by WT1.

**Recruitment of WT1 to the VLDLR promoter region by decidualization**

To clarify the mechanism by which WT1 regulates VLDLR mRNA expression during decidualization, we searched the promoter and enhancer regions of VLDLR for potential WT1-binding sites. WT1 binds to the 9-bp DNA sequence GCG(G/T)GGCG, which is similar to the consensus binding sequence recognized by early growth response-1 (EGR-1) (28, 29). Therefore, we searched ChIP-sequence data in the Encyclopedia of DNA Elements (ENCODEx) project (30) for EGR-1 binding signals around the VLDLR gene in MCF-7 cells, which is a human...
breast cancer cell line that highly expresses WT1 (31). As shown in Fig. 3A, EGR-1 binding signals were observed in the 5’ upstream promoter region (−511 to −338 bp) of the VLDLR gene. The DNA sequences around the binding signals were submitted to the JASPAR database (SCR_003030), which predicted the consensus binding sequence of WT1. Then, ChIP-qPCR primers were designed to cover the predicted sequence to examine the recruitment of WT1 (Fig. 3A). ChIP-qPCR revealed that cAMP increased the binding of WT1 to the VLDLR promoter region in a time-dependent manner, and significantly increased it at day 4 (Fig. 3B). To examine whether WT1 directly regulates VLDLR expression, ESCs were infected with retroviruses encoding WT1 (WT1 cells) or mCherry as a control (mock cells) and then the transcriptional activities of the VLDLR promoter region were examined by a luciferase assay. WT1 overexpression significantly increased the transcriptional activity of the VLDLR promoter region (Fig. 3C). These data showed that WT1 directly up-regulates VLDLR expression by binding to the promoter region during decidualization.
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**Involvement of a WT1-VLDLR pathway in LA in ESCs during decidualization**

Because VLDLR is under the regulation of WT1, we hypothesized that WT1 regulates LA by up-regulating VLDLR during decidualization. To examine whether VLDLR is involved in LA, VLDLR expression was knocked down by VLDLR siRNA (Fig. 4A, top panel). Lipid droplet accumulation and intracellular triglyceride levels were increased by cAMP, and these increases were inhibited by VLDLR knockdown (Fig. 4A), suggesting that VLDLR contributes to LA in ESCs under cAMP stimulation. Furthermore, to show that a WT1-VLDLR pathway is actually working for LA, we examined whether the decreased LA by WT1 knockdown can be rescued by compensating for VLDLR expression. For this purpose, ESCs were infected with retroviruses encoding VLDLR (VLDLR cells) or mCherry as a control (mock cells) (Fig. 4B, top panel). These cells were transfected with WT1 siRNA, and were cultured in the presence or absence of cAMP. In VLDLR cells, the decreased VLDLR expression caused by WT1 knockdown was fully rescued (Fig. 4B, top panel). As shown in Fig. 4B, VLDLR overexpression alone did not increase lipid droplet accumulation and intracellular triglyceride level in control cells (without cAMP stimulation), showing that both VLDLR expression and cAMP stimulation are needed for LA in ESCs. In mock cells, cAMP increased lipid

**Figure 2. Regulation of VLDLR expression by WT1 during decidualization.** A, mRNA expression of VLDLR was examined at days 2 and 4 after cAMP stimulation by quantitative real-time RT-PCR. Values were normalized to those of MRPL19 and expressed as a ratio of control sample at day 2. Data are mean ± S.D. of three different incubations. a, p < 0.05 versus control treatment at day 4. B, ESCs were transfected with an siRNA targeted against WT1 or with a nontargeting siRNA as a control. 48 h after siRNA transfection, ESCs were treated with or without cAMP for 4 days. mRNA expression was analyzed by quantitative real-time RT-PCR. Values of VLDLR were normalized to those of MRPL19 and expressed as a ratio of the control sample. Data are mean ± S.D. of three different incubations. a, p < 0.01 versus control treatment in the control siRNA; b, p < 0.05 versus cAMP treatment in the control siRNA. C, whole-cell lysates were prepared and subjected to Western blotting to examine VLDLR protein expression. β-Tubulin was used as an internal control. The immunoblot is representative of three different incubations. D, immunohistochemical expression of VLDLR in the late proliferative and late secretory phase endometrium. The photographs in the lower row are negative controls in each sample. Scale bars, 50 μm.

