Vitamin D3 regulates *HAND2* expression in endometrial stromal cell decidualization

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Abstract. Vitamin D3 (VD3) supplements increase pregnancy rates. In addition to its effect on fertilized eggs, the involvement of VD3 in endometrial decidualization, which is essential for embryo implantation, has been suggested; however, the detailed mechanisms involved remain unclear. The present study examined the effects of VD3 on endometrial decidualization and embryo implantation using the human endometrial stromal cell line, KC02-44D, and the choriocarcinoma cell line, BeWO. The effects of VD3 were examined using reverse transcription-quantitative PCR (RT-qPCR) and changes in the invasive capacity of BeWO cells were examined using invasion assay. The results revealed that VD3 further elevated the levels of heart and neural crest derivatives expressed 2 (*HAND2*), whose protein is a master regulator that is elevated during decidualization, and VD3 suppressed elevated prolactin (*PRL*) in decidualized KC02-44D cells, as shown by RT-qPCR analysis. The addition of VD3 to decidualization reduced the invasive capacity of BeWO cells in the invasion assay. A manual search for the vitamin D receptor binding motif suggests that *HAND2* may be directly controlled by VD3. Given that VD3 regulates *PRL*, VD3 supplementation would be appropriate to avoid the endometrial secretory phase and provide VD3 during the endometrial proliferative phase, with the expectation of an effect on fertilized eggs.

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Introduction

Vitamin D3 (VD3) is produced by the photochemical conversion of 7-dehydrocholesterol, which is synthesized from acetyl-CoA produced in the tricarboxylic acid cycle by the ultraviolet radiation of B energy in the epidermis (1-4). Conversely, VD3 of food origin is absorbed in the small intestine with other dietary fats; however, the percentage of VD3 from dietary sources in the body is low, mostly due to homeostatic synthesis (5). VD3 is hydroxylated in the liver to 25-hydroxy vitamin D3 [25(OH)D], which leaks into the blood and is hydroxylated in the kidneys, and 25(OH)D then becomes 1,25(OH)2D as active VD3 (3,6). Active VD3 is also generated by the 25-hydroxyvitamin D-1 alpha hydroxylase, mitochondrial, which is encoded by the mitochondrial cytochrome P450 family 27 subfamily B member 1 (*CYP27B1*) gene; therefore, cells expressing *CYP27B1* can produce active VD3 (7). The active form of VD3 binds to the vitamin D receptor (VDR), a known nuclear receptor, and binds upstream of specific gene sequences in the genomic DNA as a transcriptional regulator to control the transcription of downstream genes (8-10).

The oral administration of VD3 supplements has been shown to increase pregnancy rates (11). Although this supplementation was originally considered to affect fertilized eggs, VD3 supplementation has been reported to increase homeobox A10 (*HOXA10*), an indicator of endometrial embryonic receptivity (12). HOXA10 functions as a regulator of endometrial development and decidualization (13) and as a transcriptional regulator of *CYP27B1* (14). Human endometrial decidualization is caused by elevated blood progesterone levels following ovulation (15). In the secretory phase, normal progesterone delivery to the endometrium causes the decidualization of endometrial stromal cells (EnSCs) via the progesterone receptor (*PGR*). First, the upregulation of heart and neural crest derivatives-expressed transcript 2 (*HAND2*) and forkhead box O1 (*FOXO1*) (whose proteins are pivotal transcription factors that promote the decidualization of human EnSCs as an upstream of progesterone signaling) (16), occurs during the decidualization of EnSCs (17,18). Subsequently, insulin-like growth factor binding protein 1 (*IGFBP1*), prolactin (*PRL*), interleukin (*IL)15*) and other genes are initiated during their transcriptions in decidual EnSCs by HAND2 and FOXO1 (15). Translated and secreted PRL regulates extravillous trophoblast

(EVT) growth and invasion and, in concert with IL-15, is involved in the functions of uterine-specific natural killer (uNK) cells. uNK cells, in concert with EnSCs, promote spiral artery remodeling, which further promotes endometrial decidualization (16). In addition, uNK cells play a critical role in immune tolerance, which is essential for embryonic receptivity (19). Moreover, IGFBP1 promotes the migration of embryo-derived EVTs, contributing to placentation (16). Abnormalities in EnSC decidualization are known to cause preeclampsia, miscarriage implantation and fetal growth failures, as well as placenta accreta (20), EnSC decidualization is critical for the normal development of the fetus *in utero*.

