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Original Article

Long noncoding RNA H19 inhibits the growth and invasion of trophoblasts by inactivating Wnt/β-catenin signaling via downregulation of DDX3X

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Abstract: Long noncoding RNAs (lncRNAs) are implicated in a broad range of physiological and pathological processes. In this study, we aimed to explore the expression and biological functions of lncRNA-H19 in the placental villi of unexplained miscarriage. The expression of lncRNA-H19 in the villi from women with unexplained miscarriage and normal pregnancy was measured by real-time PCR analysis. The methylation status of the promoter of lncRNA-H19 was assessed. Gain-of-function studies were performed to determine the role of lncRNA-H19 in trophoblast proliferation and invasion. It was found that the level of lncRNA-H19 in the villi from women with unexplained miscarriage (n = 22) was significantly higher than that from women with normal pregnancy (n = 22). Global DNA methylation was significantly lower in the unexplained miscarriage group than that in the normal pregnancy group. Moreover, hypomethylation of the promoter of lncRNA-H19 was noted in the villi from women with unexplained miscarriage. Overexpression of lncRNA-H19 significantly inhibited the proliferation and invasion of human trophoblasts. RNA pulldown assays and mass spectrometry identified DEAD Box Helicase 3, X-Linked (DDX3X) as a novel lncRNA-H19-binding protein. Mechanistically, lncRNA-H19 suppressed the activation of Wnt/β-catenin signaling in trophoblasts by reducing the protein expression of DDX3X. In conclusion, lncRNA-H19 is upregulated in the villi from women with unexplained miscarriage and impairs the proliferation and invasion of trophoblasts through downregulation of DDX3X and inactivation of Wnt/β-catenin signaling. These findings suggest that lncRNA-H19 may be a potential therapeutic target for the prevention of miscarriage.

Keywords: Invasion, long noncoding RNA, proliferation, RNA-interacting protein, unexplained miscarriage

Introduction

Miscarriage or spontaneous abortion (SA) is the most common placental-related complication of pregnancy. Early SA occurs prior to 20 gestational weeks and is among the most common reproductive failures of human pregnancy. The pathogenesis of SA is associated with fetal chromosomal abnormalities, endocrine disorders, and immune factors [1, 2]. The exact mechanisms for early unexplained miscarriage remain unclear.

Trophoblasts are placental cells of the epithelial lineage that can invade endometrium and differentiate into highly specialized placental cells. Trophoblasts play an essential role in placental development in normal pregnancy [3]. Impaired trophoblast function is an important cause of early miscarriage [4]. Identification of key regulators of the biology of trophoblasts is of significance in uncovering the pathogenesis of unexplained recurrent miscarriage.

Long noncoding RNAs (lncRNAs) are a class of RNA transcripts longer than 200 nucleotides [5]. Global genome transcriptomic analyses have identified a large number of dynamically expressed lncRNAs, many of which are involved in various biological functions [6, 7]. It has been documented that lncRNA-H19 expression is increased in the fetal liver and placenta [8]. Knockdown of lncRNA-H19 was reported to cause proliferation-promoting effects in human trophoblastic JEG-3 cells by downregulating nodal modulator 1 via encoding of microR-

NA-675 [9]. Interestingly, lncRNA-H19 expression is elevated at the early stage of placental development, while increased miR-675 expression occurs at the late stage of placental development [8]. The distinct expression pattern may reflect a miR-675-independent pathway in the action of lncRNA-H19 expression. lncRNAs also exert their functions through direct interactions with proteins [10]. For example, the lncRNA MT1JP has the capacity to complex with the RNA-binding protein TIAR, consequently reducing tumor cell growth by the p53 pathway [11].

Therefore, in this study, we tested the hypothesis that the lncRNA-H19 may interact with an undefined RNA-binding protein to affect the biology of trophoblasts and thus participate in the pathogenesis of unexplained miscarriage.