**Figure 1. Involvement of WT1 in LA in ESCs during decidualization.** A, ESCs were treated with or without cAMP (0.5 mM) for 4 days. Cells treated without cAMP were used as the control. Lipid droplets in ESCs were stained with a lipophilic fluorescence dye, BODIPY 493/503 (green), and nuclei were stained with 4,6-diamidino-2-phenylindole (blue). Representative images of three different incubations are shown (top panel: lower magnification; scale bars, 100 μm. Middle panel: higher magnification, scale bars, 20 μm). The mean staining area of the lipid droplet per cell was calculated and quantified using BZ-X Analyzer software. Values of staining area were expressed as a ratio of control sample (bottom panel). The levels of intracellular triglyceride in ESCs were measured using an adipogenesis assay kit. Values were normalized to those of total protein concentration of the cultured cells and expressed as a ratio of control sample (lower panel). Data are mean ± S.D. of three different incubations. a, p < 0.01 versus control. B, ESCs were treated with or without cAMP (0.5 mM) for 4 days. Cells treated without cAMP were used as the control. The GPDH activities in ESCs were measured using a GPDH assay kit to analyze the ability of lipogenesis (left panel). The levels of glycerol in the culture medium of ESCs were measured using a free glycerol assay kit to analyze the ability of lipolysis (right panel). Values were normalized to those of total protein concentration of the cultured cells and expressed as a ratio of control sample. Data are mean ± S.D. of three different incubations. C, ESCs were transfected with an siRNA targeted against WT1 or with a nontargeting siRNA as a control. 48 h after siRNA transfection, ESCs were treated with or without cAMP for 4 days. Whole-cell lysates were prepared and subjected to Western blotting to confirm the knockdown of WT1 protein expression. β-Tubulin was used as an internal control. The immunoblot is representative of three different incubations (upper panel). mRNA expression of IGFBP-1 and PRL was analyzed by quantitative real-time RT-PCR. Values were normalized to those of MRPL19 and expressed as a ratio of control sample. Data are mean ± S.D. of three different incubations (lower panel). a, p < 0.01 versus control treatment in the control siRNA; b, p < 0.01 versus cAMP treatment in the control siRNA. D, lipid droplets in ESCs were stained with BODIPY 493/503 (green) and nuclei were stained with 4,6-diamidino-2-phenylindole (blue). Representative images of three different incubations are shown (top panel). Scale bars, 100 μm. Values of the staining area were expressed as a ratio of control sample (middle panel). The levels of intracellular triglyceride in ESCs were measured. Values were normalized to those of total protein concentration of the cultured cells and expressed as a ratio of control sample (bottom panel). Data are mean ± S.D. of three different incubations. a, p < 0.01 versus control in the control siRNA; b, p < 0.01 versus cAMP treatment in the control siRNA.
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Figure 3. Recruitment of WT1 to the VLDLR promoter region during decidualization. A, ChIP-sequence data on EGR-1 binding in MCF-7 cells from ENCODE. WT1 shares the same DNA consensus binding sequence with EGR-1. It showed the binding signals of EGR-1 in the upstream region (−511 to −338 bp) of the VLDLR gene. ChIP-qPCR was performed in ESCs with the primers surrounding these binding sites. Black bar at the bottom indicates the ChIP-qPCR amplicon region. B, the recruitment of WT1 to this VLDLR promoter region was examined at days 2 and 4 after cAMP stimulation by ChIP assay. Normal rabbit IgG was used as a negative control. The relative recruitment levels were analyzed by real-time PCR. Values were expressed as a ratio of control sample at day 2. Data are mean ± S.D. of three different incubations. a, p < 0.05 versus control treatment at day 4. C, ESCs were infected with retrovirus encoding WT1 to overexpress WT1 (WT1 cells). Retrovirus encoding mCherry was infected to ESCs as a control virus (mock cells). Whole-cell lysates were prepared and subjected to Western blotting to confirm overexpression of the WT1 protein. β-Tubulin was used as an internal control. The immunoblot is representative of three different transfections (upper panel). These cells were transfected with the VLDLR-promoter reporter vector and pRL-TK vector as a normalization control. After 24 h of transfection, the firefly and Renilla luciferase activities were measured. The firefly luciferase activity was normalized to those of Renilla. Values of the luciferase activities were expressed as a ratio of mock cells (lower panel). Data are mean ± S.D. of three different incubations. a, p < 0.05 versus mock cells.