The involvement of VD3 in endometrial function, i.e., embryo implantation via decidualization, has been suggested; however, the mechanisms involved remain unclear. Therefore, the present study examined the effects of VD3 on endometrial function, particularly in EnSC decidualization, using a human EnSC line.

Materials and methods

Culture of the EnSC KCO2-44D cell line and human choriocarcinoma BeWO cell line. Human EnSC KC02-44D cells (cat. no. SC-6000) (CVCL_E224) (21) and human choriocarcinoma BeWO cells (cat. no. JCRB9111) (RRID: CVCL_0044) (22) (which has been used as an EVT model) (23) were obtained from the American Type Culture Collection (ATCC) and the JCRB cell bank (Osaka, Japan), respectively. The KC02-44D and BeWO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with phenol red (Life Technologies; Thermo Fisher Scientific, Inc.) containing 100 unit/ml penicillin (Nacalai Tesque, Inc.), 100 μg/ml streptomycin (Nacalai Tesque, Inc.), 10 mM HEPES (pH 7.4) (Life Technologies; Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (FBS, Global Life Sciences Technologies Japan K.K.; Cytiva) and Ham's F12 (Life Technologies; Thermo Fisher Scientific, Inc.) containing 100 U/ml penicillin (Nacalai Tesque, Inc.), 100 μg/ml streptomycin (Nacalai Tesque, Inc.), 10 mM HEPES (pH 7.4) (Life Technologies; Thermo Fisher Scientific, Inc.) and 15% FBS (Global Life Sciences Technologies Japan K.K.; Cytiva) at 37˚C and 5% CO_2.

Decidualization and VD3 treatment of KC02-44D cells. The KC02-44D cells were seeded in 24-well plates (Corning, Inc.) until reaching confluency ($0.4x10^6$ cells per well) and then stimulated as described below. As phenol red is an estrogen-like agonist, phenol red-free DMEM (Life Technologies; Thermo Fisher Scientific, Inc.) containing 10% charcoal-stripped (CS)-FBS (activated charcoal was used to adsorb and remove other hormones in the serum), 10 mM HEPES (pH 7.4) (Life Technologies; Thermo Fisher Scientific, Inc.), 100 unit/ml penicillin (Nacalai Tesque, Inc.), 100 μg/ml streptomycin (Nacalai Tesque, Inc.) and 1% GlutaMAX (Life Technologies; Thermo Fisher Scientific, Inc.) was used as the control medium. The control group was cultured in the aforementioned medium; the VD3-treated group was cultured in the aforementioned medium with 10 nM VD3 (25-hydroxy vitamin D3, Cayman Chemical Co.); the decidualization-treated group was cultured in the aforementioned medium with 10^{-8} M estradiol (MilliporeSigma), 10^{-6} M medroxyprogesterone acetate (MPA; MilliporeSigma), an analog of progesterone and 0.5 mM 8-Bromo-cAMP (MilliporeSigma), a cell-permeable analog of cAMP that activates cyclic-AMP-dependent protein kinase and promotes decidualization; the decidualization + VD3 treatment group was cultured in the aforementioned medium with 0.5 mM 8-Bromo-cAMP, 10^{-8} M estradiol, 10^{-6} M MPA and 10 nM VD3. These stimuli were performed in triplicate, and samples were ultimately prepared for 8-9 wells per group.