Materials and methods

Human tissue collection

Villi tissues were collected from patients with unexplained miscarriage (n = 22) during the first trimester (7-9 weeks), as well as from women undergoing elective pregnancy termination (n = 22) during the first trimester (7-9 weeks) for nonmedical reasons. The selected patients were aged 23-35 years and investigated for all baseline blood parameters, chromosomal diseases, infectious diseases, and metabolic diseases. The study was approved by the Human Research Ethics Committee of Shanghai Changzheng Hospital at the Second Military Medical University (Shanghai, China), and each patient signed a written consent form.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from villous tissues obtained from cases of unexplained miscarriage or normal pregnancy or from cells treated with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed using the Reverse Transcription System Kit (Promega Corporation, Madison, WI, USA). H19 cDNA was quantified using a standard TaqMan real-time PCR protocol and a StepOne Plus System (Applied Biosystems, Foster City, CA, USA). PCR primers for H19 are as follows: forward, 5'-CTGGGAGGGTGTCTGCTTC-3' and reverse, 5'-CTGGGCAACGGAGGTGTA-3'. In parallel, 18S was employed as an endogenous control, with the primers: forward, 5'-GCTGGAATTACCGCGGCT-3' and reverse, 5'-CGGCTACCACATCCAAGGAA-3'.

Cell lines and vectors

The immortalized first trimester (6-9 weeks gestation) extravillous trophoblast (EVT) cell line HTR-8/SVneo was used. The cells were maintained in DMEM/F12 supplemented with 10% fetal calf serum (FCS), 1% L-glutamine (200 mM), and 1% penicillin/streptomycin (all from Invitrogen Life Technologies).

CCK-8 cell proliferation assay

HTR-8/SVneo cells plated at 60-70% confluency in 6-well plates were transfected with pcDNA-H19 or pcDNA3.1. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. All experiments were performed in sextuplicate. Cell proliferation curves were plotted using the absorbance at each time point.

Matrigel invasion assay

The invasion of HTR-8/SVneo cells through Matrigel was objectively evaluated in Transwell invasion chambers. HTR-8/SVneo cells transfected with pcDNA-H19 or pcDNA3.1 were plated in the upper chambers of 24-well plates at a density of 1×10^4 cells/well. The membranes were coated with Matrigel to form matrix barriers. DMEM/F12 containing 10% FCS was placed in the lower chamber as a chemo-attractant. After incubation for 48 h at 37°C, cells that had invaded to the lower surface were stained with 0.1% crystal violet and counted in 10 randomly selected microscopic fields.

RNA pulldown assays and mass spectrometry

lncRNA-H19 was in vitro transcribed from the pSPT19-H19 plasmid; biotin labelled using Biotin RNA Labeling Mix (Roche Diagnostics, Indianapolis, IN, USA) and T7/SP6 RNA polymerase (Roche Diagnostics), and then purified using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total cell lysates were extracted using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). One milligram of cell lysate was then mixed with 50 pmol of biotinylated RNA. Sixty microliters of

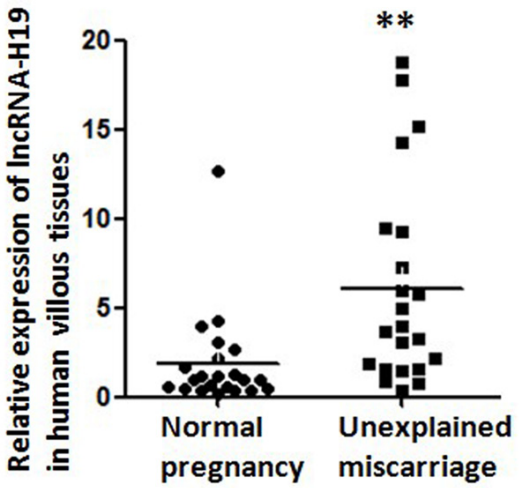

Figure 1. Upregulated lncRNA-H19 expression in human villous tissue following early unexplained miscarriage. lncRNA-H19 was highly expressed in human villous tissue obtained from patients who experienced early unexplained miscarriage (n = 22) and, to a lesser extent, from induced abortion cases (n = 22). $^{**}P < 0.01$.

washed streptavidin magnetic beads (Invitrogen Life Technologies) was added to each binding reaction, and the mixture was incubated at room temperature for 1 h. The beads were boiled in sodium dodecyl sulphate (SDS) buffer. Retrieved proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and silver stained. Specific bands were excised and analyzed by mass spectrometry (ProTech Inc., Norristown, PA, USA).

