



ORIGINAL ARTICLE

NDRG1 is expressed in human granulosa cells: An implicative role of NDRG1 in the ovary

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Abstract

Purpose: N-myc downstream-regulated gene 1 (NDRG1) is expressed in various human tissues and plays a role in regulating cellular proliferation, angiogenesis, and hypoxia sensing. However, the role of NDRG1 in the ovary remains poorly understood. Therefore, we investigated NDRG1 expression and the role of NDRG1 in the human ovary.

Methods: Follicular fluid (FF) and luteinized granulosa cells were collected from follicles during oocyte retrieval. KGN cells were cultured with cobalt chloride (CoCl₂, a hypoxia-mimicking agent) and/or echinomycin. mRNA, protein levels and secretion, and localization were assessed by real-time PCR, Western blotting, ELISA, and immunohistochemical analysis, respectively. KGN cells were also transfected with NDRG1 siRNA for 72 h.

Results: NDRG1 protein was expressed in luteinized granulosa cells. NDRG1 concentration was positively correlated with vascular endothelial growth factor (VEGF) and progesterone concentrations in FF. CoCl₂-induced hypoxic stress significantly increased NDRG1 and VEGF mRNA and protein and hypoxia-inducible factor-1 α expression compared with those in the controls. The CoCl₂-induced overexpression of NDRG1 and VEGF was suppressed by echinomycin. Transfection with NDRG1 siRNA significantly suppressed the release of progesterone in the culture medium.

Conclusions: These results indicate that ovarian NDRG1 may play important roles in follicular development, especially in the early luteinization of pre-ovulatory follicles.

KEYWORDS

angiogenesis, granulosa cells, hypoxia, N-myc downstream-regulated gene 1, ovary

1 | INTRODUCTION

The ovary continuously undergoes physiological angiogenesis. Changes in vascular formation are involved in follicular growth, atresia, ovulation, and corpus luteum formation. The ovary is distinctive

as a site of active angiogenesis, with the development of follicular microvasculature regulated by angiogenic factors.¹⁻⁴ Several mediators induce angiogenesis, including members of the vascular endothelial growth factor (VEGF) family, angiopoietin, hepatocyte growth factor, and members of the CXC chemokine family. Substances

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participating in angiogenesis are often secreted or transported into the follicular fluid (FF), and several factors detected in human FF have been suggested to play roles in the human ovary.⁵⁻¹⁰

N-myc downstream-regulated gene 1 (NDRG1) is a 43 kDa protein comprised of 394 amino acids; it is known to be highly conserved among multicellular organisms in tissues such as those of the prostate, ovaries, colon, kidney, intestines, and placenta.¹¹⁻¹⁴ Currently, there are four known members of the human NDRG family: NDRG1, NDRG2, NDRG3, and NDRG4.^{15,16} NDRG1 is a stress-responsive protein whose expression is triggered by multiple stimuli, including hypoxia, oxidative stress, heavy metals, hormones, DNA damage, and histone deacetylase inhibitors. In response to these stimuli, NDRG1 plays a role in regulating cellular differentiation, proliferation, apoptosis, angiogenesis, tumor progression, and metastasis, as well as heavy metal and hypoxia sensing.^{14,17-23} Furthermore, NDRG1 is upregulated by various hypoxia-related transcription factors—including hypoxia-inducible factor-1 α (HIF-1 α)—under hypoxic conditions.²⁵ The expression site of NDRG1 is predominantly cytosolic; it is ubiquitously expressed in tissues in response to cellular stress signals.^{13,14} Moreover, uterine NDRG1 expression was observed in the human endometrium, with localization in stromal and glandular epithelial cells.²⁶ It has been reported that NDRG1 plays an important role in decidualization during the implantation process; indeed, decreased uterine NDRG1 expression is associated with recurrent miscarriages in humans.²⁷ NDRG1 expression in the ovary has already been confirmed by microarray analysis,²⁸ but to our knowledge, no studies have been reported on the role of NDRG1 in the ovary.

VEGF acts as a key angiogenic factor in the regulation of ovarian vascularization. It potentially plays a role in developing the perfollicular capillary network and may act as a marker of follicular microenvironment quality. VEGF is produced by the theca interna and granulosa cells (GCs) in the human ovary during the pre-ovulatory phase. The regulatory role of VEGF can be observed at almost all stages of follicular angiogenesis, from the development of the antral follicle to the formation and maintenance of the corpus luteum.^{7,29} Moreover, VEGF expression in GCs is induced by hypoxic conditions and is regulated by HIF-1 α .³⁰⁻³⁴

Considering the pleiotropic functions of NDRG1, we hypothesized that hypoxia is involved in the regulation of NDRG1 in the human ovary. In this study, to assess the role of NDRG1 in regulating the female reproductive axis, we investigated the expression of endogenous NDRG1 under hypoxia and the effect of NDRG1 on steroidogenesis in human GCs.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

All human samples were obtained using a protocol designed to protect the privacy rights of human subjects approved by the Institutional Review Board of Kansai Medical University; informed

consent was obtained from all women in accordance with the Declaration of Helsinki.

2.2 | Immunohistochemistry for NDRG1

The ovarian tissues used in this study were obtained from three women (age range, 32–39 years) with regular menstrual cycles who were not taking hormonal drugs to treat uterine cancer. Immunohistochemical analysis was performed using an autostainer (Discovery ULTRA System; Roche Diagnostics) according to the manufacturer's instructions. A primary rabbit polyclonal antibody was used to detect NDRG1 (1:75; Cell Signaling Technology, Inc.). For antigen retrieval, tissue sections were autoclaved at 100°C for 16 min in a Tris-based buffer (pH 8.5; Cell Conditioning Solution CC1; Ventana Medical Systems). Thereafter, automated protocol steps for immunostaining were followed, using 3,3'-diaminobenzidine (DAB) as a colorimetric agent.

2.3 | Patients, stimulation protocol, and retrieval

FF samples were obtained from 11 women at the time of oocyte retrieval following ovarian stimulation. We analyzed the FF from women aged 38 years or younger (range 25–38; mean \pm SEM, 32.09 \pm 1.36 years) as it has been established that an elevated level of VEGF in FF correlates positively with the chronological age of women. The women underwent IVF/ICSI treatment because of male infertility. All women had both ovaries and regular menstrual cycles with normal ovulatory function as shown by cycle day 3 FSH concentration <10 IU/ml and ultrasonographic scanning indicative of ovulatory cycles.