Extraction of total RNA, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the KC02-44D cells ($0.4x10^6$ cells) cultured for 6 days with Sepasol®-RNA I Super G (Nacalai Tesque, Inc.). ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co.) was used for the reverse transcription of total RNA into cDNA. qPCR was conducted with cDNA, Thunderbird SYBR Next qPCR mix (Toyobo Co.), and primers using a Light Cycler96 (Roche Diagnostics). For PCR, following pre-incubation (95˚C, 30 sec), 45 cycles of two-step amplification (95˚C, 5 sec; 60˚C, 30 sec) were conducted, followed by a melting reaction to confirm the primer specificity. The gene names and primer sequences used are listed in Table I. A Primer3Plus web interface was used for primer design (24). As a housekeeping gene, hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) was used and the relative expression levels were calculated from the threshold cycle (Cq) values of each gene from each sample using the $2^{-\Delta\Delta Cq}$ method (25).

Cell invasion assay. Until reaching 85-90% confluency, the KC02-44D cells were cultured in the bottom part of 24-well plates (Corning, Inc.). The control, decidualization-treated and decidualization + VD3 treatment groups were stimulated for 6 days as described above, and three wells were prepared for each group. After 6 days, the insert in the BioCoat Matrigel Invasion Chamber (Corning, Inc.) was hydrated, and 50,000 BeWO cells were incubated at 37˚C and 5% CO_2 for 24 h. After 24 h, the BeWO cells that had infiltrated the bottom of the filter were stained using Diff-Quick (Sysmex Corporation), and the number of stained cells was counted using an inverted microscope (Eclipse Ts2-FL, Nikon Corporation) and MicroStudio software (version x64, 1.5.18608.20210313, Wraymer, Inc,). Finally, the infiltration frequency per unit area was calculated.

Statistical analysis. After confirming the normality of each group by performing the Shapiro-Wilk test on the data obtained for each group, a two-tailed Welch's unpaired t-test was used to estimate the difference between the means of the two groups. The Bonferroni correction was then performed to avoid a type 1 error according to multiple testing. The IBM SPSS Statistics software (version 29.0; IBM Corp., Inc.) was used for statistical analyses. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Reactivity of the KC02-44D cell line against VD3. The present study examined the expression of *VDR*, whose protein affects cellular function by binding to active VD3 in KC02-44D cells. Although *VD3* expression was found in KC02-44D

Table I. Sequences of the primers used in the present study.

Gene symbol	Definition	Position	Sequence
HPRT1	Hypoxanthine phosphoribosyltransferase 1	895F	5'-CTAGTTCTGTGGCCATCTGCTTAG-3'
		1034R	5'-GGGAACTGATAGTCTATAGGCTCATAGTG-3'
VDR	Vitamin D receptor	695F	5'-TGACCTGGTCAGTTACAGCATC-3'
		829R	5'-TTGGAGCGCAACATGATGAC-3'
CYP27B1	Cytochrome P450 family 27 subfamily B member 1	594F	5'-TGGCGGGGGAATTTTACAAG-3'
		740R	5'-TCAACAGCGTGGACACAAAC-3'
ESR1	Estrogen receptor 1	1514F	5'-TGCTGGCTACATCATCTCGGT-3'
		1665R	5'-GACTCGGTGGATATGGTCCTTC-3'
ESR2	Estrogen receptor 2	617F	5'-CTAACTTGGAAGGTGGGCCTG-3'
		767R	5'-AGCGATCTTGCTTCACACCA-3'
PGR	Progesterone receptor	2484F	5'-CCTTTGGAAGGGCTACGAAGT-3'
		2593R	5'-GAGCTCGACACAACTCCTTTTTG-3'
PRL	Prolactin	374F	5'-ATTCGATAAACGGTATACCCATGGC-3'
		623R	5'-TTGCTCCTCAATCTCTACAGCTTTG-3'
IGFBP1	Insulin-like growth factor binding protein 1	636F	5'-CTATGATGGCTCGAAGGCTC-3'
		791R	5'-TTCTTGTTGCAGTTTGGCAG-3'
IL15	Interleukin 15	165F	5'-GTTCACCCCAGTTGCAAAGT-3'
		351R	5'-CCTCCAGTTCCTCACATTC-3'
HAND2	Heart and Neural Crest Derivatives expressed 2	1479F	5'-AGAGGAAGAAGGAGCTGAACGA-3'
		1552R	5'-CGTCCGGCCTTTGGTTTT-3'
FOXO1	Forkhead box protein O1	2879F	5'-TGTTTTCTGCGGAACTGACG-3'
		2970R	5'-TTCTGTGGCAACGTGAACAG-3'
HOXA10	Homeobox A10	963F	5'-GATTCCCTGGGCAATTCCAAAG-3'
		1083R	5'-ACAGAAACTCCTTCTCCAGCTC-3'