Western blot analysis

Whole cell protein extracts were prepared via cell lysis in RIPA buffer supplemented with proteinase and phosphatase inhibitors (Beyotime Institute of Biotechnology, Haimen, China). The protein yield was quantified using the BCA protein assay (Beyotime Institute of Biotechnology). Equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were probed overnight with anti-human DDX3X, anti-human DDX3X (1:1000; Cell Signaling Technology, Beverly, MA, USA), or anti-human β-actin (1:5000, Sigma-Aldrich) antibodies. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000; Sigma-Aldrich). Proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

RNA immunoprecipitation (RIP) assay

RIP experiments were conducted with anti-DDX3X antibody (Cell Signaling Technology) using the Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. The co-precipitated RNAs were detected by reverse transcription PCR and quantitative PCR.

Global genomic DNA methylation analysis

Genomic DNA was extracted from the villi using a commercially available kit (Axygen, Union City, CA, USA). Global DNA methylation levels were determined using the Methylation Quantification Ultra Kit (Epigentek, Farmingdale, NY, USA). In this assay, 5-methylcytosine-modified genomic DNA is recognized by a 5-methylcytosine antibody, and bound DNA is colorimetrically quantified. Positive (methylated) and negative (unmethylated) control DNA was supplied with the kit. The proportion of methylated nucleotides in total genomic DNA was determined.

Bisulphite pyrosequencing

Bisulphite pyrosequencing was used to quantity DNA methylation at two sites in the DMR of the lncRNA-H19 gene. Two micrograms of DNA was subjected to bisulphate conversion using the EpiTect Bisulfite Kit (59104) (Qiagen). Pyrosequencing primers are as follows: forward, 5'-GGATGGTAAGGAATTGGTTGTAGTT-3'; reverse, 5'-ACTCCCATAAATATCCTATTCCC-3'; and sequencing, 5'-AACCCCCCTAAACCCT-3'. The degree of methylation at each CpG site was determined using Allele Quantification software.

Statistical analysis

All data are expressed as the mean ± standard deviation. Statistical analyses were performed using the Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey's test. *P*-values < 0.05 were considered statistically significant.

Results

lncRNA-H19 is upregulated in villi women with unexplained miscarriage

We first compared the expression of lncRNA-H19 in villi obtained from women with a history