All women underwent a long downregulation protocol using a gonadotrophin-releasing hormone (GnRH) agonist (buserelin acetate; Mochida Pharmaceutical Co.). Buserelin acetate 600 μ g was administered daily in the mid-luteal phase of the previous cycle, and ovarian stimulation was achieved using hMG (Fuji Pharmaceutical Co.) administered from cycle day 2 or 3 at a starting dose of 300 IU. Thereafter, the hMG dose was adjusted based on follicular response and serum E₂ concentration.

We also analyzed the FF in women \geq 40 years of age (range 40–44; mean \pm SEM, 42.08 \pm 0.37 years) to compare age-related differences. These women (\geq 40 years of age) underwent controlled ovarian stimulation according to a GnRH antagonist protocol. The hMG injections were administered daily from day 2 with a GnRH antagonist (cetrotrelix acetate 0.25 mg/day; Merck Biopharma Co.) together with gonadotropins after a leading follicle of 14–16 mm in diameter appeared. Gonadotropins and the GnRH antagonist were administered until the day of hCG injection.

Follicular development was monitored by serum E₂ concentration and vaginal ultrasound using a transvaginal probe. Finally, hCG (10,000 IU) (Fuji Pharmaceutical Co.) was administered when a consistent rise in serum E₂ concentration was associated with the

presence of two or more follicles of ≥ 18 mm in diameter. Oocyte aspiration was performed by vaginal ultrasonography 35–36 h after hCG injection.

2.4 | Collection of follicular fluid and isolation of luteinized granulosa cells

FF was aspirated separately from each follicle during oocyte retrieval. The collection tube was flushed to prevent contamination. Any follicle aspirates that were not clear (ie, potentially contaminated with blood) were discarded. After removing the oocytes, the clear FF samples were immediately centrifuged for 10 min at $1,000 \times g$ and then stored at -80°C for further analysis. Human luteinized granulosa cells (LGCs) were isolated from the FF obtained from each patient and centrifuged through a one-step Ficoll™ density gradient for 10 min at $1,000 \times g$ to remove red blood cells.

2.5 | Collection of placental tissue

Human placental tissue for RNA analysis was obtained from normal pregnancies after an abdominal delivery (age: 29 years, gestational age: 38 weeks).

2.6 | Cell culture and treatment

The KGN cell line was purchased from the RIKEN Cell Bank of Japan (Tsukuba, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 10% fetal calf serum (FCS; HyClone), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen/Thermo Fisher Scientific) in an atmosphere of 5% CO_2 at 37°C . Thereafter, KGN cells were seeded into 6-well plates (1.5×10^5 cells/well) and cultured overnight for subsequent real-time PCR analysis and Western blotting.

In the drug-induced hypoxic experiment, KGN cells were cultured in 10% FCS-DMEM supplemented with cobalt chloride (CoCl_2 , a hypoxia-mimicking agent; Wako Pure Chemical Industries, Ltd.), echinomycin (ENZO Life Sciences), and 0.01% DMSO as a vehicle control under a 5% CO_2 atmosphere. The culture medium was collected after stimulation and stored at -80°C until assayed. Each experiment was repeated at least thrice with different cell preparations.

2.7 | Biochemical assays

VEGF concentrations in cell culture medium and FF were determined using a commercially available ELISA kit (Duoset® ELISA human VEGF; R&D Systems). Concentrations of P_4 and E_2 in FF were measured using a commercially available fluorescence immunoassay (EVANET EV20; Nissui). Concentrations of NDRG1

in FF were measured using a Human Protein NDRG1 ELISA Kit (MyBioSource). Protein concentrations were assessed using a commercially available Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). All procedures were performed according to the manufacturers' instructions.

2.8 | RNA extraction and quantitative reverse transcription-PCR analysis (RT-PCR)

Total RNA was isolated from placental tissue, LGCs, and cultured KGN cells using an RNeasy Minikit (Qiagen GmbH) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA using ReverTra Ace qPCR RT Master Mix (Toyobo). Reverse transcription was performed according to the manufacturer's instruction. Quantitative RT-PCR (qPCR) was performed using Rotor-Gene Q HRM (Qiagen) and a quantitative PCR mix kit (THUNDERBIRD SYBR qPCR Mix; Toyobo) using methods described previously.³⁰ Briefly, qPCR was conducted in a final volume of 20 μl , including 10 μl THUNDERBIRD SYBR qPCR Mix, 4 μl primers (3.75 $\mu\text{mol}/\text{L}$; 2 μl each of both forward and reverse primers), 2 μl cDNA templates, and 4 μl distilled water. Each PCR run was performed as follows: initial denaturation at 95°C for 1 min, 40 amplification cycles of real-time fluorescence measurement and denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. Each experiment was performed in duplicate. *Elongation factor-1 α* (*EF-1 α*) was used as an internal control, as it is a valid reference gene for transcription profiling commonly used for real-time PCR experiments. Primer sequences and accession numbers are listed in Table 1. PCR for all standards and samples was performed using duplicated reactions, after which melting curve analysis was performed to monitor PCR product purity. To eliminate the possibility of contamination with genomic DNA during extraction of total RNA, a control reaction with each primer pair was performed simultaneously under identical conditions without reverse transcription; no amplification was detected. Relative mRNA expression levels from real-time PCR were calculated using the comparative threshold cycle ($\Delta\Delta\text{Ct}$) method, as described previously.³⁵

2.9 | Western blot analysis

To analyze protein levels, cultured cells were homogenized in lysis buffer containing mammalian protein extraction reagent (Thermo Fisher Scientific) and protease inhibitor cocktail (Calbiochem Corp.). Protein concentrations were quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Equivalent quantities of lysate protein (20 $\mu\text{g}/\text{lane}$) were electrophoresed using an Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immun-Blot® PVDF Membrane (Bio-Rad Laboratories). Non-specific binding sites were blocked with 10% skim milk powder in Tris-buffered saline for

1 h. The blots were then incubated overnight at 4°C with rabbit polyclonal NDRG1 antibody (1:1,000; Cell Signaling Technology), rabbit polyclonal p-NDRG1 antibody (1:1,000; Cell Signaling Technology), rabbit monoclonal HIF-1 α antibody (1:1,000; Abcam), or mouse

monoclonal β -actin antibody (1:5,000; Sigma-Aldrich) as the primary antibody and anti-rabbit immunoglobulin IgG peroxidase-labeled secondary antibody (1:2,000; GE Healthcare Life Sciences) or anti-mouse IgG peroxidase-labeled secondary antibody (1:10,000; GE Healthcare Life Sciences) as the secondary antibody. Immune complexes were visualized using enhanced chemiluminescence plus Western blotting detection reagents (GE Healthcare Life Sciences).

