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Research Article

Regulation of HAND2 Expression by LncRNA *HAND2-AS1* in Ovarian Endometriosis Involving DNA Methylation

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Abstract

HAND2 is a critical mediator of progesterone receptor signaling in endometrium. Silencing of *HAND2* expression is associated with female infertility and endometrial cancers. We recently observed that lncRNA *HAND2-AS1* and *HAND2* are expressed coordinately in human endometrial stromal cells. To investigate involvement of *HAND2-AS1* and *HAND2* in pathogenesis of endometriosis, we employed immunohistochemistry, in situ hybridization, and quantitative real-time PCR to assess their expression in normal endometrium and the ectopic lesions obtained from patients with ovarian endometriosis. *HAND2* promoter methylation was also monitored in these samples. Our results revealed that *HAND2* and *HAND2-AS1* expression levels were reduced but promoter methylation was enhanced significantly in ectopic endometrium when compared with the normal controls. Fluorescence in situ hybridization showed that *HAND-AS1* is predominantly localized in the nuclei of endometrial stromal cells in contrast to the cytoplasmic distribution in epithelial cell compartment. To further investigate regulation of *HAND2* expression by *HAND2-AS1*, *HAND2-AS1* was silenced or overexpressed in human endometrial stromal cells. Our studies showed that expression levels of *HAND2* and its direct target *IL15* were attenuated markedly in *HAND2-AS1* silenced cells but enhanced significantly in the overexpressed human endometrial stromal cells. Silencing of *HAND2-AS1* also impaired endometrial stromal cell decidualization as indicated by downregulation of decidual biomarkers *IGFBP1* and *PRL*. In addition, *HAND2* promoter methylation was also enhanced upon *HAND2-AS1* silencing. RNA immunoprecipitation studies further revealed that *HAND2-AS1* is capable of binding to DNA methyltransferase DNMT1, indicating that *HAND2-AS1* governs *HAND2* expression epigenetically involving DNA methylation.

Key Words: endometrium, endometriosis, lncRNA *HAND2-AS1*, *HAND2*, DNA methylation

Abbreviations: AZA, 5-aza-2′-deoxycytidine; DMEM/F-12, Dulbecco Modified Eagle Medium/Ham's F-12; DNMT, DNA methyltransferase; E, 17β-estradiol; EC, ectopic endometrium; EM, normal endometrium; ESR, estrogen receptor; EU, eutopic endometrium; FBS, fetal bovine serum; FGF, fibroblastic growth factor; FISH, fluorescence in situ hybridization; *HAND2-AS1*, HAND2-antisense 1; *HAND2*, heart and neural crest derivatives expressed transcript 2; IGFBP, insulin-like growth factor binding protein; *IL15*, interleukin 15; lncRNA, long noncoding RNA; P, progesterone; PGR, progesterone receptor; PRL, prolactin-related protein; qRT-PCR, quantitative real-time polymerase chain reaction; RIP, RNA immunoprecipitation.

Endometriosis is a gynecological disorder affecting 6% to 10% of women of reproductive age, with a prevalence as high as 35% to 50% in women experiencing chronic pelvic pain and/or infertility. The estimated treatment costs are over $22 billion per year in the United States [1, 2]. A well-accepted etiology of endometriosis is the entry of endometrial cells into the peritoneal cavity following retrograde menstruation; there endometrial cells evade immune surveillance, invade into the peritoneal surface, and grow as endometriotic lesions with extensive adhesion, inflammation, and blood vessel formation [2, 3].

Like the endometrium, the cyclic change of ectopic endometrium is tightly controlled by steroid hormone 17β-estradiol (E) and progesterone (P). E stimulates proliferative and inflammatory responses of endometrial cells. P, on the other hand, counteracts the stimulatory actions of E and inhibits endometrial epithelial cell proliferation and inflammatory response [4]. Hence, progestins are often used clinically to alleviate pain and reduce the risk of recurrence in endometriosis [5]. In the pregnant uterus, P promotes differentiation of endometrial epithelial cells that renders the uterine receptive for embryo implantation, as well as differentiation of endometrial stromal cells into decidual cells that provide nutrients and protection to the implanted embryo. Interestingly, ample evidence suggests that a large portion of women with endometriosis are refractory to progesterone therapy (P resistance) [6, 7]. Progesterone receptor (PGR)-mediated actions are reduced dramatically in the

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eutopic endometrium and ectopic lesions of endometriosis patients, including reduction in expression of PGR-regulated target genes and impairment in stromal cell differentiation, which are the leading causes for the infertility in women with endometriosis [6-8]. The molecular mechanisms underlying P resistance are far from clear.

Our previous studies have shown that transcriptional factor heart and neural crest derivatives expressed transcript 2 (HAND2) is a critical mediator of PGR-signaling in endometrial cells [9]. Loss of *Hand2* expression in mouse uterus results in an unopposed E action that leads to sustained epithelial proliferation, thus impairs uterine receptivity in female mice [9]. Further analysis revealed that loss of *Hand2* expression results in upregulation of fibroblastic growth factors (FGFs) in uterine stromal cells. These FGFs bind to FGF receptor (FGFR) on epithelial cell surface, activating the fibroblast growth factor receptor substrate 2 (FRS2)-mediated mitogen-activated protein kinase (MAPK) signaling cascade, which eventually enhances epithelial cell proliferation [9]. In addition, HAND2 plays a pivotal role in endometrial stromal cells decidualization via induction of downstream target genes, such as interleukin 15 (*IL15*) [10, 11]. Interestingly, our studies and others have also shown that epigenetic silencing of *HAND2* expression by DNA methylation in endometrial cells is associated with early events of endometrial cancers [12-14]. Hence, *HAND2* DNA methylation functions as an epigenetic mark to predict the development and recurrence of endometrial cancers, as well as the efficacy of P therapy to those diseases.

It is well known that long noncoding RNAs (lncRNAs), RNA transcripts longer than 200 bp with non-protein coding, regulate gene expression at transcriptional and posttranscriptional levels [15]. Aberrant lncRNA expression is often involved in various human diseases including endometrial cancers [16]. Early studies have shown that lncRNA *HAND2-AS1* (*HAND2*-antisense 1) is associated with development of endometrial cancers, and its expression is often silenced by promoter methylation [17]. *HAND2-AS1* was found located at the *HAND2* genomic locus sitting on the opposite strand in Head-to-Head orientation and separated by a 228 bp bidirectional promoter [18]. Sequences analysis revealed the bidirectional promoter lacks the classical TATA box but is enriched with CpG content [18]. In cardiac development, *HAND2-AS1* regulates *HAND2* expression via maintaining histone modification at heart-specific enhancer upstream *HAND2* promoter [19]. In the endometrium, our previous studies showed that *HAND2* expression is under the control of PGR and mediates PGR-signaling [9]. Interestingly, we observed recently that *HAND2-AS1* and *HAND2* are coordinately expressed in normal endometrial stromal cells but attenuated simultaneously in the ectopic endometrial stromal cells. The molecular mechanism of *HAND-AS1* in regulation of HAND2 expression in the endometrium is not fully clear yet.

In this study, we investigated expression and localization of *HAND2-AS1* and HAND2 in the eutopic and ectopic endometrium obtained from patients with ovarian endometriosis. The methylation status of *HAND2* bidirectional promoter was also monitored in these tissues. Herein we provide evidence that the downregulation of *HAND2* and *HAND2-AS1* expression in the ectopic endometrial biopsies is associated with establishment of endometriotic lesions. *HAND2-AS1* governs *HAND2* transcription, at least in part, via modulating *HAND2* promoter methylation.