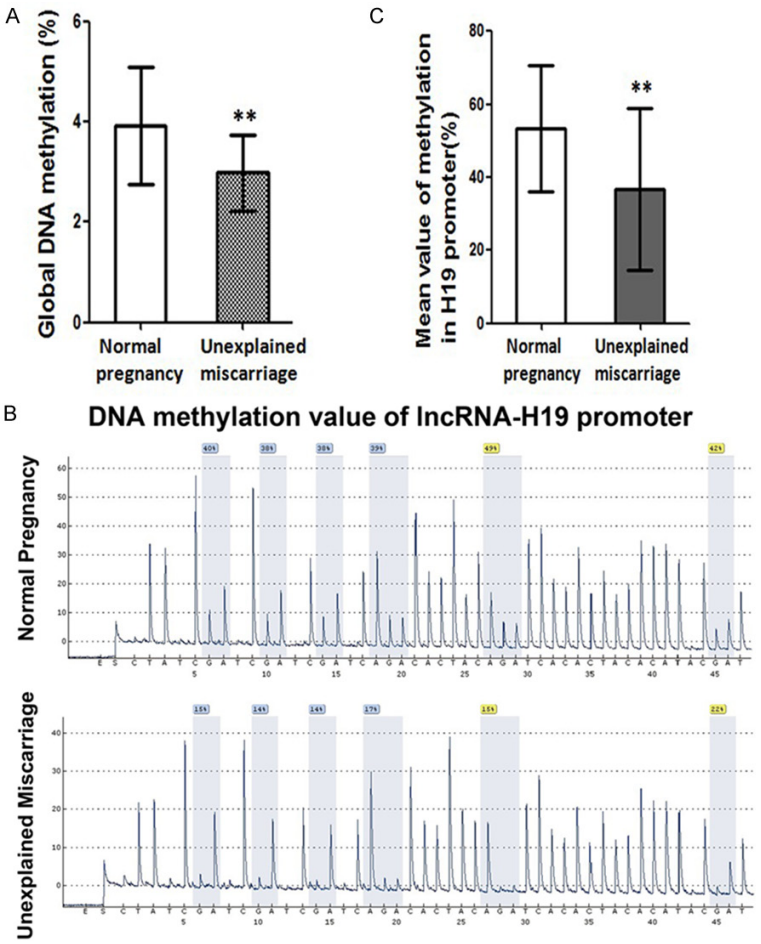

Figure 2. Promoter hypomethylation of lncRNA-H19 in villous tissue from women with early unexplained miscarriage. A. Global DNA methylation was significantly lower in villous tissue obtained from women (n = 22) with early unexplained miscarriage than that in the normal group (n = 22) $^{**}P < 0.01$. B, C. The promoter region of the lncRNA-H19 gene exhibited lower DNA methylation levels in villous tissue from women (n = 10) with early unexplained miscarriage than from those in the control group (n = 10). $^{**}P < 0.01$.

of unexplained miscarriage (n = 22) or IA (n = 22). The results of qRT-PCR analysis showed that lncRNA-H19 was expressed at higher levels in villi obtained from women with unexplained miscarriage than in that from women with normal pregnancy ($P < 0.01$; **Figure 1**). However, no difference in miR-675 expression was observed in the 2 groups (Figure S1).

Hypomethylation of the promoter of lncRNA-H19 is detected in villi from women with unexplained miscarriage

As shown in **Figure 2A**, global DNA methylation in villi was significantly lower in women with unexplained miscarriage than in those with normal pregnancy. Moreover, compared to the normal group, women with unexplained miscarriage showed hypomethylation of the promoter of lncRNA-H19 (**Figure 2B** and **2C**).

Overexpression of lncRNA-H19 inhibits trophoblast proliferation and invasion

Next, we assessed the biological functions of lncRNA-H19 in trophoblast proliferation and invasion. To this end, we overexpressed lncRNA-H19 in HTR-8/Svneo human trophoblast cell line (**Figure 3A**). Enforced expression of lncRNA-H19 significantly inhibited the proliferation (**Figure 3B**) and invasion (**Figure 3C**) of HTR8/Svneo cells, compared to vector-transfected cells.

lncRNA-H19 decrease the protein expression of DDX3X

RNA pulldown assays revealed a novel lncRNA-H19-binding protein (**Figure 4A**), which was identified as DEAD Box Helicase 3, X-Linked (DDX3X) by mass spectrometry (data not shown). Western blot analysis confirmed the presence of DDX3X in the lncRNA-H19-immunoprecipitated complex (**Figure 4B**). We also performed RIP experiments using an antibody against DDX3X in HTR8/SVneo trophoblast extracts. We observed enrichment of lncRNA-H19 mRNA but not of GAPDH mRNA (**Figure 4C**). Overexpression of lncRNA-H19 led to a significant decline in the level of DDX3X protein (**Figure 4D**), but without altering the level of DDX3X mRNA (**Figure 4E**). Taken together, lncRNA-H19 is upregulated in trophoblast cells, and binds to DDX3X and decreases the protein level of DDX3X.