TABLE 1 Primer sequences used for real-time PCR and amplicon sizes

Gene	Primer sequence 5'→3'	Product size (bp)
NDRG1	Forward: AGGCAGGTGACAGCAGGGAC	300
	Reverse: CGTGGCAGACGGCAAAGT	
VEGF	Forward: CGAAACCATGAACTTTCTGC	301
	Reverse: CCTCAGTGGGCACACACTCC	
EF	Forward: TCT GGTTGGAATGGTGACAACATGC	329
	Reverse: AGAGCTTCACTCAAAGCTTCATGG	
StAR	Forward: AAACTTACGTGGCTACTCAGCATC	66
	Reverse: GACCTGGTTGATGATGCTCTTG	
P450scc	Forward: CAGGAGGGGTGGACACGAC	64
	Reverse: AGGTTGCGTGCCATCTCATAC	

2.10 | NDRG1 and VEGF silencing with small interfering RNA

To silence NDRG1 and VEGF expression, two small interfering RNAs (siRNAs) directed against distinct areas of the gene sequence (catalog numbers NDRG1: HSS115920 and HSS173605, VEGF: HSS111274 and HSS187670; Invitrogen) and a nonsilencing RNA negative control recommended by the manufacturer (catalog no. HSS12935-112 Human Stealth Select RNAi™; Invitrogen) were purchased from Life Technologies/Thermo Fisher Scientific. KGN cells were seeded at 1.5×10^5 cells/well in 6-well plates and precultured in phenol red-free DMEM/F12 supplemented with 10% dextran-coated charcoal-stripped (DCS)-FCS. The cells were cultured until 30% confluency, as determined by optimization of the experimental conditions, and

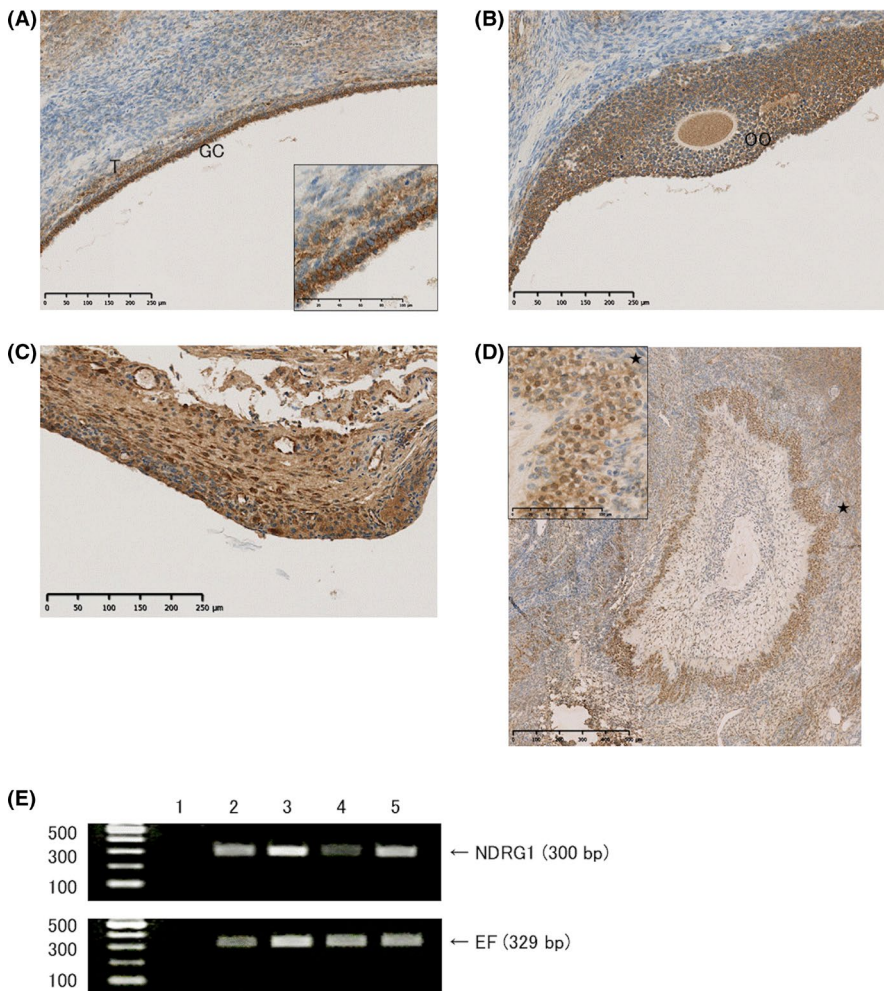


FIGURE 1 Expression of NDRG1 in human GCs. Immunohistochemical detection of NDRG1 protein in the human ovarian follicle (A, GCs and theca cells (T) in antral follicle; B, oocyte (OO) in antral follicle; C, corpus luteum; D, corpus albicans). (D) RT-PCR revealed the presence of *NDRG1* mRNA in placental tissue (positive control; Lane 2), human ovary (Lane 3), human LGCs (Lane 4), KGN cells (Lane 5), negative control (Lane 1). GCs, granulosa cells; LGCs, luteinized granulosa cells

then transfected with each siRNA (10 nmol/L) using Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. After 72 h, the medium was removed, and RNA was extracted from the cells. The medium after knockdown of NDRG1 was frozen and stored at -80°C until hormone assays. NDRG1 gene silencing was confirmed by qPCR, Western blotting, and P_4 assays. Each experiment was repeated at least thrice with different cell preparations.

2.11 | Measurement of hormone secretion

Concentrations of P_4 and E_2 in cell culture medium were determined using commercially available Progesterone and Estradiol ELISA Kits (Cayman Chemical Co.). Measurements were performed in triplicate according to the manufacturer's instructions. The data are expressed as the quantity of steroids secreted (pg/ml). To avoid possible bias due to the proliferative ability of KGN cells, P_4 and E_2 concentrations were corrected for protein concentration.

2.12 | Statistical analysis

All experimental results are expressed as the mean \pm standard error of the mean (SEM). For experiments involving only two groups, the data were analyzed using a *t* test. Multiple comparisons were assessed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). Statistical significance was defined as $p < 0.05$.

3 | RESULTS

3.1 | Localization of NDRG1 protein in the human ovary

First, we aimed to demonstrate the localization of NDRG1 protein in the human ovary using immunohistochemistry. NDRG1 protein was detected in the cytoplasm of GCs and theca-interstitial cells, and oocytes in antral follicles (Figure 1A,B). As shown in Figure 1C,D, the corpus luteum and the corpus albicans exhibited positive NDRG1 immunostaining. To confirm the expression of NDRG1, human LGCs were obtained from women undergoing *in vitro* fertilization (IVF); RT-PCR analysis showed the expression of NDRG1 mRNA in human LGCs and KGN cells as a single specific band corresponding to the expected size of 300 bp (Figure 1E).