droplet accumulation and intracellular triglyceride level, and WT1 knockdown inhibited them with the down-regulation of VLDLR. On the other hand, in VLDLR cells, they were increased by cAMP, and these increases were not altered by WT1 knockdown. These results indicated that VLDLR overexpression rescued the suppression of LA caused by WT1 knockdown. Therefore, it is likely that a WT1-VLDLR pathway regulates LA during decidualization. To examine the possibilities that genes other than VLDLR were involved in LA in ESCs, we checked the microarray data for other genes involved in the lipid uptake that were up-regulated by cAMP and down-regulated by WT1 knockdown. Fatty acid-binding proteins (FABPs), fatty acid transport protein (FATPs), and CD36 are known to participate in lipid uptake and LA (26, 32, 33). Among these proteins, FABP3, FATP1, FATP4, and FATP5 were detected at the mRNA level in ESCs by microarray analysis. Their expression levels were neither increased by cAMP treatment nor decreased by WT1 knockdown (Fig. 4C), suggesting that VLDLR is the main regulator of the lipid uptake in ESCs during decidualization.

Effect of VLDLR knockdown on the expression of decidualization marker genes

To investigate whether the LA affects the expression levels of decidualization marker genes, VLDLR expression was knocked down and then the expression levels of IGFBP-1 and PRL, which are specific markers of decidualization, were examined. mRNA levels of IGFBP-1 and PRL were increased by cAMP but were not affected by VLDLR knockdown (Fig. 5), suggesting that LA is not involved in the induction of mRNA expressions of IGFBP-1 and PRL during decidualization.

Discussion

WT1 is an essential transcription factor regulating mammalian urogenital development (1). We recently reported that WT1 induces the expression of IGFBP-1 and PRL by binding to their promoter regions in human ESCs undergoing decidualization (7). During decidualization, ESCs undergo dramatic changes of cellular functions including energy metabolisms (11, 13, 14). Although WT1 is known to regulate cell metabolisms in other types of cells (15, 16), it has been unclear whether it regulates cellular functions of ESCs undergoing decidualization. In cells, lipids are stored in lipid droplets, which are monolayer membrane-encased organelles (34). We showed that decidualization stimulated lipid droplet accumulation, in agreement with a previous report (21). In addition, we showed that decidualization also increased intracellular triglyceride levels in ESCs. Because the abilities of lipogenesis and lipolysis were not altered in ESCs by cAMP, LA during decidualization results from the increase of lipid uptake into ESCs. To date, there are...
no reports showing which transcription factors are involved in LA in ESCs. The present results demonstrate that WT1 enhances LA by up-regulating VLDLR in ESCs during decidualization. They are the first to show that WT1 has a role in regulating lipid storage in human cells.

VLDLR is a member of the low-density lipoprotein (LDL) receptor family. It mediates the uptake of lipoproteins by endocytosis and contributes to LA in cells (26, 27). Knockdown of VLDLR in mice cardiomyocytes completely blocked hypoxia-induced LA (26). Our gene ontology analysis showed that VLDLR was up-regulated by WT1 during decidualization, and knockdown of VLDLR inhibited the LA during decidualization. VLDLR is highly expressed in heart, muscle, adipose tissue, macrophages, endothelial cells, and brain (27). However, expression of VLDLR has not yet been reported in human endometrium. Therefore, this is the first report that VLDLR is expressed in ESCs and increased by decidualization in vivo and in vitro. How VLDLR mRNA expression is regulated is not well-understood.