F, forward; R, reverse.

cells, no significant differences were observed among the VD3-(P=0.398) and decidualization-treated groups (P=0.366 and 0.641) compared with the control group (Fig. 1). *CYP27B1* expression was also examined; the protein converts VD3 to its active form, and it was found that *CYP27B1* expression was significantly elevated in the decidualization-treated groups compared with the control group (P=0.014 and 0.009) (Fig. 1). This indicates that EnSCs locally produce active VD3 during decidualization, suggesting the need for active VD3 in decidualization and the regulation of VDR target gene expression in EnSCs.

Effects of VD3 on the decidualization of EnSCs. The present study examined the changes due to the effects of VD3 by adding 100 μM inactive VD3 to decidualized KC02-44D cells using RT-qPCR. The results revealed no significant differences in either *VDR* (P=0.281) or CYP27B1 (P=0.478) between the decidualization and decidualization + VD3 treatment groups (Fig. 1). Similar to *VDR*, no significant differences were found in the nuclear receptors, such as estrogen receptor 1 (*ESR1*) in the VD3-(P=0.075) and decidualization-treated groups (P=0.692 and 0.975), *ESR2* in the VD3-(P=0.986) and decidualization-treated groups (P=0.415 and 0.894), and *PGR* in the VD3-(P=0.091) and decidualization-treated groups (P=0.019 and 0.032), compared with the control.

The *PRL* levels were not significantly altered in the VD3-treated group compared with the control group (P=0.371); however, a significant increase was found between the decidualization-treated (P=0.000007) and decidualization + VD3 treatment groups (P=0.000001), and the control, as well as between the decidualization-treated group and VD3-treated group (P=0.000007) or decidualization + VD3 treatment groups (P=0.003) (Fig. 2). *IGFBP1* expression was significantly elevated in the decidualization (P=0.00001) and decidualization + VD3 treatment groups (P=0.000001; P<0.05) compared with the control, although there was no significant difference between the VD3-treated group and the control group (P=0.075) (Fig. 2). There were no significant differences in *IGFBP1* expression between the decidualization and decidualization + VD3 treatment groups (P=0.282) (Fig. 2). The results also revealed that the expression of *IL15* was significantly increased in the decidualization (P=0.001) and decidualization + VD3 treatment groups (P=0.001) compared with the control, although there was no significant difference between the VD3-treated group and the control group (P=0.489; P<0.05) (Fig. 2). There were no significant differences in the expression of *IL15* between the decidualization and decidualization + VD3 groups (P=0.564). *HAND2* expression was significantly increased in the decidualization-treated (P=0.005) and decidualization + VD3 treatment