lncRNA-H19 inhibits Wnt/β-catenin signaling by downregulating DDX3X

Since DDX3X is required for activation of Wnt/β-catenin signaling in mammalian cells [12], we

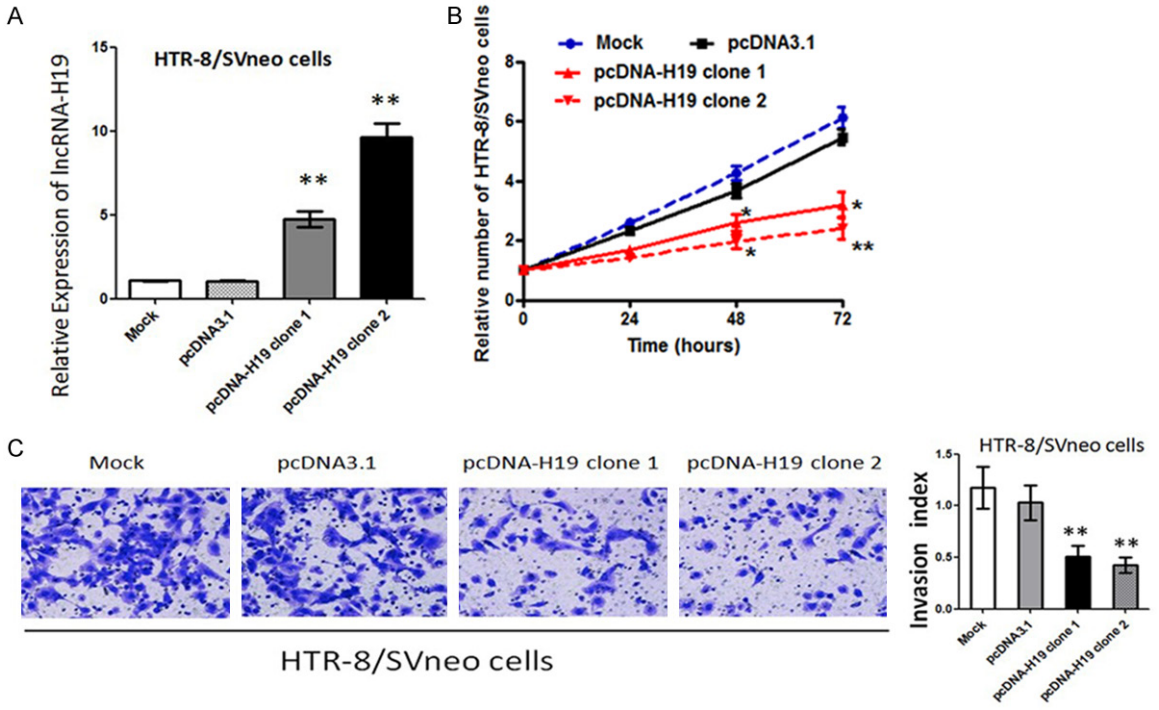

Figure 3. Overexpression of lncRNA-H19 inhibits trophoblast proliferation and invasion. A. Measurement of lncRNA-H19 expression in HTR8/Svneo trophoblasts transfected with indicated constructs. B. lncRNA-H19 upregulation inhibited HTR8/SVneo trophoblast proliferation. C. Transwell invasion assay. lncRNA-H19 overexpression decreased HTR8/SVneo trophoblast invasion. $^{**}P < 0.01$ vs. mock.