Materials and Methods

Tissue Collection

Thirty cases of normal endometrium specimens and 35 cases of endometriosis specimens archived from the Department of Pathology, Heping Hospital, Changzhi Medical College from 2017 to 2020 were chosen for immunohistochemistry. A set of 18 cases of fresh ectopic specimens were collected from patients with ovarian endometriosis without hormonal therapy history at Heping Hospital, Changzhi Medical College. Additionally, 23 cases of normal endometrium at the secretory phase of menstrual cycle were collected by routine endometrial cell sampling operations in Heping Hospital for RNA and DNA purification. All the specimens were verified by histopathological analysis. Institutional review board approval was obtained from Changzhi Medical School and Heping Hospital (No. RT2019038), and all patients provided informed consent for the investigation and experiments. There were no significant differences in basic characteristics between the patient groups and the control group.

Quantitative Real-time Polymerase Chain Reaction Analysis

For quantitative real-time polymerase chain reaction (qRT-PCR) analysis, total RNA was isolated from normal endometrium, ectopic lesions, and cultured endometrial stromal cells using RNeasy Mini kit (Qiagen). Single-strand cDNA was synthesized following Goscript reverse transcription system (Promega, Madison, WI, USA). The qRT-PCR analysis was performed as described previously [20]. Primer sequences corresponding to the specific target genes used in qRT-PCR are listed in Table 1. For each target gene, the delta Ct value was determined by the geometric mean of Ct values derived from 3 independent measurements after normalization to the geometric mean of Ct values obtained from 3 different housekeeping genes (*RPLP0*, *GAPDH*, and *ACTB*). The delta delta Ct was calculated as the difference between the delta Ct values of the experimental and control samples. The fold change of gene expression in each sample relative to a control was computed as $2^{-\text{delta delta Ct}}$. The relative gene expression level was expressed as the average fold change ± SD from at least 3 independent experiments.

Immunohistochemical Staining for HAND2

The formalin-fixed paraffin-embedded tissues were cut into 4-μm-thick sections. The sections were then deparaffinized, rehydrated, and boiled for 10 minutes in antigen retrieval buffer (10 mm sodium citrate, pH 6.0). After cooling for 25 minutes at room temperature, the tissues were incubated with 3% hydrogen peroxidase for 10 minutes to inactivate endogenous peroxidases. After washing, the sections were incubated with rabbit anti-human HAND2 antibody (ab200040, Abcam, Shanghai, China. RRID:AB_2923502) overnight at 4 °C and were then detected with VECTASTAIN Elite ABC system (VECTOR Laboratories, Shanghai, China) following the manufacturer's protocol. After staining with DAB chromogen, the sections were counterstained briefly with hematoxylin. Bright-field images were captured under the same microscopic conditions. For each section, 4 to 5 different views were chosen; all the images were processed using ImageJ software. The intensity of staining and the percentage of positive cell number were counted and analyzed.

Table 1. Primer sequences for real-time PCR to detect gene expression and DNA methylation

Gene expression		
Gene	Forward (5′−3′)	Reverse (5′−3′)
β-actin	CGGGAAATCTGCGTGACAT	AAGGAAGGCTGGAAGAGTGC
GAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG
HAND2-AS1	GATCTGGGGCCCTGAATGAG-3	TCTTGCTGGGAAAATCCGCT
HAND2	CTGCTCTCCCAACTCGCAT	CTGGAAAGCTGGCGACCTAA
IGFBP1	CCAAACTGCAACAAGAATG	GTAGACGCACCAGCAGAG
PGR	TGTATTTGTGCGTGTGGGTG	TACAGCCCATTCCCAGGAAG
PRL	CTACATCCATAACCTCTCCTCA	GGGCTTGCTCCTTGTCTTC
ESR1	GAATCTGCCAAGGAGACTCGC	ACTGGTTGGTGGCTGGACAC
CYP19A1	CAGGAGCTATAGATGAACCTTTTAGGG	CTTGTGTTCCTTGAC CTCAGAGG
MMP9	ACGACGT CTTCCAGTACCGAG	AGGGCACTGCAGGATGTCATA
PCNA	GGCTCCATCCTCAAGAAGGT	AGTCCATGCTCTGCAGGTTT
NR5A1	GCCCTGAAACAGCAGAAGAA	GCCCTGTCTCCAGCTTGAA
IL 15	GTTCACCCCAGTTGCAAAGT	CCTCCAGTTCCTCACATTC
DNA Methylation		
Bidirectional promoter	GCTACATCTTTAGGGCCGCTC	GGTAGCCAATCCTGGAAGAGG

Table 2. Oligo sequences for *HAND2-AS1* FISH probes

5′−3′ sequence	
Probe 1	AGGGA CACCA GTCCC TACGA AGACC TTGGG CGATT TTGAA
Probe 2	CTGGT ATCGG TGTTC CCCTG GTTTA ACTAG CCTGT TTGAA
Probe 3	CCTGG GGATC TTCAC TTTCG CAGTC TACGA CTGCC TGTGA

Fluorescence In Situ Hybridization for *HAND2-AS1*

Fluorescence in situ hybridization (FISH) was employed to analyze *HAND2-AS1* expression in normal endometrial specimens and endometriotic specimens following the manufacturer's instructions (Boster, Wuhan, China). Briefly, the formalin-fixed paraffin-embedded tissues were cut into 4-μm-thick sections, dewaxed, rehydrated, and digested with proteinase pepsin. Three 5′-digoxigenin-labeled oligonucleotide probes (Table 2) or nonrelevant negative control probe were mixed with hybridization solution and incubated with a coverslip at 38 °C overnight. After washing, the sections were incubated with biotin-labeled mouse anti-digoxin monoclonal antibody followed by FITC-labeled avidin and biotin conjugated complex. The sections were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and photographed under fluorescence microscopy.

Isolation of Human Endometrial Stromal Cells and Culture In Vitro

Human endometrial tissue was washed in phosphate-buffered saline and then minced in a medium containing Dulbecco Modified Eagle Medium/Ham's F-12 (DMEM/F-12) supplemented with 100 mg/mL penicillin and 100 mg/mL streptomycin. The tissue fragments were digested with collagenase type 3 (300 μg/mL; Sigma) and deoxyribonuclease type I (40 μg/mL; Sigma) at 37 °C for 90 minutes, and then dissociated into a single cell by vortexing briefly. After removal of the glandular and epithelial components, the cell suspension passed sequentially through meshes of 100, 70, and 40 sieves (BD Biosciences), respectively. Cell viability and numbers were assessed by trypan blue staining. The purified endometrial stromal cells were seeded at 2×10^5 cells per well of 6-well cell culture plates in DMEM/F-12 supplemented with penicillin and streptomycin (Invitrogen) and 5% fetal bovine serum (FBS), then they were incubated in 5% CO_2 at 37 °C. The medium was changed every 2 to 3 days until they reached 80% to 100% confluency. The stromal cell identity was confirmed using Vimentin staining in confluent cells (>99% by our estimation) by immunohistochemical analysis as described previously.

Transfection of siRNA Oligos or Overexpression Plasmid Vectors in Human Endometrial Stromal Cells

Human endometrial stromal cells were seeded into 6-well cell culture plates at a density of 2.5×10^5 per well and cultured as described above. When the cells grew to 70% to 80% confluency, cell culture medium was replaced with fresh DMEM/F12 medium supplemented with 2% charcoal-stripped FBS and transfected with siRNA oligos or expression vectors as described previously [20, 21]. For siRNA oligo transfection, cells were incubated with the mixture of 10 nm of nonspecific RNAi oligos, single or a combination of *HAND2-AS1*-specific siRNA oligos (AS1-a, -b, or -c) siRNA oligos (Gene Pharma, Shanghai, China) and Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific) for 24 hours. For plasmid transfection, cells were incubated with 20 nm of empty vector or HAND2-AS1 overexpression vector in pEX2 (Gene Pharma, Shanghai, China) mixed with Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) for 24 hours, respectively. Transfection reagent alone served as mock control. After washing, the cells were further cultured in fresh culture medium containing 10 nm of E and 1 μm of P for 3 days. Total RNA and genomic DNA were purified for qPCR analysis. The siRNA oligo sequences are listed in Table 3.