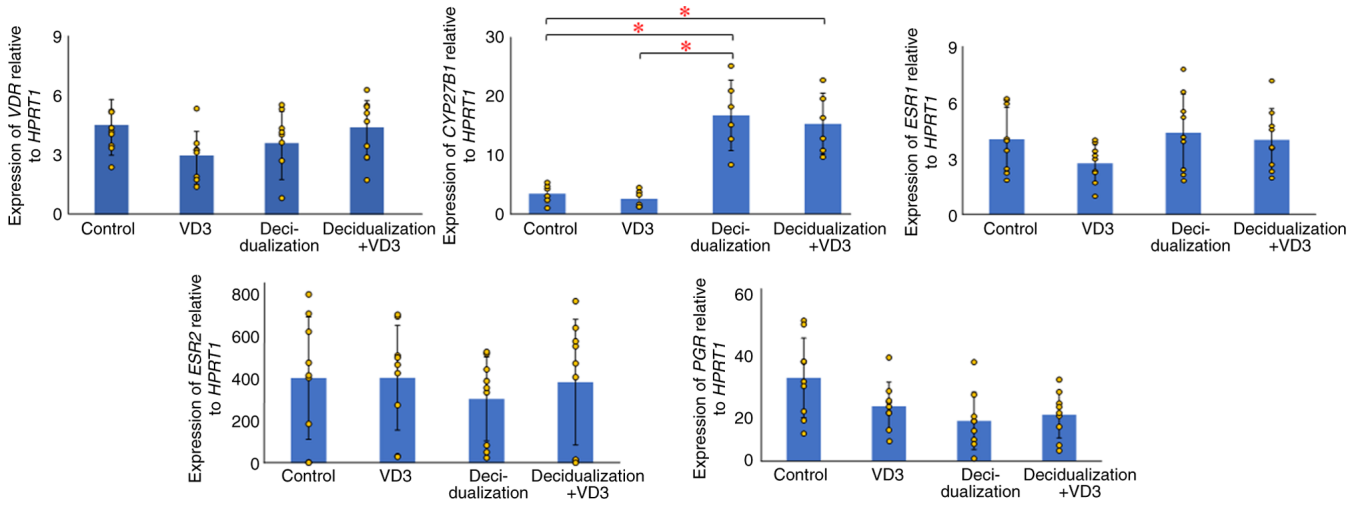

Figure 1. Changes in the levels of nuclear receptors and *CYP27B1* expression in decidualized KC02-44D cells with/without VD3. The values for each group are presented as bars (mean) and error bars (standard deviation). Significant increases in *CYP27B1* levels (P<0.05) were observed in the decidualized KC02-44D cells with/without VD3. *P<0.05, indicates significant differences between groups using Welch's t-test with the Bonferroni correction. VD3, vitamin D3; *HPRT1*, hypoxanthine phosphoribosyltransferase 1; *VDR*, vitamin D receptor; *CYP27B1*, cytochrome P450 family 27 subfamily B member 1; ESR, estrogen receptor; PGR, progesterone receptor.