3.2 | Relationship among the concentrations of NDRG1, VEGF, and hormones in follicular fluid

Follicular fluid provides a critically important microenvironment for the development of ovarian follicles and oocytes. Therefore,

we examined the relationship between NDRG1 and VEGF, P_4 and E_2 levels in FF. A total of 138 clinical samples were obtained from 11 women (≤ 38 years of age) who underwent IVF. To avoid the effect of follicle size, we analyzed FF in follicles with a diameter of 15–20 mm. The mean \pm SEM concentrations of NDRG1, VEGF, P_4 , and E_2 in the FF were 17.1 ± 0.29 ng/ml, 386.4 ± 16.00 pg/ml, 7285.8 ± 252.28 ng/ml, and 629.8 ± 23.32 ng/ml, respectively. Interestingly, FF NDRG1 levels were positively correlated with FF VEGF ($r = 0.568$, $p < 0.01$) and FF P_4 levels ($r = 0.400$, $p < 0.01$) (Figure 2A,B). There was no correlation between the concentrations of NDRG1 and E_2 in FF ($r = 0.257$; Figure 2C).

3.3 | Relationship between the concentrations of NDRG1 and VEGF in follicular fluid and age

We measured the concentrations of NDRG1 and VEGF in the FF of the dominant follicles. A comparison of the mean NDRG1 and VEGF concentrations in FF between the two groups (≤ 38 years of age vs. ≥ 40 years of age) is shown in Figure 2D,E. The FF NDRG1 and VEGF concentrations in women ≤ 38 years of age were 7.1 ± 0.53 ng/ml and 378.7 ± 59.63 pg/ml, respectively. For women ≥ 40 years of age, the FF NDRG1 and VEGF concentrations were 16.5 ± 1.35 ng/ml and 889.7 ± 116.73 pg/ml, respectively. These differences were significant ($p < 0.01$).

3.4 | Influence of hypoxia on the expression levels of NDRG1 and VEGF in KGN cells

To examine the effect of CoCl_2 -induced hypoxic stress on NDRG1 and VEGF expression, KGN cells were cultured with 10 or 100 μM CoCl_2 for 24 h. We then determined the expression levels of NDRG1 and VEGF mRNA in KGN cells under CoCl_2 -induced hypoxic stress. As shown in Figure 3A,B, CoCl_2 significantly increased NDRG1 and VEGF mRNA expression levels compared with those of the controls in a concentration-dependent manner after 24 h of stimulation. Next, we investigated the expression of VEGF protein in the culture medium using ELISA. VEGF secretion was enhanced by CoCl_2 treatment (Figure 3C). Western blot results confirmed the stimulatory effects of CoCl_2 on both NDRG1 protein and phosphorylation levels. As shown in Figure 3D, NDRG1 expression and its phosphorylation were upregulated upon exposure to CoCl_2 .

We performed a time-course study of changes in the mRNA expression levels of NDRG1 and VEGF. As expected, 100 μM CoCl_2 enhanced NDRG1 and VEGF expression levels in KGN cells in a time-dependent manner. CoCl_2 caused a significant increase in NDRG1 and VEGF mRNA expression after 1 h of culture compared with the control; furthermore, this increase continued until the end of the experimental period at 48 h (Figure 3E,F). Similarly, CoCl_2 caused a significant increase in VEGF production after 6 h of culture compared with that of the control; this effect also continued to increase until the end of the study at 48 h (Figure 3G).