Table 3. siRNA oligo sequences

Gene	5′–3′ sequence
hand2-AS1 siRNA-a	GCCAAGGUACAUCUCAGAUTT
hand2-AS1 siRNA-b	CCUCUUGACUUGCCAGUAUTT
hand2-AS1 siRNA-c	GCUGGUACUGUCACUUAUATT
HAND2 siRNA	UUCUUGUCGUUGCUGCUCACUGUGC

Induction of In Vitro Decidualization of Human Endometrial Stromal Cells

Human endometrial stromal cells were seeded into 6-well cell culture plates at a density of 2.5×10^5 per well and cultured as described above. When cells grew to 70% to 80% confluency, cell culture medium was replaced with fresh DMEM/F12 medium supplemented with 2% charcoal-stripped FBS. To induce in vitro decidualization, the cells were treated with medium containing vehicle or a hormonal cocktail: 10 nM of E, 1 μM of P, and 0.5 mM of 8-bromo-cyclic AMP (E + P + cAMP) for 3 days. Three batches of human endometrial stromal cells isolated from different individuals were examined.

DNA Methylation Analysis

DNA methylation analysis was performed as described previously [22]. Briefly, genomic DNA samples were purified from eutopic endometrium, ectopic lesions, and the cultured endometrial stromal cells that were transfected with siRNA oligos following the protocol of Allprep DNA/RNA purification kit (Qiagen), then digested with methylation-sensitive, methylation-dependent, both, and non, respectively. Equal amounts of the digested DNAs were then subjected to qRT-PCR following the EpiTect Methyl II Assay protocol (Qiagen) using specific primers flanking CpG islands in the bidirectional promoter (Table 1). The methylated or nonmethylated human genomic DNA (New England Biolabs) served as positive or negative controls. Levels of DNA methylation were determined by the average ΔCt values obtained from qPCR amplification of mock-digested, methylation-sensitive enzyme-digested, methylation-dependent enzyme-digested and double-digested DNA samples following product protocol. The fold induction of DNA methylation in the ectopic lesions compared with that in the normal endometrium was determined. The representative samples were submitted to bisulfite sequencing using the primers specific to the promoter region to validate the methylation results obtained from quantitative real-time PCR.

RNA Immunoprecipitation

The interaction between *HAND2-AS1* LncRNA and DNMT1 protein was assessed by RNA immunoprecipitation (RIP) following the manufacturer's instructions (Cat. No. P0101, GENESEED, Biotech, China, Ltd). Briefly, human endometrial stromal cells were lysed in RIP assay buffer followed by a centrifugation at 12 000*g* for 10 minutes at 4 °C. The supernatant was collected and mixed with magnetic beads coated with anti-DNMT1 antibody (catalog number 5032, Cell Signaling Technology, RRID:AB_10548197) or negative control (NC) non-immunoglobulin G (IgG) antibody (ab172730, 1:100, Abcam) and incubated at 4 °C overnight. After washing with RIP wash buffer, the immunoprecipitated complex was eluted from magnet beads. RNA was purified by passage through a purification column after DNA removal and subjected to qRT-PCR analysis after reverse transcription. The relative level of RNA enrichment in DNMT1 group is calculated by $2^{-\Delta\Delta Ct}$ following equation $^{\Delta\Delta}Ct = (Ct\ DNMT1\text{-}Ct\ Input)\text{-}(Ct\ IgG\text{-}Ct\ Input)$.

Statistical Analysis

All the numerical values were obtained from at least 3 independent samples or more and were analyzed by one-way ANOVA followed by Dunnett post hoc test when comparisons were made between a control group and more than one experimental group, or by Student *t* test for single comparison (GraphPad Prism 5.0, GraphPad Software, Inc., San Diego, CA). Data were expressed as mean ± SD. Statistical significance was defined as $P < .05$.

Results

HAND2 Expression Is Downregulated in the Ectopic Endometrium of Endometriosis

Our early studies have shown that HAND2, a critical PGR-mediator in both human and mouse endometrial stromal cells, plays an essential role in suppression of E-driven epithelial proliferation [9]. HAND2 is predominately expressed in the endometrial stromal cells at the time of embryo implantation and decidualization [10]. Attenuation of HAND2 expression is correlated with early events of endometrial carcinogenesis [12] To investigate whether HAND2 is involved in pathogenesis of endometriosis, the endometrial tissues obtained from healthy women and the ectopic endometrial tissues obtained from women with ovarian endometriosis at the secretory phase of menstrual cycles were subjected to qRT-PCR and immunohistochemical staining to assess *HAND2* expression. As shown in Fig. 1A, in comparison to the normal endometrial tissues (EM), *HAND2* mRNA expression level is markedly down regulated in the ovarian ectopic endometrial tissues (EC) ($P < .001$). Consistently, when compared to the normal endometrial tissues, the relative intensity of HAND2 nuclear immunostaining as well as the number of positive cells are significantly reduced in endometrial stromal cell compartment (Fig. 1B, solid arrows), no specific staining was observed in endometrial epithelial cell compartment on any tissue section (Fig. 1B, open arrows). These results indicate that downregulation of *HAND2* gene expression is correlated with development of endometriosis.

Downregulation of *HAND2-AS1* Expression in Ectopic Endometrium of Ovarian Endometriosis

We next assessed *HAND2-AS1* expression by qRT-PCR in the endometrium of healthy women and the ectopic endometrium from patients with ovarian endometriosis. Compared with the normal endometrium, *HAND2-AS1* expression was markedly attenuated in the ectopic endometriotic lesions (Fig. 2A). To further investigate whether *HAND2* mRNA expression is associated with *HAND2-AS1*, the expression levels of *HAND2* and *HAND2-AS1* transcripts were assessed by qRT-PCR in endometrial stromal cells purified from the normal endometrium and endometriotic lesions. As shown in Fig. 2B, *HAND2* and *HAND2-AS1* expression was markedly reduced in the ectopic endometrial stromal cells, indicating that downregulation of *HAND2-AS1* expression in

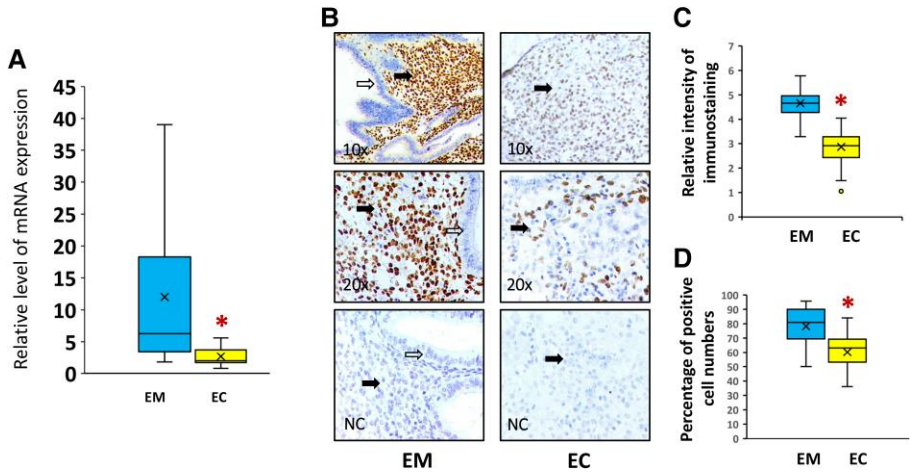

Figure 1. HAND2 expression is downregulated in the ectopic endometrium of endometriosis. A, The normal endometrial tissues (EM, n = 23) and the ectopic lesions (EC, n = 18) of ovarian endometriosis were subjected to qRT-PCR to assess *HAND2* mRNA expression. B, The endometrial tissue sections obtained from heathy women (EM, n = 30) or ectopic lesions from women with ovarian endometriosis (EC, n = 35) were subjected to immunohistochemical staining to assess HAND2 protein expression. The representative images of HAND2 immunostaining with different magnification are shown. The brown color in the nuclei indicates HAND2-specific immunostaining. NC indicates the non-IgG control. Open arrows indicate endometrial epithelial cells. Solid arrows indicate endometrial stromal cells. 4 to 5 Bright-field images were photographed from each slide, the relative intensity of immunostaining (C) and the number of positive nuclear staining (D, bottom) in each image were analyzed by Image J softwate. The numeral data was represented as a box-and-whisker plot in which X shows the mean. **P* < .001, EC vs EM.