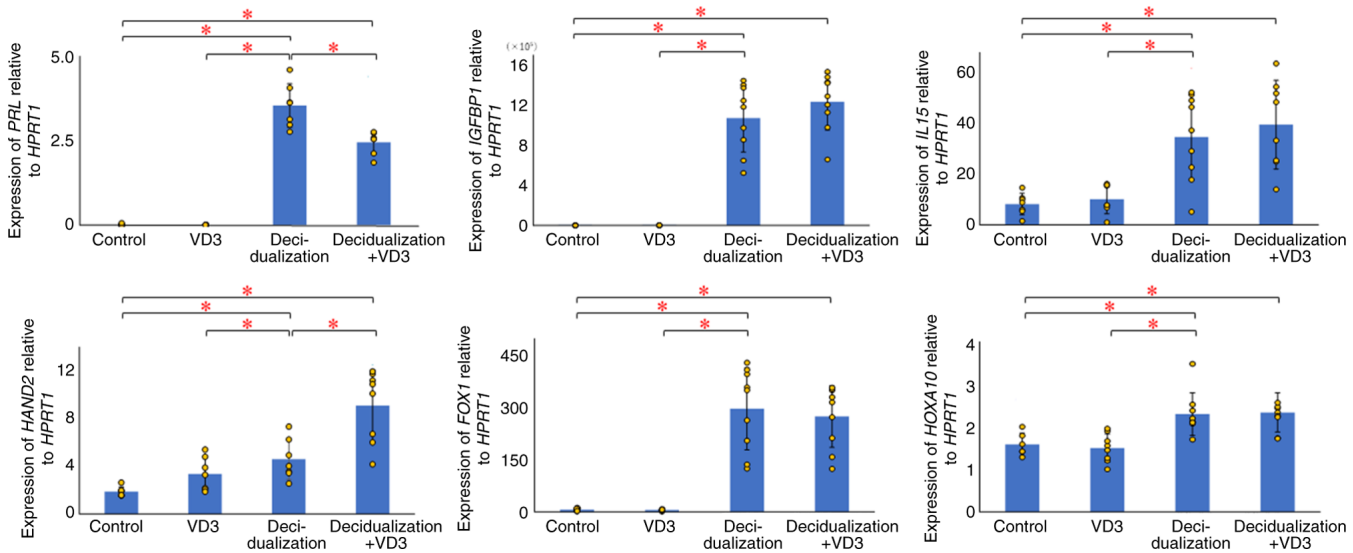

Figure 2. Effects of VD3 on decidualization markers in KC02-44D cells. The values for each group are presented as bars (mean) and error bars (standard deviation). The significant upregulation of *PRL*, *IGFBP1*, *IL15*, *HAND2*, *FOXO1* and *HOXA10* was observed in the decidualized KC02-44D cells. Additional VD3 stimulation affected *PRL* and *HAND2* in the decidualized KC02-44D cells. *P<0.05, indicates significant differences between groups using Welch's t-test with the Bonferroni correction. VD3, vitamin D3; *HPRT1*, hypoxanthine phosphoribosyltransferase 1; *PRL*, prolactin; *IGFBP1*, insulin-like growth factor-binding protein 1; *IL15*, interleukin 15; *HAND2*, heart and neural crest derivatives expressed 2; *FOXO1*, forkhead box protein O1; *HOXA10*, homeobox A10.

groups (P=0.0002) compared with the control, although there was no significant difference between the VD3-treated and control group (P=0.032) (Fig. 2). By contrast, the addition of VD3 during decidualization significantly increased *HAND2* expression compared with the decidualization group (P=0.004) (Fig. 2). *FOXO1* expression was significantly elevated in the decidualization (P=7.69541E-05) and decidualization + VD3 treatment groups (P=1.76083E-05) compared with the control, although there was no significant difference in the VD3-treated group compared with the control group (P=0.538) (Fig. 2). There were no significant differences in *FOXO1* expression between the decidualization and decidualization + VD3 treatment groups (P=0.659). As regards *HOXA10*, there was no significant difference in *HOXA10* expression between the control and VD3-treated groups (P=0.607); however, *HOXA10* expression was significantly upregulated in the decidualization-treated (P=0.003) and decidualization + VD3 treatment groups compared with the control (P=0.001) (Fig. 2). There were no significant differences in *HOXA10* expression between the decidualization and decidualization + VD3 treatment groups (P=0.873).

VD3 decreases the invasive capacity of EVTs. Following implantation, placentation occurs as the EVTs invade the decidua of the endometrium. The present study then performed an invasion assay to examine the effects of VD3 on the invasive