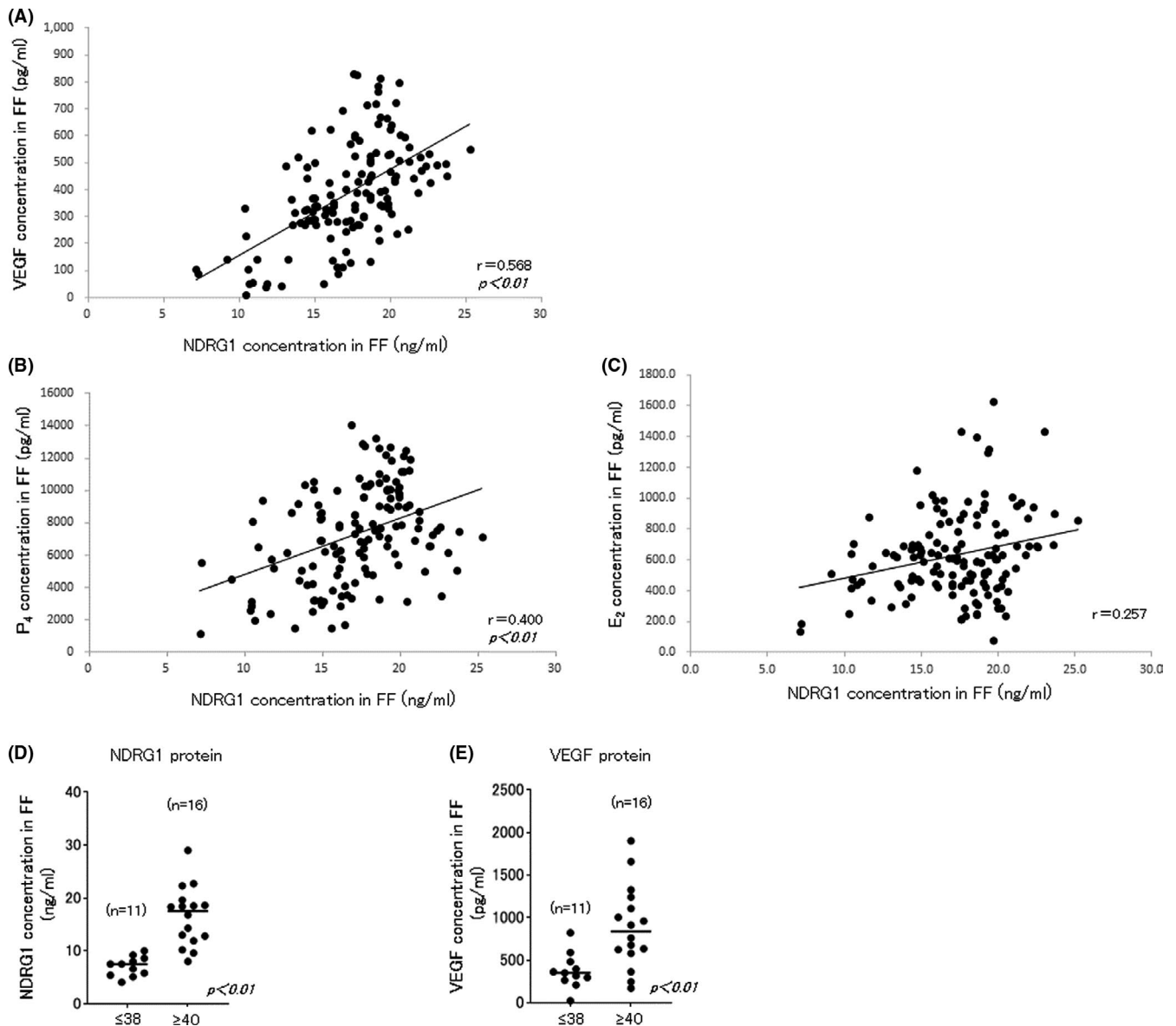


FIGURE 2 NDRG1 concentrations in FF. NDRG1, VEGF, P₄, and E₂ levels in FF were examined using ELISA, and Pearson's correlation analysis was performed to assess their relationship. FF was analyzed for women aged 38 years or younger. FF NDRG1 levels were positively correlated with FF VEGF ($r = 0.568$, $p < 0.01$) (A) and FF P₄ levels ($r = 0.400$, $p < 0.01$) (B). There was no correlation between the concentrations of NDRG1 and E₂ in FF ($r = 0.257$) (C). NDRG1 and VEGF levels in FF were examined for different ages. FF NDRG1 (D) and VEGF (E) levels were significantly different in the combined group of women ≥ 40 years compared with women ≤ 38 years of age ($p < 0.01$). Only the first FF aspirate without blood was used for analysis. FF, follicular fluid

3.5 | Regulation of NDRG1 expression by transcription factor HIF-1 α

We then evaluated the potential mechanism underlying the effect of CoCl₂ on NDRG1 and VEGF production in KGN cells. Using Western blotting, we determined whether the addition of 100 μ M CoCl₂ led to an increase in the levels of HIF-1 α protein in KGN cells. The temporal release of HIF-1 α , NDRG1, and p-NDRG1 from KGN cells in response to hypoxia is shown in Figure 3H. The highest level of HIF-1 α protein was observed at 6 h, followed by a gradual decline after 48 h. In contrast, the protein levels of NDRG1 and p-NDRG1 were upregulated in KGN cells after 24 h of stimulation and increased up to 48 h.

3.6 | Effect of echinomycin on NDRG1 and VEGF expression in KGN cells

To demonstrate the role of HIF-1 α in CoCl₂-mediated NDRG1 and VEGF protein production, we used echinomycin—a small-molecule inhibitor of HIF-1 α activity. We monitored the secretion of NDRG1 and VEGF under hypoxic conditions induced by CoCl₂ with the addition of echinomycin in KGN cells. A concentration of echinomycin 1 nM or higher inhibited the induction of NDRG1 and VEGF mRNA under hypoxic conditions induced by CoCl₂ (Figure 3I,J). The expression of VEGF protein was inhibited by echinomycin concentrations of 0.1 nM or higher (Figure 3K). As