**A**

![Image showing control siRNA, VLDLR siRNA, and WT1 siRNA effects on VLDLR and β-tubulin expression](image)

**B**

![Image showing area of lipid droplets and intracellular triglyceride levels](image)

**C**

![Image showing relative mRNA expression levels](image)
understood. In other cells, hypoxia-inducible transcription factor 1α and peroxisome proliferator-activated receptor γ were shown to bind to the VLDLR promoter region (35, 36). In this study, using ChIP assay, we identified a novel WT1-binding region in the upstream promoter region of the VLDLR gene. Although there are other potential WT1-binding regions based on public ChIP-seq data, we did not observe an increase of WT1 recruitment in these regions by cAMP stimulation in ESCs (data not shown). The WT1-binding region we identified is therefore the one that is most likely associated with the regulation of VLDLR expression during decidualization. Our time course experiments revealed that WT1 binding levels to the VLDLR promoter region and VLDLR mRNA expressions showed similar time course patterns. In addition, WT1 overexpression significantly increased the transcriptional activity of the VLDLR promoter region, which indicates that WT1 is sufficient to induce VLDLR expression in ESCs. Taken together, the cAMP-WT1 pathway directly increases VLDLR expression by binding to the promoter region during decidualization.

Overexpression of VLDLR in ESCs clearly demonstrated that VLDLR rescued the suppression of LA caused by WT1 knockdown (Fig. 4B). These data directly demonstrate the existence of a WT1-VLDLR pathway that accumulates lipids under cAMP stimulation. In addition, expression levels of other genes that are well-known to participate in lipid uptake were neither increased by cAMP treatment nor decreased by WT1 knockdown. Taken together, these findings strongly suggest that VLDLR is a key regulator of LA under the control of WT1 in human ESCs during decidualization. However, VLDLR overexpression alone could not increase LA (Fig. 4B, mock cells-control versus VLDLR cells-control), which indicates that both cAMP stimulation and VLDLR expression are essential for LA during decidualization. We also speculate that cAMP stimulation can fully induce LA in ESCs, and further increase of VLDLR expression did not alter it (mock cells-cAMP versus VLDLR cells-cAMP).

We previously reported that glucose uptake is increased by decidualization (11, 14), which results in a high glucose content in decidualized ESCs (18). Glucose uptake is essential for the inductions of decidualization marker genes (IGFBP-1 and PRL) during decidualization of ESCs (11, 14, 37). On the other hand, the decrease in LA by VLDLR knockdown did not affect the gene expression levels of the decidualization markers (Fig. 5). This result indicates that LA is not involved in the inductions of IGFBP-1 and PRL expressions in ESCs. Therefore, ESCs may store lipid during decidualization for some other reason. Blastocyst implantation and subsequent placentation is tightly regulated by a trophoblast-endometrial microenvironment (17).

![Figure 5. Effect of VLDLR knockdown on expression of the decidualization marker genes.](image)

ESCs were transfected with an siRNA targeted against VLDLR or with a nontargeting siRNA as a control. 48 h after siRNA transfection, ESCs were treated with or without cAMP for 4 days. mRNA expression of IGFBP-1 and PRL was analyzed by quantitative real-time RT-PCR. Values were normalized to those of MRPL19 and expressed as a ratio of control sample. Data are mean ± S.D. of three different incubations. a, p < 0.01 versus control treatment in the control siRNA.
Decidualized ESCs have been implicated as an important source of histotrophic support for the early conceptus (18, 19). Therefore, we speculate that ESCs increase the lipid storage during decidualization so that they can supply enough nutrition to the conceptus, which contributes to successful implantation. Further studies are needed to clarify the function of LA in decidualized ESCs.

The present study is the first to show that WT1 enhances LA in human ESCs during decidualization and that it is mediated by up-regulating VLDLR expression. Given that one of the roles of decidualized ESCs is to supply nutrition to the conceptus, WT1 helps to accomplish this goal by enhancing lipid storage through the up-regulation of VLDLR.

**Experimental procedures**

**Reagents**

Dulbecco’s modified Eagle’s medium (DMEM), l-glutamine, 1X trypsin-EDTA, streptomycin, and penicillin were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from Biological Industries Ltd. (Beit Haemek, Israel). Collagenases and dibutyryl cAMP were obtained from Sigma.