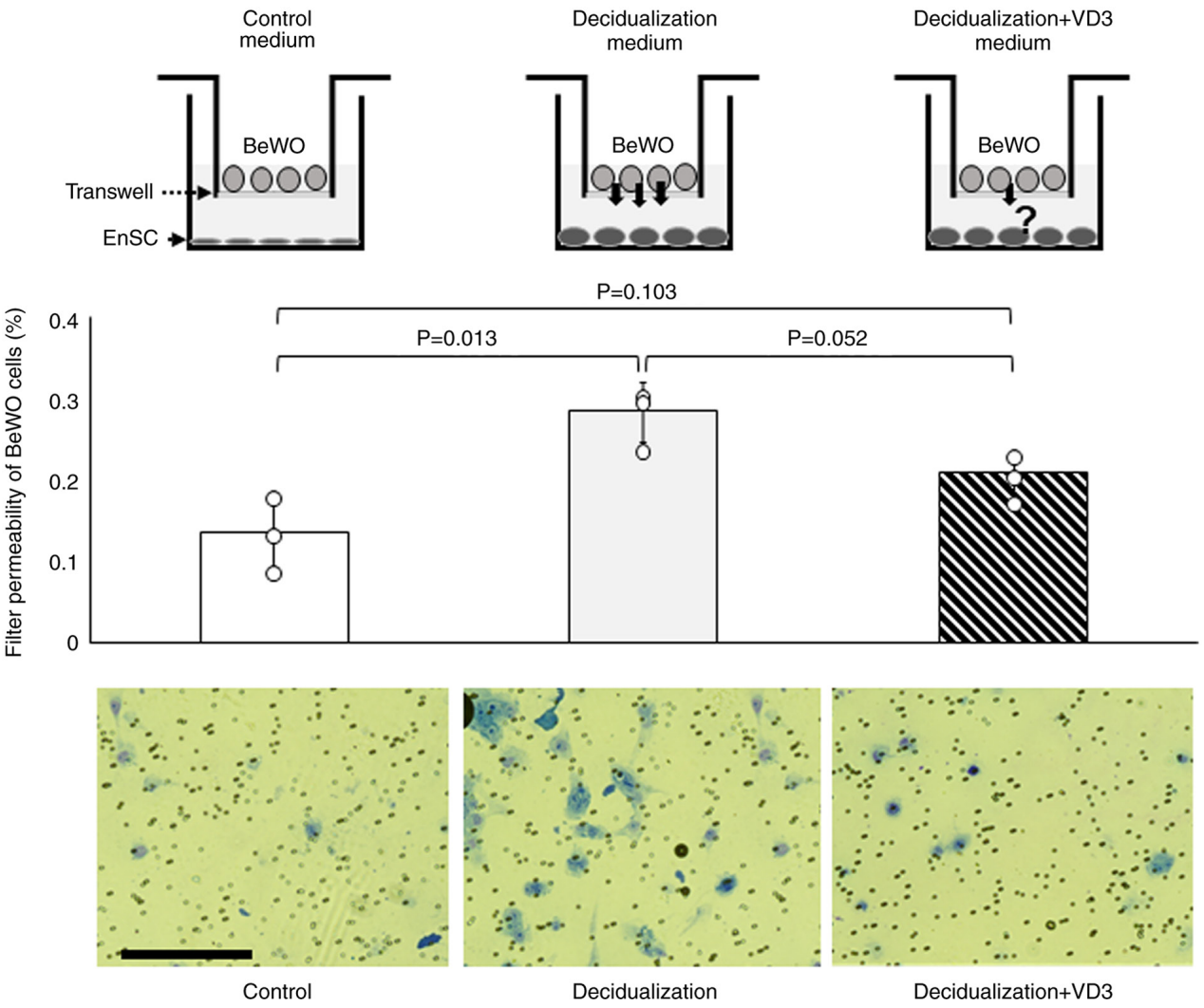

Figure 3. Effects of VD3 on extravillous trophoblast invasion. A schematic diagram of the invasion assay system is presented in the upper panel. Representative images of the invasion assay are presented in the lower panel. The invasive ability of the BeWO cells was significantly increased in the decidualization-conditioned medium with KC02-44D cells compared with the control medium ($P<0.05$). The values for each group are presented as bars (mean) and error bars (standard deviation). Scale bar, 50 μm. EnSC, endometrial stromal cell; VD3, vitamin D3.

ability of the human choriocarcinoma cell line, BeWO, with or without decidualization and VD3. The results revealed that the invasive ability of BeWO cells was significantly increased in decidualization-conditioned medium with KC02-44D cells compared to that in the control medium (P=0.013) (Fig. 3), whereas no difference was observed in the decidualization + VD3-added medium compared with the control medium (P=0.103) (Fig. 3).

Discussion

In the present study, an increase in *HOXA10* expression and a subsequent increase in *CYP27B1* (7) expression during decidualization in KC02-44D cells, an EnSC line, were observed. The activation of VD3 by the CYP27B1 enzyme is considered to facilitate the translocation of VDR into the nucleus and cause changes in its target gene expression during decidualization. Indeed, the observed upregulation of *HAND2* and downregulation of *PRL* upon the addition of VD3 during decidualization suggests that these genes may be transcriptionally regulated, either directly or indirectly, by the VD3-VDR complex. The VD3-VDR complex may also be involved in EVT invasiveness via *PRL* by VD3, as observed in the invasion assay herein.