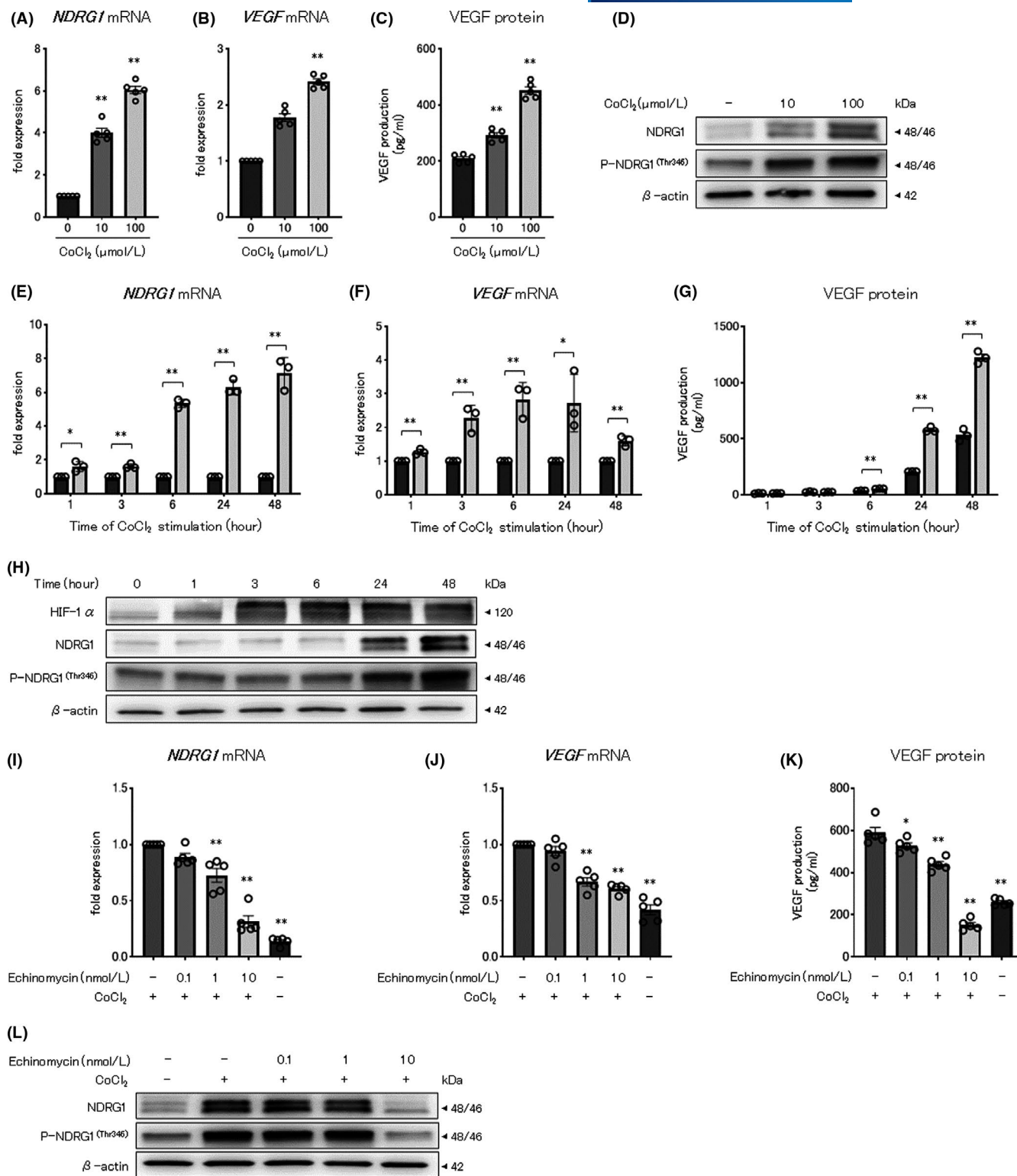


FIGURE 3 Influence of CoCl₂-induced hypoxic stress on the expression of NDRG1 and VEGF in KGN cells. The dose-dependent effect of CoCl₂ on (A) *NDRG1* mRNA, (B) *VEGF* mRNA, (C) VEGF protein, and (D) NDRG1 and p-NDRG1 protein. KGN cells were incubated in medium containing 10 or 100 μmol/L CoCl₂ for 24 h ($n = 5$). The time-dependent effect of CoCl₂ on (E) *NDRG1* mRNA, (F) *VEGF* mRNA, (G) VEGF protein, (H) HIF-1α, NDRG1, and p-NDRG1 proteins. KGN cells were treated with 100 μmol/L CoCl₂ for 0, 1, 3, 6, 24, or 48 h ($n = 3$). The effect of echinomycin on (I) *NDRG1* mRNA, (J) *VEGF* mRNA, (K) VEGF protein, and (L) NDRG1 and p-NDRG1 protein. KGN cells were cultured in medium containing 100 μmol/L CoCl₂ with or without varying concentrations of echinomycin (0.1–10 nmol/L) for 24 h ($n = 5$). mRNA levels of *NDRG1* and *VEGF* in KGN cells were assessed by quantitative RT-PCR and calculated after normalization to *EF1α* mRNA levels. Fold differences are shown compared with those of the control, for which the value was defined as 1.0. The data are presented as the mean ± SEM. The protein levels of HIF-1α, NDRG1, and p-NDRG1 were quantified by Western blotting, and β-actin was used as the control. VEGF protein levels were analyzed by ELISA. The data are presented as the mean ± SEM. Significant differences are indicated by brackets: * $p < 0.05$ versus the control group. ** $p < 0.01$ versus the control group

shown in Figure 3L, the levels of NDRG1 and p-NDRG1 were suppressed by echinomycin.

3.7 | Effect of NDRG1 silencing on hormone production

To directly examine the critical role of NDRG1 in hormone production, a siRNA-based gene silencing approach was used to knock down NDRG1 expression. Transfection of KGN cells with NDRG1 siRNA for 72 h significantly downregulated endogenous NDRG1 mRNA and protein levels (Figure 4A,B).

Transfection of KGN cells with NDRG1 siRNA for 72 h significantly reduced the mRNA levels of *StAR* and *P450scc* (Figure 4C,D) and suppressed the release of P_4 in the culture medium (Figure 4E). However, we could not find any significant effect of siRNA-mediated NDRG1 knockdown on E_2 production in KGN cells (Figure 4F).

3.8 | Interaction of NDRG1 with VEGF

To examine the interaction between NDRG1 and VEGF, we investigated whether NDRG1 affects VEGF expression. NDRG1 silencing did not alter the expression levels of VEGF mRNA (Figure 5A) or protein (Figure 5B). Next, we analyzed whether VEGF regulates NDRG1 expression. VEGF silencing significantly suppressed VEGF mRNA levels (Figure 5C) and VEGF protein levels (Figure 5D). However, transfection of KGN cells with VEGF siRNA had no obvious effect on NDRG1 mRNA and protein levels (Figure 5E,F).

4 | DISCUSSION

In this study, we report that NDRG1 is present in human ovarian follicles and KGN cells. In KGN cells, NDRG1 expression is regulated through the HIF-1 α signaling pathway under hypoxic conditions, which is likely regulated by HIF-1 α stabilization. Furthermore, NDRG1 is involved with luteinization-related genes and P_4 secretion, suggesting that NDRG1 affects ovarian folliculogenesis and luteinization.

NDRG1 is involved in diverse cellular characteristics such as specific stress and hormone responses, cell growth, and differentiation.^{14,17-23} Here, we demonstrated the expression of NDRG1 protein in human GCs, theca-interstitial cells, corpus luteum, and oocytes using immunohistochemistry.

Members of the VEGF family may play important roles in folliculogenesis, as they have marked angiogenic and/or vasodilator effects in addition to potential dual effects on cell growth and proliferation.³⁶ In the present study, we confirmed that NDRG1 was present in FF and was positively correlated with VEGF levels in FF. High NDRG1 expression levels have been reported to correlate with angiogenic activity in several cancers.^{37,38} Therefore, angiogenesis during folliculogenesis may depend on a synergistic angiogenic pathway mediated by NDRG1 and VEGF.

We found that the mean NDRG1 and VEGF concentrations in FF were increased in older women. Friedman et al. reported that VEGF in the FF of women of advanced reproductive age was elevated compared with that in younger women. They suggested that the increase in VEGF levels was caused by a hypoxic environment within the follicles of older women. Indeed, VEGF protein levels were increased by both hypoxic and $CoCl_2$ treatments in human GCs.³¹ Gaulden proposed that the developing ovarian follicle is surrounded by deficient microcirculation with aging.³⁹ The elevated NDRG1 and VEGF concentrations in FF suggested an age-associated reduction in GC-mediated angiogenesis.

Based on our clinical results, we performed experiments at the cellular level. In the present study, $CoCl_2$ -induced hypoxic stress enhanced both NDRG1 and VEGF mRNA and protein levels in a concentration- and time-dependent manner. Our results are consistent with those of previous studies in which NDRG1 expression was strongly induced by hypoxia or $CoCl_2$.⁴⁰⁻⁴² It has been shown that hypoxia-induced NDRG1 expression is initiated by HIF-1 α .

HIF-1 α is stably expressed during hypoxia and is a transcription factor known to play a critical role in the cellular response to hypoxia. HIF-1 α activation in the hypoxic microenvironment contributes to the induction or downregulation of the expression of genes involved in various cellular functions such as angiogenesis, cell survival, oxygen homeostasis, proliferation, glucose metabolism, and apoptosis.^{43,44} HIF-1 α has been shown to bind directly to hypoxia-responsive elements in the promoters of genes encoding VEGF and NDRG1.^{33,34,41,45} Therefore, we hypothesized that HIF-1 α might regulate NDRG1 and VEGF expression.

In the present study, $CoCl_2$ -induced NDRG1 and VEGF expression was suppressed by echinomycin in a concentration-dependent manner. Echinomycin specifically inhibits the DNA-binding ability of HIF-1 α by directly interacting with the hypoxia response element of its target gene.⁴⁶ These results suggest that HIF-1 α initiates NDRG1 expression during hypoxia and that the HIF-1 signaling pathway contributes substantially to the regulation of NDRG1 under hypoxic conditions in KGN cells.