**ESC isolation**

Human endometrial tissues were obtained at hysterectomy from patients with a normal menstrual cycle, aged 40–45 years, who underwent surgery for myoma uteri or early stage cervical cancer. The patients were not on hormonal therapy at the time of surgery. Informed consent was obtained from all participating patients, and ethical approval was obtained from Institutional Review Board of Yamaguchi University Hospital. All experiments were performed in accordance with Tenets of the Declaration of Helsinki. Endometrial samples utilized for ESC isolation were histologically diagnosed as being in the late proliferative phase according to published criteria (38). Tissue samples were washed with phenol red-free DMEM containing 4 mM glutamine, 50 μg/ml of streptomycin, and 50 IU/ml penicillin, and minced into pieces of <1 mm³. ESCs were isolated as reported previously (39, 40). In brief, tissues were minced, enzymatically digested with 0.2% collagenase in a shaking water bath for 2 h at 37 °C, and filtered through a 70-μm nylon mesh. Stromal cells in the filtrates were washed three times with the medium, and the number of viable cells was counted by trypan blue dye exclusion. Under the microscope, all of the cells reacted with the stromal-reacting antibody vimentin (data not shown), indicating that they were homogeneous. The cells were verified to be negative for the epithelial cell-reacting antibody cytokeratin (data not shown). Cells were seeded at 10⁵ cells/cm² in 75-cm² tissue culture flasks and incubated in phenol red-free DMEM containing glutamine, antibiotics, and 10% dextran-coated charcoal-stripped FBS at 37 °C, 95% air, and 5% CO₂. At confluence, cells were treated with 1X trypsin-EDTA and subcultured into 6-well-plates. At 80% confluence after the first passage, the cell culture medium was changed to the treatment medium.

**Cell culture**

To induce decidualization, ESCs were incubated with treatment medium (phenol red-free DMEM supplemented with glutamine, antibiotics, and 2% dextran-coated charcoal-stripped FBS) containing cAMP (0.5 mM) for 2 or 4 days. The cells were then used for the following experiments described below. cAMP is considered as a second messenger of progesterone for decidualization because progesterone increases intracellular cAMP concentrations in ESCs (22). The concentration of Bt₂cAMP and the period of incubation used in this study were based on our previous report (22). The medium was changed every other day. Cells isolated from one patient were incubated one time in triplicate. Cells from three individuals were incubated in a single experiment.

**Microarray analysis**

Transcriptome data were obtained by performing microarray analysis as reported previously (41). ESCs were transfected with control or WT1 siRNA and then treated in the presence or absence of cAMP for 4 days. Total RNAs extracted from these three samples (control siRNA with no treatment, control siRNA with cAMP treatment, and WT1 siRNA with cAMP treatment) were subjected to microarray analysis. cDNA was prepared from 100 ng of total RNA. Gene expression was analyzed using a Clariom™ D assay, human (Affymetrix, Santa Clara, CA). Hybridization to the microarrays, washing, staining, and scanning were performed using the GeneChip system (Affymetrix). The scanned data were processed using the Partek Genomics Suite software program (Partek, Munster, Germany). When gene expression levels were more than 1.4-fold different between two samples, they were defined as differentially expressed genes. 1.4-Fold has been widely used for the cutoff level in genome-wide analyses because 1.4-fold corresponds to 0.5 when transformed to log2 ratio (11, 42, 43).

**cAMP measurement**

Intracellular cAMP concentrations of ESCs were measured using a cAMP kit (Cayman, Ann Arbor, MI) according to manufacturer’s protocol. The total protein concentration of the cultured cells was measured with a protein assay kit (Bio-Rad) and used as an internal control.

**Lipid droplet staining**

ESCs cultured on 48-well-plates were fixed with 4% paraformaldehyde and permeabilized by 0.1% Triton X-100. Lipid droplets in ESCs were stained with a lipophilic fluorescence dye, BODIPY 493/503 (Invitrogen), as reported previously (21). 4,6-Diamidino-2-phenylindole was used to identify nuclei. Fluorescent images of lipid droplets were obtained using a BZ-X700 (KEYENCE, Osaka, Japan). The mean staining area of the lipid droplet per cell was calculated using BZ-X Analyzer software (KEYENCE) as reported previously (44).