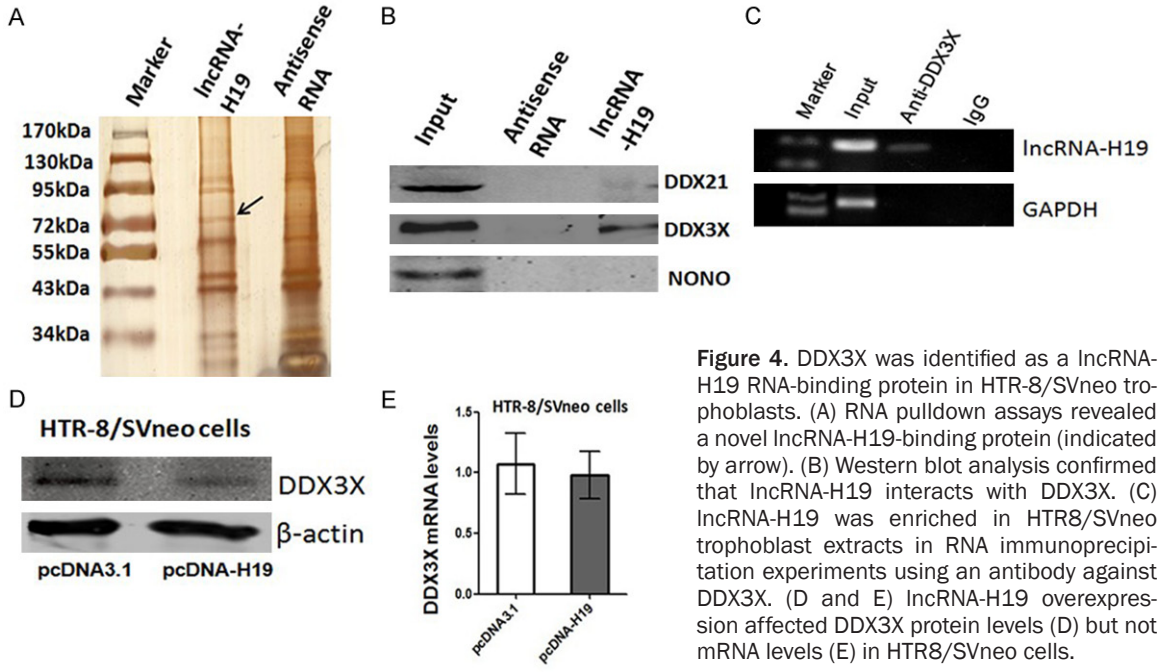

Figure 4. DDX3X was identified as a lncRNA-H19 RNA-binding protein in HTR-8/SVneo trophoblasts. (A) RNA pulldown assays revealed a novel lncRNA-H19-binding protein (indicated by arrow). (B) Western blot analysis confirmed that lncRNA-H19 interacts with DDX3X. (C) lncRNA-H19 was enriched in HTR8/SVneo trophoblast extracts in RNA immunoprecipitation experiments using an antibody against DDX3X. (D and E) lncRNA-H19 overexpression affected DDX3X protein levels (D) but not mRNA levels (E) in HTR8/SVneo cells.

tested whether lncRNA-H19 regulates Wnt/β-catenin signaling in trophoblast cells by facilitating DDX3X degradation. To this end, we overexpressed DDX3X in HTR8/SVneo cells and showed that DDX3X overexpression decreased E-cadherin protein expression (**Figure 5A**).