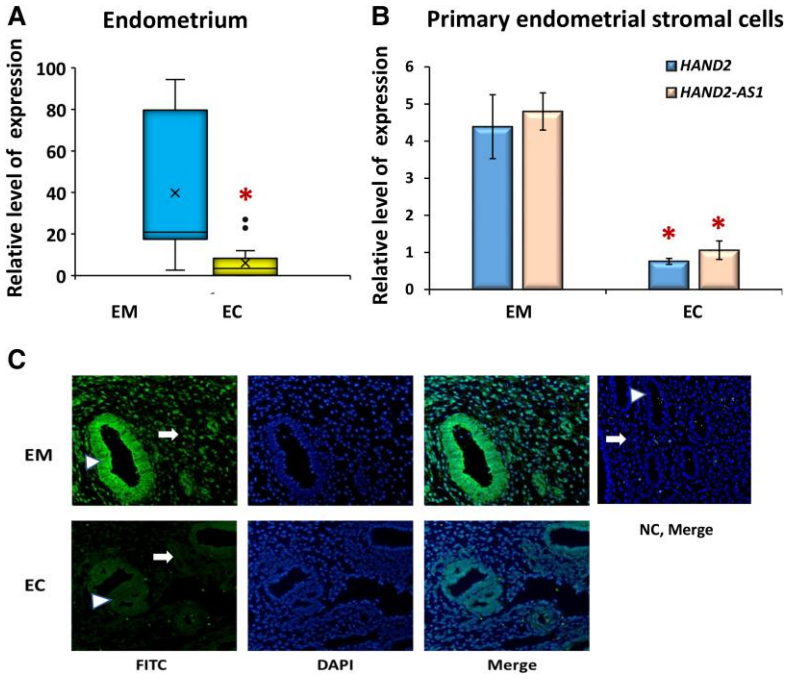

Figure 2. Downregulation of *HAND2-AS1* expression is associated with establishment of endometriosis. A, The normal endometrial tissues (EM, n = 23) and the ectopic lesions (EC, n = 18) of ovarian endometriosis were subjected to qRT-PCR to assess *HAND2-AS1* expression. The data are represented as a box-and-whisker plot in which X indicates the mean. B, Endometrial stromal cells purified from the endometrium of healthy women (EM) and the ectopic lesions of patients with ovarian endometriosis (EC) were cultured in vitro in the presence of 10 nm of E and 1 μm of P for 3 days (n = 3). Total RNAs were isolated and subjected to qRT-PCR to assess gene expression. The relative levels of *HAND2* and *HAND2-AS1* expression in the ectopic endometrial stromal cells compared to the normal endometrial stromal cells are shown. **P* < .001. C, The endometrial tissue sections obtained from heathy women (EM, n = 3) or ectopic lesion from women with ovarian endometriosis (EC, n = 3) were subjected to FISH to assess *HAND2-AS1* expression and localization. The green color indicates *HAND2-AS1*-specific hybridization signal. The sections were counterstained with DAPI to visualize the cellular nuclei. Arrow heads indicate the endometrial epithelial cells; Arrows indicate the endometrial stromal cells. NC indicates negative control.

endometrial stromal cells is also associated with the development of endometriosis.

To further confirm downregulation of *HAND2-AS1* expression in endometriosis, we performed FISH analysis on the endometrial sections obtained from the healthy women and the ectopic endometrial tissues from endometriosis patients at the secretory phase of the menstrual cycle. *HAND2-AS1* hybridization signal (green fluorescence) was

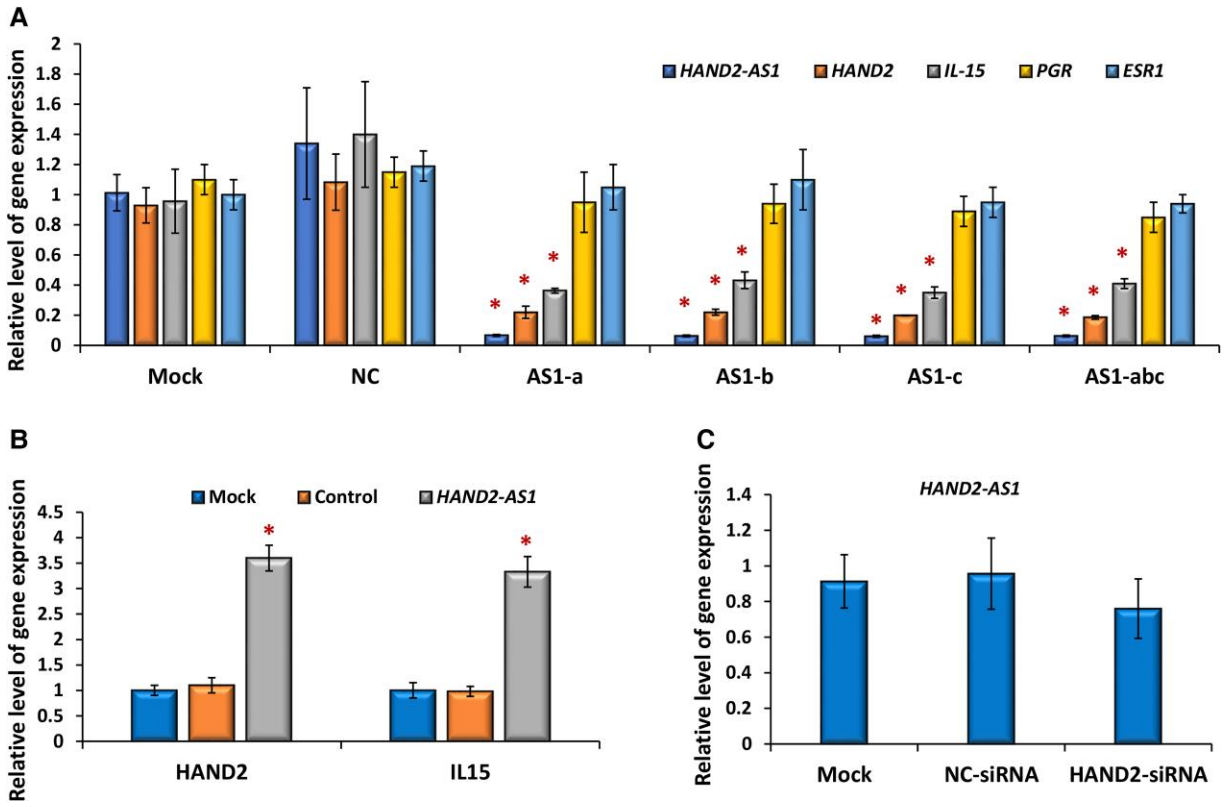

Figure 3. *HAND2-AS1* regulates *HAND2* expression in human endometrial stromal cells. Human endometrial stromal cells were transfected with *HAND2-AS1*-specific siRNA oligos (A), *HAND2-AS1*-overexpressing vector (B), or *HAND2*-specific siRNA oligos (C) respectively. These cells were then cultured in presence of 10 nm of E and 1 μm of P for 3 days (n = 3). Total RNAs were isolated from these cells and subjected to qRT-PCR to assess gene expression. The relative expression levels of *HAND2*, *HAND2-AS1*, *IL15*, *PGR*, and *ESR1* compared with the mock controls after normalization to the internal control genes are shown. Abbreviations: AS1-a, -b, or -c, *HAND2-AS1*-specific siRNA oligo; Mock, transfection reagent control; NC, nonspecific control oligo. $^*P < .001$, n = 3.

abundantly detected in normal endometrium, but it subsided dramatically in the ectopic endometrium obtained from patients with ovarian endometriosis (Fig. 2C, upper panels vs lower panels). No specific signal was observed in the negative controls (Fig. 2C, right panel). Interestingly, distinct intracellular localization of *HAND2-AS1* RNA was observed in endometrial tissues. While a diffused cytoplasmic localization was noticed in endometrial epithelial cells (Fig. 2C, arrowhead), *HAND2-AS1* is primarily expressed in the nuclei of endometrial stromal cells (Fig. 2C, arrow).