**Triglyceride assay**

The levels of intracellular triglyceride in ESCs were measured using an adipogenesis assay kit (Biovision Inc., Milpitas, CA) according to the manufacturer’s protocol. The total protein concentration of the cultured cells was measured with a protein assay kit (Bio-Rad) and used as an internal control.
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**Lipogenesis assay**

To determine the ability of lipogenesis, the GPDH activities in ESCs were measured using a GPDH assay kit (Cosmo-bio, Tokyo, Japan) according to the manufacturer’s protocol. The total protein concentration of the cultured cells was measured with a protein assay kit (Bio-Rad) and used as an internal control.

**Lipolysis assay**

To determine the ability of lipolysis, the levels of glycerol in the culture medium of ESCs were measured using a Free Glycerol assay kit (Cell BIOLABS, Inc., San Diego, CA) according to the manufacturer’s protocol. The total protein concentration of the cultured cells was measured with a protein assay kit (Bio-Rad) and used as an internal control.

**Real-time RT-PCR**

Total RNA was isolated from the cultured cells with an RNeasy 
Mini Kit (Qiagen, Inc., Valencia, CA). The RNA was reverse transcribed as reported previously (45). Amplicons of VLDLR, IGFBP-1, PRL, and MRPL19 (internal control) were amplified by real-time RT-PCR as reported previously (46) with the previously reported sequence-specific primer sets (7, 10, 47). Ct values obtained from quantitative real-time RT-PCR are shown in Table S3.

**Western blotting**

Whole cell lysates were prepared using loading buffer reagents (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) without trypsin treatment. Equal amounts of total protein were electrophoresed on a 10% SDS-polyacrylamide gel. The proteins were transferred to polyvinyldene difluoride membranes (ATTO, Tokyo, Japan). The membranes were blocked with blocking solution (5% skimmed milk with 0.1% Tween 20 dissolved in Tris-buffered saline (pH 7.5)), incubated with the first antibody for WT1 (ab89901) (Abcam plc, Cambridge, UK), VLDLR (sc-18824) (Santa Cruz Biotechnology), and β-tubulin (T4026) (Sigma), which was diluted in blocking solution, incubated with the peroxidase-conjugated secondary antibody diluted in blocking solution, visualized with the ECL-Western blotting detection system (Amersham Biosciences, Aylesbury, UK) according to the manufacturer’s protocol, and used to expose hyperfilm-ECL (Amersham Biosciences). To reuse the blot, the membranes were stripped in Restore Western stripping buffer (Pierce).

**Transfection of small interfering RNA (siRNA) duplexes**

WT1 ON-TARGET plus SMART pool, VLDLR ON-TARGET plus SMART pool, and ON-TARGET plus Non-Targeting pool siRNA were purchased from Dharmacon (Lafayette, CO). siRNA target sequences are shown in Table S4. siRNA was transfected to ESCs as reported previously (48). In brief, ESCs were plated in medium lacking antibiotics at ~1 × 10^5 cells in 6-well-plates. At 50% confluence, siRNA duplexes (20 nm) and RNAi MAX (2.5 μl/well; Invitrogen) diluted in Opti-MEM (Invitrogen) were transfected to ESCs. The medium was changed 4 h later. After 48 h of transfection, cells were incubated in the presence or absence of cAMP for 4 days.

**Immunohistochemistry**

Tissue samples from the late proliferative phase and late secretory phase were immunostained as reported previously (7). The endometrial tissues were fixed in formalin and embedded in paraffin and sectioned (5 μm thick). The tissue sections were deparaffinized in xylene and dehydrated in a graded series of ethanol. The sections were stained with Histofine simple stain MAX-PO(R) (Nichirei Co. Ltd., Tokyo, Japan) using a rabbit polyclonal antibody to VLDLR (sc-18824) (Santa Cruz Biotechnology). Peroxidase activity was visualized by incubating the sections with 3,3'-diaminobenzidine-4 HCl (Nakalai Tesque Co. Ltd., Tokyo, Japan) in 0.05 m Tris-HCl buffer (pH 7.6) containing 0.01% H2O2 for 3 min. Control sections were incubated with normal mouse IgG. The sections were counterstained with Meyer’s hematoxylin.