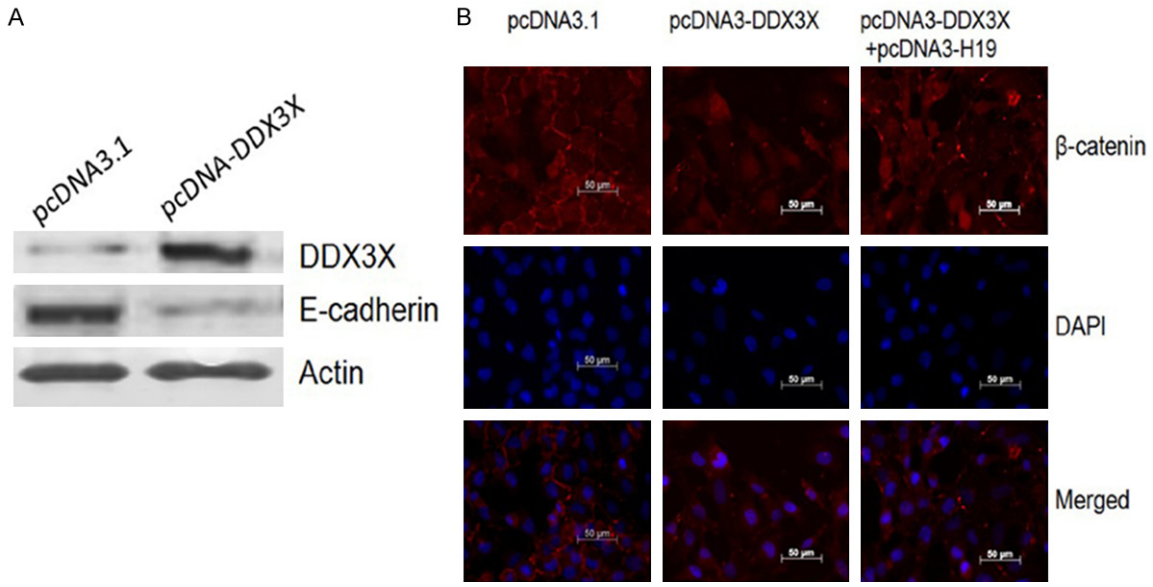

Figure 5. lncRNA-H19 inhibits Wnt/β-catenin signaling by downregulating DDX3X. A. DDX3X overexpression decreased E-cadherin protein expression in HTR8/SVneo cells. B. DDX3X overexpression in HTR8/SVneo cells promoted the nuclear translocation of β-catenin. lncRNA-H19 upregulation counteracted the effect of DDX3X on β-catenin distribution.

Notably, DDX3X overexpression promoted the nuclear translocation of β-catenin, confirming that the Wnt/β-catenin pathway is activated by DDX3X in HTR8/SVneo cells (**Figure 5B**). In contrast, ectopic expression of lncRNA-H19 impaired β-catenin nuclear translocation induced by DDX3X (**Figure 5B**). Taken together, lncRNA-H19 negatively regulates the activation of Wnt/β-catenin by suppressing DDX3X expression.

Discussion

Many pathogenic factors (e.g., chromosomal abnormalities, endocrine disorders, and immune factors) can affect placental development and trophoblast function and can lead to unexplained miscarriage and SA [13, 14]. Epigenetic abnormalities secondary to genetic and non-genetic etiology are the main elements in the molecular regulatory network of early villi and trophoblasts [15, 16]. Villous trophoblastic cells are the main cellular components of the placenta, and these cells secrete hormones, maintain maternal-fetal exchange, and play an important role in implantation and embryonic development [17]. In this study, we identified differences in the global genomic methylation in placental villi obtained from cases of unexplained miscarriage or normal pregnancy. Consistent with genome-wide hypomethylation, DNA methylation of the lncRNA-H19 promoter was lower in unexplained miscarriage samples than in normal pregnancy samples, leading to lncRNA-H19 upregulation in placental villi from women with unexplained miscarriage. Furthermore, we demonstrated that lncRNA-H19 overexpression inhibits the growth and invasion of trophoblasts. We also identified DDX3X as the lncRNA-H19 RNA-binding protein in trophoblasts; this finding facilitated the elucidation of the mechanism of action of lncRNA-H19 during unexplained miscarriage. We confirmed that lncRNA-H19 inhibits the growth and invasion of trophoblasts by inactivating the Wnt pathway, which is activated in early placental villi development.

Unexplained miscarriage is a common medical problem. Aberrant DNA methylation has been identified as a primary cause of unexplained miscarriage [18]. Our data showed that the methylation percentage of lncRNA-H19 was lower in unexplained miscarriage samples than in normal pregnancy samples, which may provide an explanation for the finding that lncRNA-H19 expression was elevated in unexplained miscarriage samples. The lncRNA-H19 gene belongs to a highly conserved imprinted gene cluster that plays important roles in embryonic development and growth control [19, 20]. The

lncRNA-H19 gene transcript is present in the placenta from the early developmental stage to the late developmental stage in mice. The main physiological role of lncRNA-H19 in mice is to limit placental growth at the late developmental stage [8]. In the present study, we demonstrated that lncRNA-H19 upregulation is associated with the occurrence of unexplained miscarriage. We also demonstrated that lncRNA-H19 overexpression inhibits the proliferation and invasion of HTR-8/SVneo cells, which is an immortalized first trimester EVT cell line. However, we did not observe a difference in miR-675 expression between unexplained miscarriage and normal pregnancy samples (Figure S1). These findings provide a rationale for exploring the novel mechanism of action of lncRNA-H19 in human trophoblasts during the early development of the placenta.