HAND2-AS1 Regulates *HAND2* Expression in Human Endometrial Stromal Cells

The coordinated expression pattern of *HAND2* and *HAND2-AS1* prompts us to postulate that *HAND2-AS1* may control *HAND2* expression in endometrial stromal cells. To test this possibility, *HAND2-AS1* expression in endometrial stromal cells was artificially altered by siRNA-mediated silencing or overexpression, respectively, as described above. Transcription levels for *HAND2* and HAND2-regulated downstream target gene *IL15* were monitored by qRT-PCR. Our results showed that in response to silencing of *HAND2-AS1* expression, the transcription levels of *HAND2* and *IL-15* were reduced significantly, whereas expression levels of *PGR* and estrogen receptor 1 (*ESR1*) were not altered (Fig. 3A). On the other hand, when *HAND2-AS1* was overexpressed in endometrial stromal cells, expression levels for both *HAND2* and *IL-15* were augmented markedly (Fig. 3B). To test whether HAND2 may reversely affect *HAND2-AS1* transcription, HAND2 expression was knocked down by siRNA-mediated silencing in endometrial stromal cells. We observed that *HAND2-AS1* expression is only marginally affected in response to *HAND2* silencing (Fig. 3C).

Early studies have shown that HAND2 plays a critical role in human endometrial stromal cell decidualization [11]. To investigate the regulatory role of *HAND2-AS1* in the differentiation process of human endometrial stromal cells, *HAND2-AS1* expression was either silenced or overexpressed in these cells prior to induction of in vitro decidualization. Expression of decidual biomarker genes was assessed by qRT-PCR. Consistent with that observed in *HAND2*-silenced human endometrial stromal cells, our results showed that expression levels of both insulin-like growth factor binding protein 1 (*IGFBP1*) and prolactin-related protein (*PRL*) were attenuated significantly in response to silencing of *HAND2-AS1* expression (Fig. 4A) but increased in *HAND2-AS1* overexpressed cells (Fig. 4B). No significant difference in expression level of *PGR* and *ESR1* was observed in these cells. Collectively, these results indicate that *HAND2-AS1* controls the transcriptional activity and physiological functions of HAND2 in endometrial stromal cells.

Endometriotic cells are characterized by the enhanced proliferative potential and upregulation of estrogen biosynthesis machinery [23]. To investigate the role of *HAND2-AS1* in pathogenesis of endometriosis, expression levels of 2 enzymes

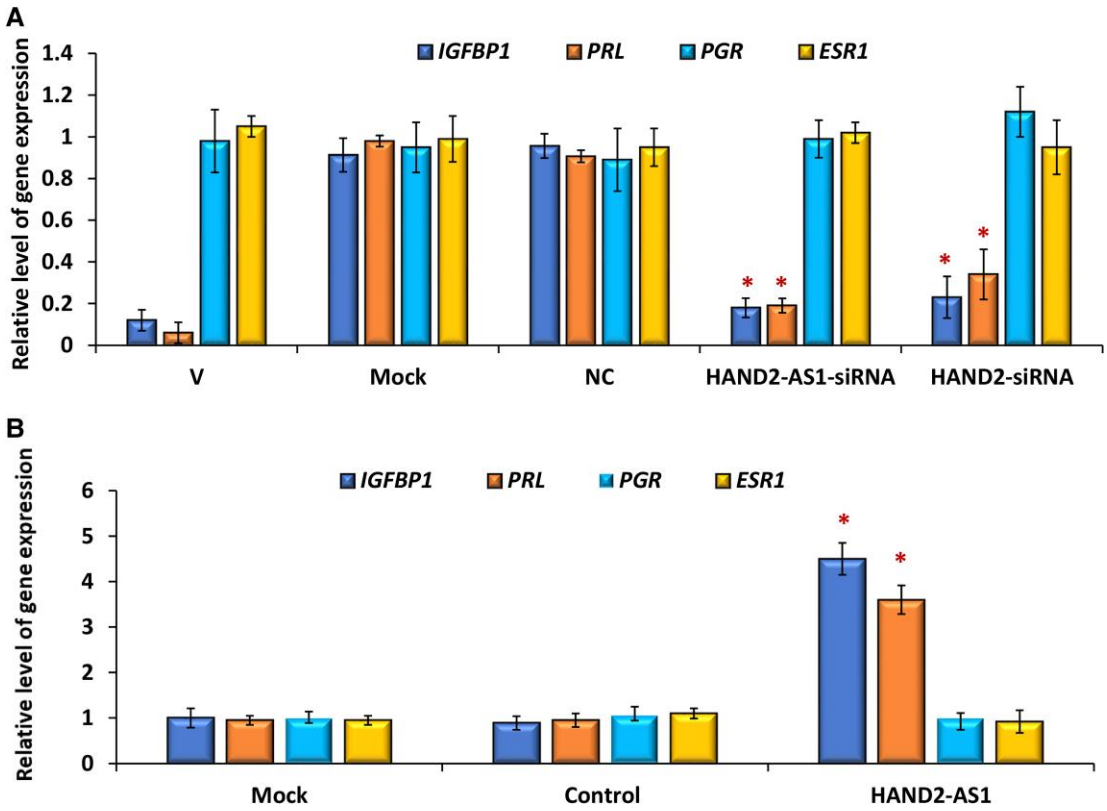

Figure 4. The role of *HAND2-AS1* in decidualization of human endometrial stromal cells. Human endometrial stromal cells transfected with *HAND2-AS1*- or *HAND2*-siRNA oligos (A) or overexpression vectors (B) were cultured in the presence of hormonal cocktail: 10 nm of E, 1 μm of P and 0.5 mM 8-Br-cAMP for 3 days. Total RNA was isolated and subjected to qRT-PCR to assess gene expression. The relative expression levels of *IGFBP1*, *PRL*, *PGR*, and *ESR1* compared to the mock control after normalization to the internal control genes are shown. Abbreviations: Mock, transfection reagent control; NC, nonspecific control oligo; V, vehicle-treated control. $*P < .001$, n = 3.

that are critical for estrogen biosynthesis, aromatase (encoded by *CYP19A1*) and steroidogenic factor 1 (SF-1, encoded by *NR5A1*) were examined by qRT-PCR in human endometrial stromal cells in response to silencing of *HAND2-AS1* expression. Interestingly, *CYP19A1* but not *NR5A1* expression was enhanced (about 2.5-fold) in cells transfected with *HAND2-AS1* specific siRNA oligos when compared with the corresponding negative controls (Fig. 5A and 5B). The proliferative potential of these cells was also assessed by qRT-PCR to examine the expression level of *PCNA* (proliferating cell nuclear antigen), a cell proliferation biomarker. As shown in Fig. 5C, the expression level of *PCNA* mRNA was enhanced significantly in response to *HAND2-AS1* silencing, while expression level of *MMP-9* (matrix metalloproteinase-9, a cell invasive biomarker) was not altered (Fig. 5D).