HAND2 is a master regulator that acts upstream of progesterone signaling and promotes the establishment of pregnancy as a key to decidualization (15,26). The addition of VD3 during decidualization significantly increased *HAND2* expression, suggesting that the VDR activated by VD3 binding cooperates with the PGR to regulate *HAND2* transcription, an essential function for decidualization. The authors manually searched for the VDR binding motif [-AGGGTCA-GAGTTC(-GTTG GT-AGAGAGGG)] (27) in the 2k-basepairs upstream region of *HAND2* gene (ACC no. NC_000004.12; *Homo sapiens* chromosome 4, GRCh38.p14 Primary Assembly, from 173524091 to 173530229, 2024/04/15 version). Consequently, a VDR binding candidate motif (GGGTCA) was found at position-562/-556 from the transcription start site, as well as another candidate VDR-binding motif (GAGTTC) at -1493/-1488. As a limitation, changes in HAND2 protein levels were not evaluated in the present study, as the antibodies used in a previous study by the authors (goat dHAND antibody (M-19), cat. no. sc-9409; Santa Cruz Biotechnology, Inc.,

Dallas, TX) (18) are no longer available, and no other suitable antibodies have been found since then. Additionally, only a candidate binding sequence was found, and further functional analysis are thus necessary to confirm the details of the regulation of *HAND2* expression by the VDR. Furthermore, the epigenetic changes in the *HAND2* promoter region need to be determined, since the VDR-binding sequence in the vicinity of the *HAND2* promoter region may become a euchromatin region due to decidualization, and gene expression may be actively underway.

HAND2 is known to be an upregulator of *PRL* expression (28), which is inconsistent with the present results showing *HAND2* upregulation but *PRL* downregulation. Additionally, given that no VDR-binding candidate motif was found in the *PRL* promoter region, it may be necessary to consider other factors regulated by the VDR in the regulation of *PRL* expression during decidualization.

PRL is an indicator of EnSC decidualization, and the action of PRL in the endometrial microenvironment stimulates EVT functions, prevents the rejection of embryos, regulates the survival of uNK cells and facilitates angiogenesis (16). Elevated blood levels of PRL inhibit the secretion of gonadotropin-releasing hormone from the hypothalamus and luteinizing hormone from the pituitary gland and suppress *ESR1* expression in the pituitary gland, causing hypogonadotropic hypogonadism with amenorrhea (29). In the ovary, elevated blood levels of PRL cause anovulation (30), suppress follicle maturation and lead to inadequate corpus luteum formation, with decreased luteinizing hormone receptor affinity in the corpus luteum and concomitant decreased progesterone production and secretion (30). In the uterus, hyperprolactinemia has been implicated in hyperproliferative myoma (31), as well as endometriosis and consequent infertility (30). Taken together, the findings presented herein suggest that VD3 may prevent endometriosis and uterine fibroids owing to excess PRL in the endometrial microenvironment by decreasing *PRL* expression.

The present study found that VD3 regulates *HAND2* expression, the master regulator of decidualization, and *PRL*, which is critical for the uterine microenvironment in decidualization. In light of the effects on *PRL* in the present study, further research is required to decide the optimal timing of VD3 supplementation. By contrast, in patients with cellular tumor antigen p53-positive gastrointestinal cancers, vitamin D supplementation has been shown to reduce the risk of recurrence/mortality (32). In addition, nutritional approaches, including VD3 for the management of gynecological cancers molecularly classified by polymerase epsilon and cellular tumor antigen p53, particularly endometrial and ovarian cancers (33), may become useful.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ST conceptualized the study and was involved in the study methodology. ST also provided the methodology, research environment and reagents, etc., supervised the study, and was also involved in project administration and in funding acquisition. NY, KT and ST were involved in data validation and data curation, as well as in the writing, review and editing of the manuscript and in the preparation of the figures. NY, KT and AT were involved in the formal analysis and in the investigative aspects of the study. NY and ST were involved in the writing and preparation of the original draft of the manuscript. NY and ST confirm the authenticity of all the raw data. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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