We further demonstrated that $CoCl_2$ -induced hypoxic stress enhanced NDRG1 phosphorylation, and $CoCl_2$ -induced p-NDRG1 levels were suppressed by echinomycin. Phosphorylation of NDRG1 occurs under hypoxic conditions. NDRG1 phosphorylation at Thr 346 is known to be important in cell differentiation, cell growth, and some signaling pathway regulation.^{47,48} In this investigation, we did not perform detailed mechanistic studies examining the expression of downstream genes involved in NDRG1 phosphorylation. In the future studies, it will be interesting to assess the role of NDRG1 phosphorylation under hypoxic conditions in GCs.

In the present study, we investigated the relationship between NDRG1 and VEGF in KGN cells. However, we were unable to confirm this interaction between NDRG1 and VEGF. Conflicting views currently exist regarding the relationship between these two factors. In cervical adenocarcinoma, an increase in NDRG1 expression is associated with VEGF expression and angiogenesis.³⁷ Moreover, a cell line with NDRG1 knockdown showed a marked decrease in

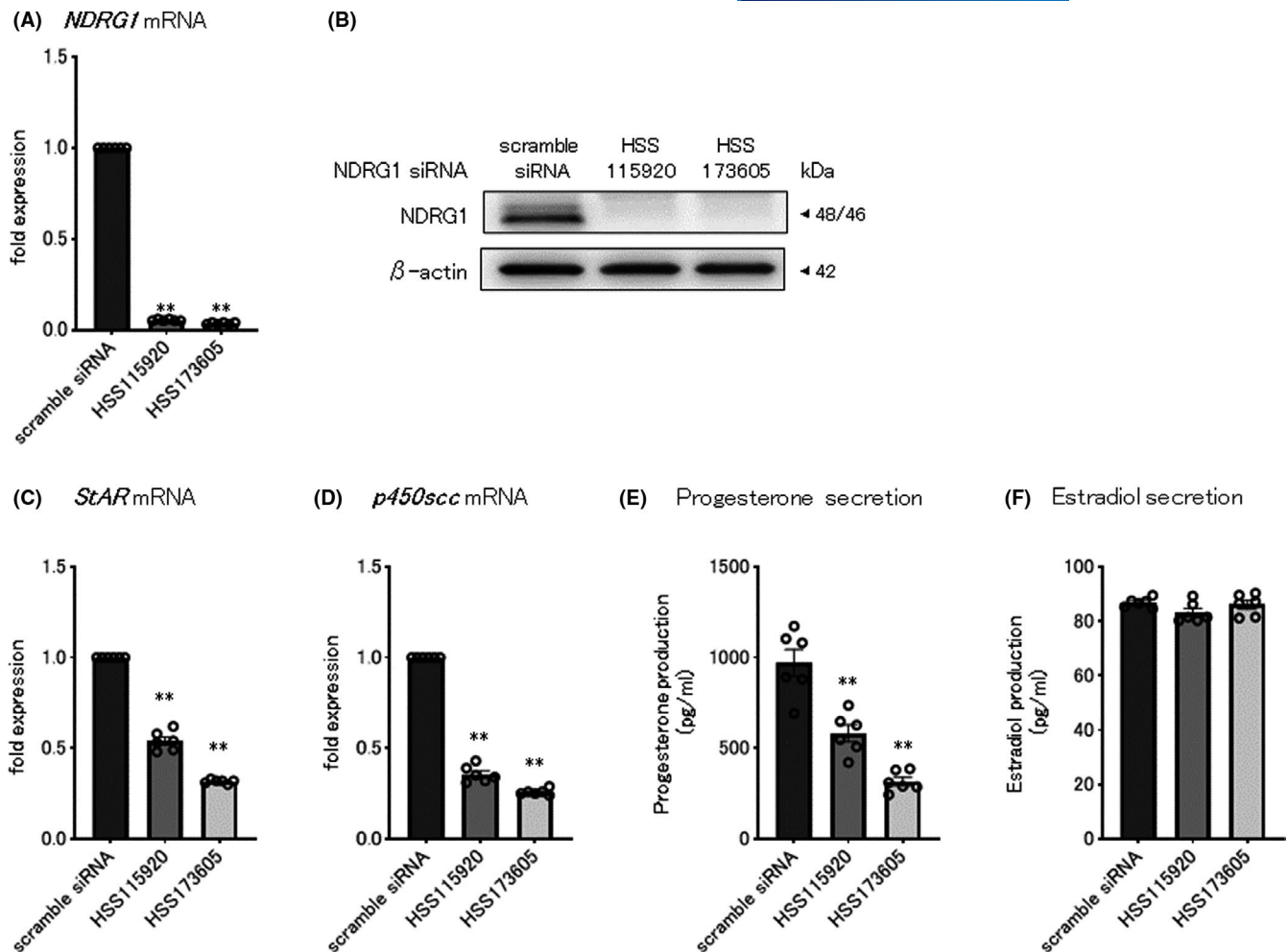


FIGURE 4 Effect of NDRG1 silencing on hormone production. KGN cells were transfected with siRNA targeting *NDRG1* or control scramble siRNA for 72 h. *NDRG1* knockdown efficiency was examined by quantitative RT-PCR (A) and Western blotting (B). Effect of *NDRG1* expression on molecules related to luteinization, *StAR* mRNA (C), and *p450scc* mRNA (D) was investigated by quantitative RT-PCR. (E) Progesterone and (F) estradiol secretion was measured by ELISA in the culture medium of KGN cells after 72 h of siRNA-mediated knockdown of *NDRG1*. Data are expressed as the amount of steroids secreted. Results are expressed as the mean \pm SEM, $n = 6$. Significant differences are indicated by brackets: * $p < 0.05$ versus the control group. ** $p < 0.01$ versus the control group

VEGF production in human lung cancer cells.⁴⁹ In contrast, pancreatic cancer cells with high *NDRG1* levels had a significant reduction in VEGF protein levels.³⁸ This discrepancy is likely due to different cell types or tissue-specific functions. Although it remains to be clarified how *NDRG1* promotes or suppresses angiogenesis in folliculogenesis, HIF-1 may separately regulate the expression of *NDRG1* and VEGF by which the additive angiogenic pathway is mediated.

In the present study, transfection of KGN cells with *NDRG1* siRNA significantly reduced the mRNA levels of *StAR* and *P450scc*, suggesting that *NDRG1* promotes steroidogenesis and luteinization. Although we investigated this, we could not find any significant difference in E_2 production between the control and *NDRG1* knockdown groups. *StAR* transports cholesterol into the mitochondria, and *P450scc* converts cholesterol to pregnenolone, the first committed step and rate-limiting process in steroid hormone synthesis.⁵⁰ In humans and other primates, *StAR* is essential for developing and maintaining the corpus luteum.⁵¹ Luteinization

is associated with the upregulation of *StAR* in LGCs.⁵² The expression of these markers decreased substantially after *NDRG1* gene silencing, suggesting that *NDRG1* promotes luteinization—a process of terminal differentiation of GCs—in the ovary.