Methylation of *HAND2-AS1/HAND2* Bidirectional Promoter Is Enhanced in Endometriosis

Earlier studies reported that *HAND2* DNA methylation is involved in pathogenesis of endometrial cancers [12, 17]. To investigate the involvement of *HAND2* DNA methylation in endometriosis, methylation status in the bidirectional promoter between *HAND2* and *HAND2-AS1* was assessed in the normal endometrium, the eutopic endometrium and ectopic lesions obtained from patients with ovarian endometriosis by digestion of genomic DNA with DNA methylation-sensitive/-dependent enzymes, followed by qRT-PCR. Our results showed that the level of the bidirectional promotor methylation in the ectopic endometrium is significantly higher than that in the endometrial tissues collected from healthy women or the eutopic endometrium (EU) from patients with ovarian endometriosis (Fig. 6A, EC vs EM and EC vs EU, $P < .01$). Compared with the normal endometrium, DNA methylation is also slightly enhanced in the eutopic endometrial tissues of patients (Fig. 6A, EU vs EM, $P < .05$). We also compared methylation status in the same region of promoter in the endometrial stromal cells isolated from normal endometrium and ectopic lesions. Once again, our real-time PCR results showed that the level of DNA methylation is markedly increased in the ectopic endometrial stromal cells (Fig. 6B, EC vs EM). To further confirm the changes in HAND2 prompter methylation, the representative endometrial DNA samples obtained from healthy women and endometriosis were subjected to bisulfite sequencing using primer specific to the bidirectional promoter that contains 18 CpG dinucleotides. As shown in Fig. 6C, most of the CpGs are methylated in the ectopic endometrium (EC, solid circle), but retained unmethylated status in the normal endometrium (EM, open circle).

HAND2-AS1 Modulates DNA Methylation in *HAND2* Bidirectional Promoter in Human Endometrial Stromal Cells

lncRNA may control target gene expression via various epigenetic mechanisms, including DNA methylation [24]. To investigate whether DNA methylation is involved in regulation of *HAND2* expression in endometriosis, human endometrial stromal cells were first treated with 5-aza-2′-deoxycytidine

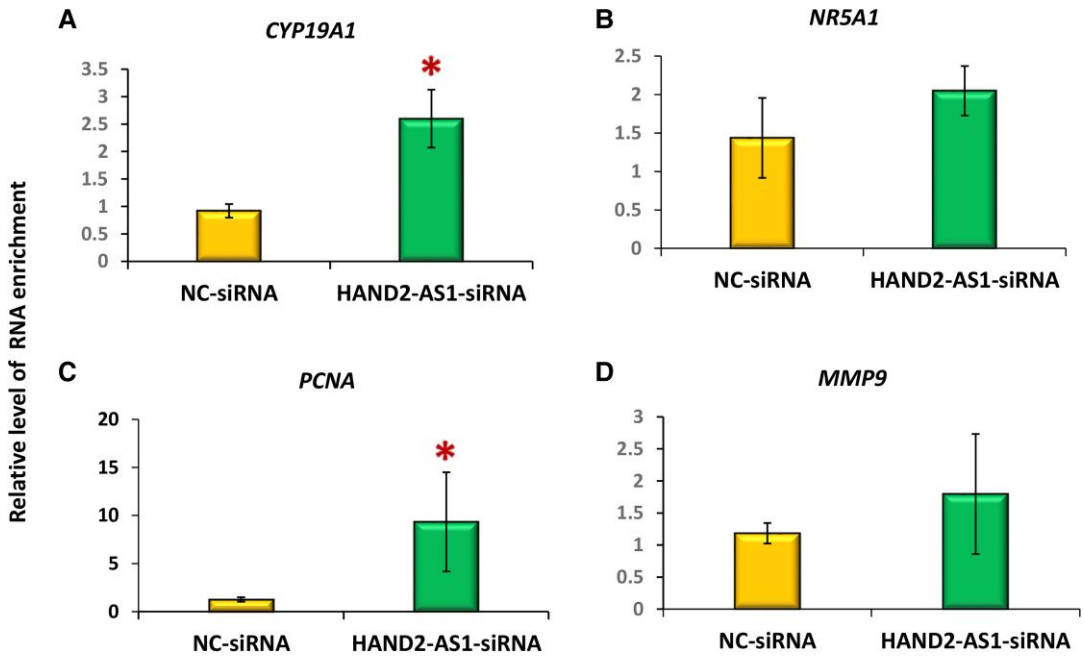

Figure 5. Expression of endometriosis biomarkers. Human endometrial stromal cells transfected with *HAND2-AS1* or nonspecific control siRNA oligos were cultured in the presence of E and P for 3 days. Total RNAs were isolated and subjected to qRT-PCR to assess gene expression. The relative expression levels of *CYP19A1* (A), *NR5A1* (B), *PCNA* (C), and *MMP9* (D) in cells transfected with *HAND2-AS1* siRNA oligo (*HAND2-AS1-siRNA*) compared to the nonspecific control (*NC-siRNA*) after normalization to the internal control genes are shown. $^{*}P < .01$, n = 3.

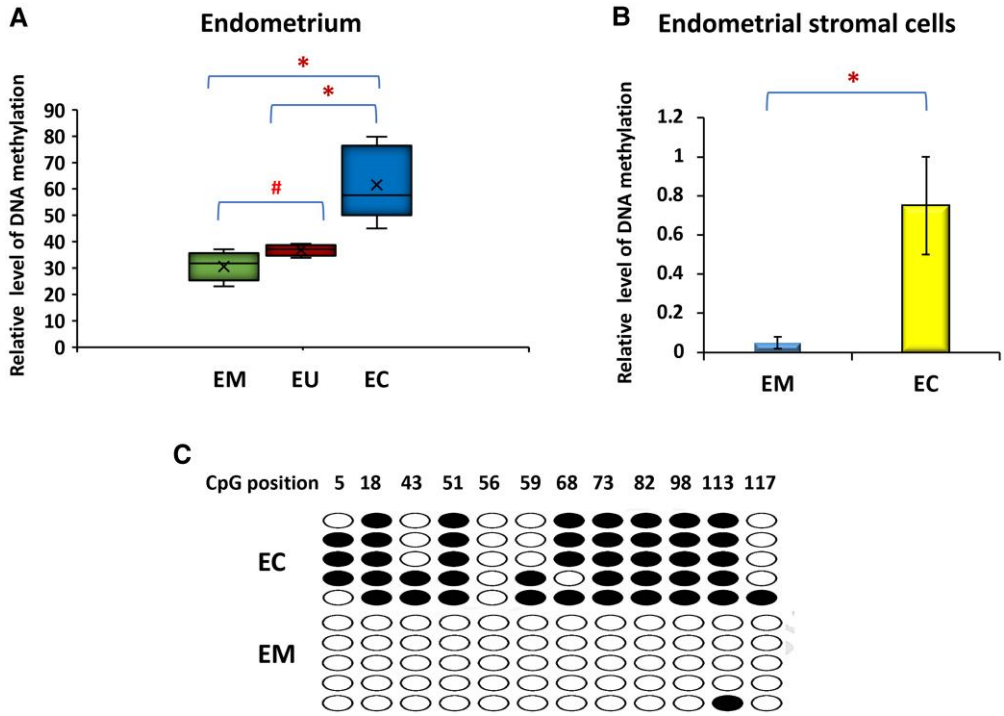

Figure 6. DNA methylation in *HAND2-AS1-HAND2* bidirectional promoter is enhanced in endometriosis. A, Genomic DNA was purified from the normal endometrial tissues (EM, n = 18), the eutopic (EU, n = 4), and ectopic lesions (EC, n = 23) of patients with ovarian endometriosis. B, Genomic DNA was purified from endometrial stromal cells isolated from normal endometrium (EM) and ectopic lesions (EC). DNA methylation levels were assessed by qRT-PCR using primers specific to the bidirectional promoter after digestion with DNA methylation-sensitive and/or dependent enzymes (n = 3). The relative levels of DNA methylation in ectopic endometrial stromal cells compared to the normal endometrial stromal cells are shown. $^{*}P < .01$; $^{\#}P < .05$. C, Representative endometrial DNA samples were subjected to bisulfite sequencing (n = 5). The numbers indicate the CpG locations in bidirectional promoter; Solid circles represent the methylated CpG sites. Open circles represent the unmethylated CpG sites.