In hepatocellular carcinoma, hnRNPU was identified as an lncRNA-H19 RNA-binding protein [21]. To further clarify the mechanism for the action of lncRNA-H19 in human trophoblasts, lncRNA-H19 RNA-binding proteins were screened via an RNA pulldown assay in HTR-8/SVneo cells. DDX3X was identified as the lncRNA-H19 RNA-binding protein in human trophoblasts; DDX3X is a highly conserved DEAD-box RNA helicase that participates in RNA transcription, RNA splicing, and mRNA transport, translation, and nucleo-cytoplasmic transport [22]. This protein is highly expressed in metaphase II (M II) oocytes and is the predominant DDX3 variant in the ovary and embryo [23]. Interestingly, a recent study has identified DDX3X as a Wnt-β-catenin regulator in human embryonic kidney 239T cells [24]. In a Wnt-dependent manner, DDX3X binds CK1ε and directly stimulates its kinase activity, thus promoting phosphorylation of the scaffold protein [25, 26]. These findings suggest that lncRNA-H19 is associated with the regulation of Wnt-β-catenin signaling through the binding of DDX3X. In humans, Wnt proteins promote decasualization, endometrial function and trophoblast differentiation [27]. The interaction between DDX3X and CK1ε was confirmed by co-immunoprecipitation of endogenous proteins [28]. Here, we confirmed that DDX3X promotes Wnt-β-catenin signaling pathway activation. Moreover, ectopic expression of lncRNA-H19 prevented DDX3X-induced β-catenin nuclear translocation. Therefore, we suggest that lncRNA-H19 plays an inhibitory role in early trophoblasts by interacting with DDX3X.

In conclusion, lncRNA-H19 is upregulated in the villi from women with unexplained miscarriage, which is associated with hypomethylation of its promoter. lncRNA-H19 restricts the growth and invasion of early trophoblasts via interaction with DDX3X and inactivation of Wnt/β-catenin signaling. Therefore, lncRNA-H19 represents a potential therapeutic target for the treatment of unexplained miscarriage.

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Disclosure of conflict of interest

None.

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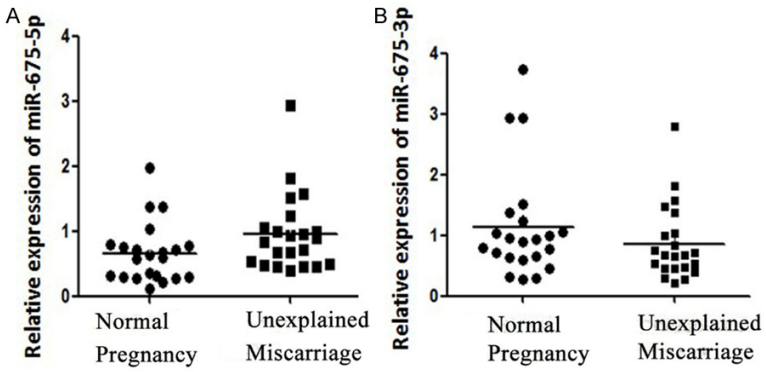

Figure S1. Upregulated expression of micro-675-5p and micro-675-3p in human villous tissue following early unexplained miscarriage. The results showed that neither micro-675-5p nor micro-675-3p was significantly highly expressed in human villous tissue from early unexplained miscarriage patients (n = 22) compared to that (to a lesser extent) in human villous tissue from normal pregnancy patients (n = 22).