In fact, P_4 secretion decreased after *NDRG1* gene silencing. These results suggest a role for *NDRG1* in promoting luteinization of the ovary. Following ovulation, GCs undergo luteinization and form the corpus luteum, which secretes P_4 . P_4 production from the corpus luteum provides the necessary support for early pregnancy.⁵³ In addition, we showed that the concentration of *NDRG1* was closely related to P_4 concentrations in FF. Along with the data obtained from *in vitro* experiments, these clinical results confirmed the effect of *NDRG1* on the expression of luteinization-related molecules and the production of P_4 in the human ovary. These results strongly support the *in vivo* stimulatory effect of *NDRG1* on steroidogenesis in the human ovary. However, further *in vivo* experiments are needed to demonstrate the role of *NDRG1* in follicle development and luteinization.

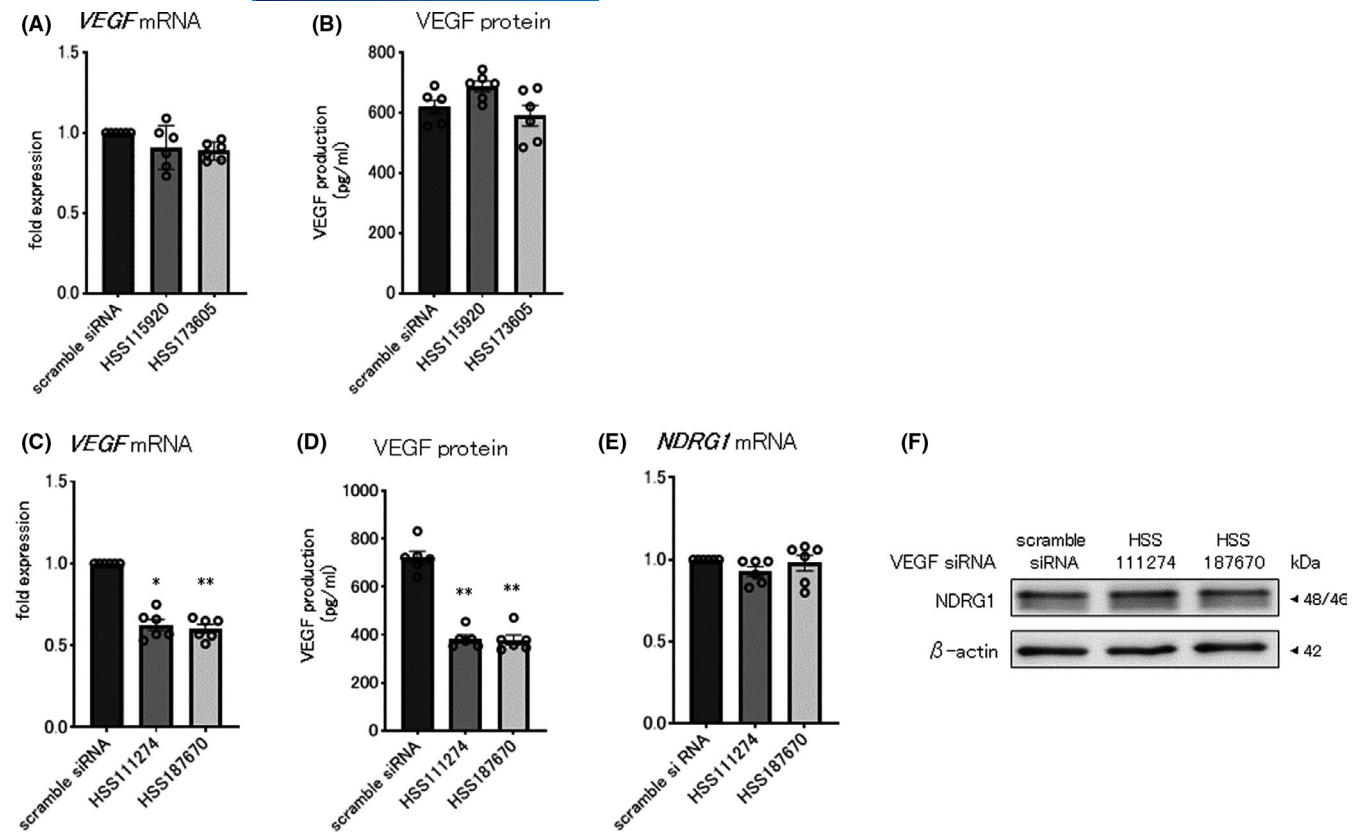


FIGURE 5 Interaction of NDRG1 with VEGF. The effect of *NDRG1* knockdown on *VEGF* mRNA (A) and VEGF protein (B). KGN cells were transfected with siRNA targeting *NDRG1* or control scramble siRNA for 72 h. To avoid possible bias due to the proliferative ability of KGN cells, the concentrations of VEGF protein were corrected for protein concentration. The effect of *VEGF* knockdown on *VEGF* mRNA (C), VEGF protein (D), *NDRG1* mRNA (E), and *NDRG1* protein (F). KGN cells were transfected with siRNA targeting *VEGF* or control scramble siRNA for 72 h. mRNA levels of *NDRG1* and *VEGF* in KGN cells were assessed by quantitative RT-PCR and calculated after normalization to *EF1 α* mRNA levels. Fold differences are shown compared with those of the control, for which the value was defined as 1.0. The data are presented as the mean \pm SEM, $n = 6$. The protein level of NDRG1 was quantified by Western blotting, and β -actin was used as the control. VEGF protein levels were analyzed by ELISA. The data are presented as the mean \pm SEM, $n = 6$. Significant differences are indicated by brackets: * $p < 0.05$ versus the control group. ** $p < 0.01$ versus the control group

In conclusion, our findings suggest that NDRG1 plays a key role in terminal differentiation of GCs and activating luteinization in pre-ovulatory follicles. The activation of NDRG1 may have important clinical implications in maintaining ovarian homeostasis; however, further studies are necessary to confirm the physiological implications of NDRG1 in the ovary. This research will provide new insights into our understanding of ovarian physiology, including the processes of ovarian luteinization. Ultimately, our findings may affect future treatments of infertility while offering an enhanced understanding of the pathogenesis of various ovarian diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

HUMAN AND ANIMAL RIGHTS

All the procedures were followed in accordance with the ethical standards of the institutional ethics committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all the women who underwent IVF treatment in the study. This study was approved by the Institutional Review Board at Kansai Medical University. This article does not contain any study that was performed by any of the authors that included animal participants.

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