**ChIP assay**

Recruitment of WT1 to the VLDLR promoter region were examined by ChIP assay according to the protocol for the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) as reported previously (10, 49, 50) with some modifications. Cells were cross-linked by addition of formaldehyde into the medium at a final concentration of 1% and incubated for 10 min at 37 °C. Cross-linking was terminated by addition of glycine (0.125 m, final concentration). Cells lysates were sonicated using a Bio- ruptor ultrasonicator (Cosmo-bio), precleared with salmon sperm DNA-protein A at 4 °C for 4 h. Five percent of the chromatin solution was kept as input controls (INPUT). Dynabeads Protein A (Invitrogen) were incubated with antibodies for WT1 (sc-192X) (Santa Cruz Biotechnology) and normal rabbit IgG (Invitrogen) overnight at 4 °C. The precleared chromatin was incubated with antibody-bound Dynabeads for 6 h at 4 °C. Immune complexes were collected and cross-linking of the immunoprecipitated (IP) chromatin complex and input control (INPUT; 2% of the total soluble chromatin) were reversed by heating the samples overnight at 65 °C and subjected to proteinase K treatment. The DNA was purified using a QIAquick PCR purification kit (Qiagen) and subjected as a template for PCR amplification. The following primers were used for PCR analysis to amplify the VLDLR promoter regions (Fig. 3A): forward (5’-CTTGTTGCCCCCTGAAACT-3’) and reverse (5’-GTCGCCCTCCCTACAGTCATT-3’). Real-time PCR was used to determine the relative levels of WT1 recruitment to the VLDLR promoter region. The ratio of IP DNA to the INPUT DNA sample (%INPUT) was calculated as reported previously (48). The relative recruitment levels were expressed as a ratio of the control sample at day 2.

**Establishment of ESCs overexpressing WT1 or VLDLR**

The coding sequences of WT1 and VLDLR were amplified by PCR with Prime STAR GXL DNA polymerase (TaKaRa, Ohtsu, Japan) using cDNA of ESCs as a template. The ampiclon was inserted at the multiple cloning site of pMXs-ires-Blasticidin retroviral vector (Cell BIOLABS) using an In-Fusion HD Cloning kit (TaKaRa). A mock control in which the mCherry coding sequence was inserted was also constructed. For retrovirus production, these vectors were co-transfected with packaging plasmids into HEK293 cells using Lipofectamine 2000 (Invitrogen).

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WT1 regulates lipid accumulation during decidualization

Retroviral supernatants were collected 48 h after transfection and passed through a 0.45-μm filter. The virus-containing supernatant was concentrated with PEG-it Virus Precipitation Solution (System Biosciences, Palo Alto, CA). ESCs were seeded at 1 × 10^5 cells in 6-well plates. The following day, virus concentrate was added to the medium with 8 μg/ml of final concentration of Polybrene (Sigma). The plate was centrifuged at 800 × g for 60 min at 33 °C, washed with PBS, and changed to fresh medium. The stable ESC lines overexpressing mCherry (mock cells) or WT1(WT1 cells) or VLDLR (VLDLR cells) were established by sorting with 5 μg/ml of blasticidin S (Thermo Fisher Scientific) for 4 days.

Luciferase assay

Luciferase assay was performed to examine the transcriptional activity of the VLDLR promoter region. The promoter region was amplified by PCR from the human genomic DNA. The PCR products were subcloned upstream of the luciferase gene into firefly luciferase vectors pGL4.23 (Promega, Madison, WI), which contains minimal promoter. Mock cells and WT1 cells were cultured on a 24-well-plate (5 × 10^4 cells/well) for 24 h and then transfected with VLDLR-promoter reporter vector and pRL-TK vector (Promega) as a normalization control using Lipofectamine LTX (Invitrogen). After 24 h of transfection, the firefly and Renilla luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

Statistical analysis

Statistical significance was determined by one-way analysis of variance. After analysis of variance, the Tukey-Kramer test was applied to analyze differences between groups. An unpaired t test was applied to analyze the difference between two groups. All statistical analyses were performed using SPSS for Windows version 11 (SPSS Inc., Chicago, IL). Differences were considered significant at p < 0.05.

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