(AZA), a widely used inhibitor of DNA methylation [25], for 3 days. Expression levels of *HAND2-AS1* and *HAND2* were assessed by qRT-PCR. As shown in Fig. 7A, in comparison to the vehicle-treated controls, *HAND2-AS1* expression was moderately elevated in response to AZA administration (about 1.8-fold). The level of *HAND2* and *IL15* mRNA,

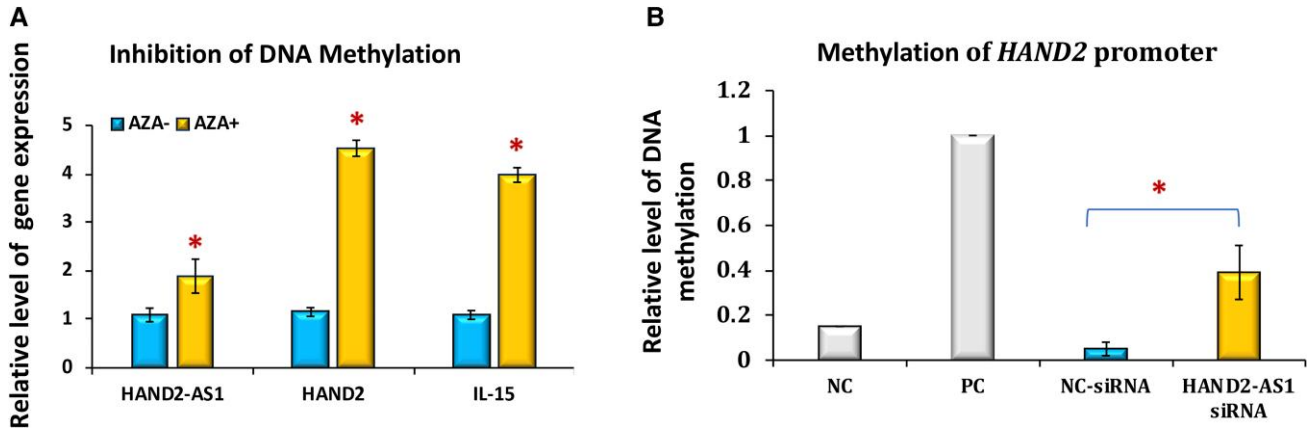

Figure 7. HAND2-AS1 modulates DNA methylation in HAND2 promoter in human endometrial stromal cells. A, Human endometrial stromal cells were cultured in presence of 10 nM of E and 1 μM of P with 15 μM of 5-aza-2′-deoxycytidine (AZA+) or vehicle control (AZA-) for 3 days. Total RNAs were isolated and subjected to qRT-PCR to assess gene expression. The relative levels of *HAND2, HAND2-AS1*, and *IL15* expression compared to AZA– controls after normalization to internal control genes are shown. **P* < .001, n = 3. B, Human endometrial stromal cells were transfected with *HAND2-AS1*-specific siRNA oligo (*HAND2-AS1* siRNA) or nonspecific siRNA control (NC-siRNA), and then cultured in presence of 10 nM of E and 1 μM of P for 3 days. Genomic DNAs were purified from these cells and subjected to digestion with DNA methylation sensitive and/or dependent enzymes, followed by qRT-PCR using primers specific to *HAND2* bidirectional promoter. The unmethylated and methylated human genomic DNA were served as the negative (NC) and positive controls (PC), respectively. *, *P* < .01 (n = 3).

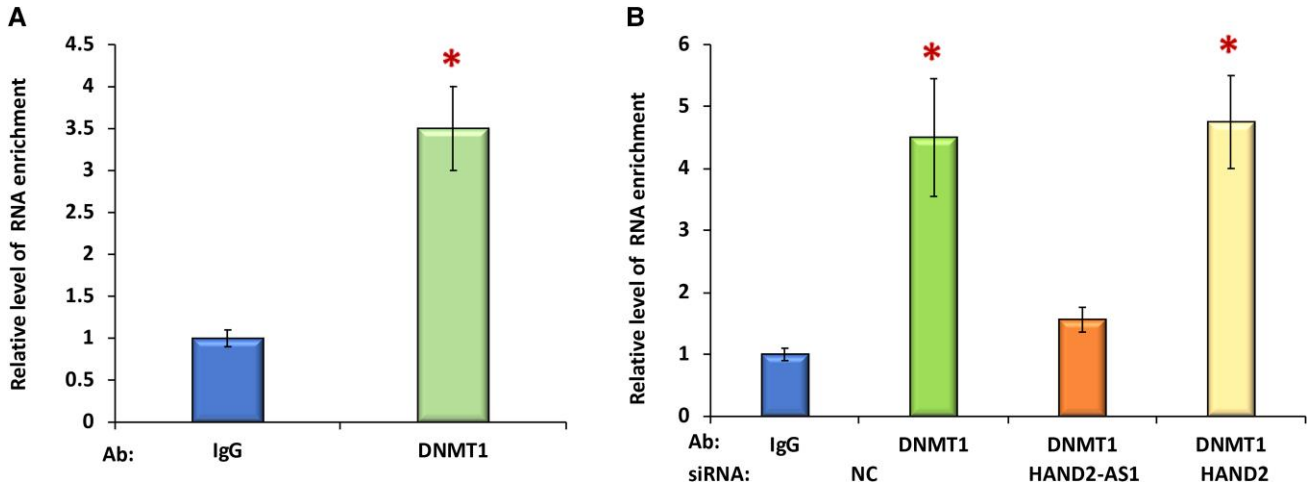

Figure 8. *HAND2-AS1* interacts with DNMT1 in human endometrial stromal cells. Human endometrial stromal cells transfected without (A) or with nonspecific siRNA oligos (NC), *HAND2-AS1*, or *HAND2*-specific oligos, respectively, (B) were cultured in the presence of 10 nM of E and 1 μM of P for 3 days. Cell lysate was subjected to RNA immunoprecipitation using rabbit mAB against DNMT1 and non-immune IgG, respectively. *HAND2-AS1* RNA enrichment was assessed by qRT-PCR using primers specific to *HAND2-AS1* transcript. The relative levels of *HAND2-AS1* enrichment compared to the IgG control are shown **P* < .01 (n = 3).

however, was significantly enhanced when DNA methylation was inhibited in these cells (about 4- to 5-fold). To further explore whether *HAND2-AS1* governs *HAND2* expression through modification of the local epigenetic background, the methylation status of the bidirectional promoter was assessed by enzymatic digestion followed by qRT-PCR. The unmethylated (NC) and methylated (PC) human genomic DNA served as the negative and positive controls, respectively. Compared with the nonspecific siRNA-transfected cells, the level of DNA methylation in *HAND2* promoter was increased moderately in *HAND2-AS1*-silenced human endometrial stromal cells (Fig. 7B), indicating that *HAND2-AS1* controls HAND2 transcription, at least in part, through DNA methylation.

To further explore the underlying molecular mechanism of *HAND2-AS1* in regulation of *HAND2* promoter methylation, RNA immunoprecipitation technology (RIP) was employed to investigate the interaction of this lncRNA with DNMT1, a critical DNA methyltransferase in maintenance of all of the methylation marks in genome, in human endometrial stromal cells. The cell lysate was incubated with anti-DNMT1 antibody or nonspecific IgG, respectively, and the binding RNAs were then immunoprecipitated and subjected to qRT-PCR with *HAND2-AS1*-specific primers. As shown in Fig. 8A, in comparison to nonspecific IgG control, there is about a 3- to 4-fold increase in the level of *HAND2-AS1* enrichment in DNMT1-binding RNA transcripts. To further confirm the binding specificity, we next performed RIP in *HAND2-AS1* or *HAND2*-silenced human endometrial stromal cells with anti-DNMT1 antibody or non-immune IgG, respectively. Consistently, a significant amount of *HAND2-AS1* transcript

was detected in DNMT1 antibody-immunoprecipitates in cells transfected with nonspecific RNAi oligos. This enrichment, however, was absent in *HAND2-AS1*-silenced cells, but remained unaltered in *HAND2*-silenced endometrial stromal cells (Fig. 8B).

Discussion

HAND2-AS1 and *HAND2* are a pair of genes that are present at the same genomic locus in Head-to-Head orientation and are separated by a 228-bp bidirectional promoter. Their transcriptional activities are driven in opposite direction but more profoundly in *HAND2* direction [18]. In this study, we report that *HAND2-AS1* and *HAND2* are coordinately expressed in normal endometrial stromal cells and silenced simultaneously in the ectopic endometrial stromal cells, indicating that both genes are involved in regulation of the same physiological functions of endometrium and pathological events in endometriosis. Since abundant CpG islands are present at their genomic locus, including gene bodies and the bidirectional promoter [18], administration of a DNA methylation inhibitor enhances expression of both *HAND2* and *HAND2-AS1*, but more in *HAND2* transcription, in human endometrial stromal cells. Ample evidence suggests that chronic inflammation increases DNA methyltransferase activity and induces aberrant DNA methylation [26]. It is reasonable to envisage that the increased levels of inflammatory factors from the infiltrated immune cells in the endometriotic lesions is responsible for silencing of their expression [27]. Interestingly, our results for the first time showed that *HAND2-AS1* is critical for *HAND2* expression in normal endometrial stromal cells, and more importantly, *HAND2-AS1* could modify the local DNA methylation background in *HAND2* promoter via binding to DNMT1. Based on these observations, we propose a working model in which *HAND2-AS1* expression maintains an active transcriptional activity on *HAND2* promoter in normal human endometrial stromal cells via suppression in DNA methylation throughout *HAND2* genomic locus. In endometriosis, however, loss of *HAND2-AS1* expression leads to an increase in DNA methylation at the *HAND2* promoter that attenuates *HAND2* expression.

Gain-of-function and loss-of-function studies have revealed that *HAND2-AS1* is involved in cancer cell proliferation, migration, invasion, apoptosis, glucose metabolism, and inflammation [28]. Clinically, *HAND2-AS1* is associated with tumor cell differentiation, tumor size, lymph node metastasis, and overall survival, indcating that *HAND2-AS1* is a potential prognostic marker or a novel therapeutic target for these diseases [28]. Although the role of *HAND2-AS1* in endometriosis remains unclear, our studies provided compelling evidence that downregulation of this lncRNA is tightly associated with establishment of ectopic lesions in endometriosis (see Fig. 2). In addition, *HAND2-AS1* expression is positively correlated with that of *HAND2* in normal endometrial stromal cells. Silencing *HAND2-AS1* expression results in downregulated *HAND2, IL15*, and the decidual biomarkers *IGFBP1* and *PRL*, overexpression of *HAND2-AS1* reverses their expression (see Figs. 3 and 4). Our early studies revealed that HAND2 is a critical mediator of PGR action in endometrial stromal cells [9]. Silencing of *HAND2* expression in these cells leads to enhanced production of the paracrine growth factors that act on epithelial cells to promote proliferation and impair uterine receptivity for embryo implantation [9]. It is conceivable that aberrant *HAND2-AS1* and *HAND2* expression in endometrial stromal cells is one of the major causal factors for P resistance in endometriosis. The unopposed E action due to P resistance may enhance endometrial cell proliferation, adhesion, invasion, and inflammation at the ectopic sites via production of FGFs, thus promoting establishment and growth of ectopic lesion and impairing endometrial stromal cell differentiation in the eutopic endometrium at the time of decidualization. This notion is supported by the observation in endometrial stromal cells in which silencing of *HAND2-AS1* expression promotes cell proliferation, and in endometrial cancer cells in which *HAND2-AS1* overexpression suppresses the migration and invasion of endometrial epithelial cancer cells [17]. Although more samples need to be included, it is of interest to note that *HAND2* promoter methylation is enhanced in the eutopic endometrium of patients with ovarian endometriosis. Further investigation of *HAND2-AS1* and *HAND2*-regulated molecular targets in the eutopic endometrium of endometriosis will provide novel insights into the dysfunctional endometrium that causes infertility in women with endometriosis.

The molecular mechanisms by which *HAND2-AS1* regulates *HAND2* expression is not completely understood. Early studies showed that *HAND2-AS1* transcription, not the mature transcript, is required for *HAND2* expression in the heart, indicating that *HAND2-AS1* controls HAND2 in cis in transcription-dependent but transcript-independent fashion [19]. *HAND2-AS1* elongation maintains H3K27ac signature at the super enhancer in *HAND2* promoter [19]. Recently, lncRNA *HANDSDOWN* (*HDN*) was identified in early heart cells that is critical for *HAND2* expression. In contrast to *HAND2-AS1*, transcriptional activity of the *HDN* locus, independent of its RNA, suppresses *HAND2* expression [29]. In mouse uterus, however, ablation of the heart-specific enhancer in mouse uterus does not affect *Hand2* transcriptional activity (our unpublished data). We found that in response to P stimulation, *Hand2* expression is augmented exclusively in PGR-positive stromal cells, not in PGR-positive epithelial and myometrial cells [9]. A recent study showed that *HAND2-AS1* transcription is enhanced in mouse uterine stromal cells in response to P stimulation and *HAND2-AS1* plays a critical role in mouse uterine stromal cell differentiation [30]. In this study, we report for the first time that *HAND2-AS1* controls *HAND2* expression via modulation of local DNA methylation. These results indicate that multiple mechanisms are likely involved in regulation of *HAND2* expression in diverse tissue types.

Several lines of evidence suggest that lncRNA could physically interact with DNA methyltransferase and control DNA methylation [24, 31-33]. Recent studies by Boque-Sastre have shown that lncRNA *VIM-AS1* forms an RNA:DNA hybrid (R-loop) at *VIM* promoter that facilitates binding of transcription factors. Silencing of *VIM-AS1* leads to an increase in *VIM* promoter methylation [34]. Interestingly, our study revealed that *HAND2-AS1* could modulate DNA methylation in *HAND2* promoter, and the direct interaction between *HAND2-AS1* and DNMT1 was also observed in human endometrial stromal cells (see Fig. 8). It is postulated that *HAND2-AS1* sequesters/impedes DNMT1 binding from *HAND2* promoter to keep a lower level of DNA methylation in the *HAND2* promoter. It is worth noting that distinct intracellular locations of *HAND2-AS1* was observed in endometrium. In contrast to the cytoplasmic distribution in epithelial

cells, *HAND2-AS1* expression is primarily localized in the nuclei of endometrial stromal cells (see Fig. 2). Since HAND2 is exclusively induced in endometrial stromal cells, not in epithelium and myometrium, it is likely that *HAND2-AS1* controls HAND2 transcriptional activity solely in endometrial stromal cells. In epithelial cells, however, *HAND2-AS1* may function as "miRNA sponges" to control target mRNA stability and translation. In support of this notion, many recent studies revealed that *HAND2-AS1* is aberrantly expressed in many epithelium-originated cancer cells, including endometrial cancer cells, in which *HAND2-AS1* acts as a competitive endogenous RNA (ceRNA) and competes with endogenous RNA for miRNAs binding [35-37]. It will be interesting to identify the novel *HAND2-AS1*-binding targets in human endometrial epithelial cells in our future studies.

In conclusion, our studies reveal a new model of mechanism in which lncRNA *HAND2-AS1* controls target gene *HAND2* expression via DNA methylation, leading to P resistance in endometriosis. This is also likely a common phenomenon in other endometrial diseases, such as endometrial cancer, adenomyosis, and infertility. Based on this notion, DNA methylation status in *HAND2* promoter could serve as a potential prognostic marker to predict occurrence and treatment efficacy of P-related drugs in these endometrial diseases. Restoration of *HAND2-AS1* and *HAND2* expression would be a promising therapeutic strategy. Further identification of *HAND2-AS1* and HAND2-regulated molecular targets will help us to understand better the molecular basis of pathogenesis in endometriosis.

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Disclosures

The authors declare no conflict of interest.

Data Availability

Original data generated and analyzed during this study are included in this published article.

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