The function and possible mechanism of lncRNA LINC00881 in regulating fetal growth restriction

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Abstract

Background: Long noncoding RNAs (lncRNAs) were found to play important roles in the pathogenesis of FGR. Herein, we aimed to elucidate the role of LINC00881 in regulating various biological events of FGR.

Methods: LINC00881 expression level was examined in the normal pregnancy placentas and FGR patients by quantitative reverse transcription PCR (qRT-PCR). The assays of CCK8, transwell and flow cytometry were performed to evaluate the functional effects of overexpression and silencing of LINC00881 on the HTR8/SVneo trophoblast cell line. RNA Pull down and immunoprecipitation (RIP) assays were futher used to verify the putative relationship between LINC00881 and its potential target of FSCN1.

Findings: LINC00881 expression was signifcantly downregulated in 20 FGR placental tissues compared to 20 healthy placental tissues. LINC00881 overexpression promotes proliferation, migration, invasion and apoptosis of HTR8/SVneo trophoblast cell line in vitro. Downregulation of LINC00881 leads to the opposite results. RNA pull-down assay and RIP assays reveals FSCN1 as a possible target of LINC00881. Futhermore, FSCN1 overexpression eliminated the effect of LINC00881 overexpression in HTR-8 cells.

1. Introduction

Fetal growth restriction (FGR), also known as intrauterine growth retardation, means that the fetus is affected by unfavorable factors and fails to reach the desired growth rate. FGR is associated with infant morbidity and mortality as well as typical clinical manifestations of delay in both intellectual and physical[1]. However, the precise molecular mechanisms underlying placental development remain unclear.

The placenta is located between the fetus and the mother. It has important functions such as nutrient transport, defense, synthesis and immunity to maintain the growth and development of the fetus. Placental development defects or dysfunction often lead to FGR[2]. The invasion of trophoblast-derived cells into maternal luterine tissue is a specific and constant feature of human placentation[3]

Long noncoding RNAs (lncRNAs) are a class of nonprotein-coding RNA transcripts with over 200 nucleotides, which are involved in the regulation of many cellular processes, such as proliferation, migration, invasion and apoptosis[4,5]. lncRNA has rarely been reported in the field of fetal growth and development research. It has been found that lncRNAs such as NEAT1 and H19 are abnormally expressed in FGR placental tissues[6,7]. Silencing of H19 can down-regulate transforming factor receptor III (TβR3) gene expression, thereby inhibiting trophoblast migration and invasion, and participating in the occurrence of FGR. The above studies initially revealed the role of lncRNAs in placental cell proliferation, invasion/apoptosis. The exact molecular mechanisms involved in lncRNA-mediated modulation of FGR still remains unclear. Many reported lncRNAs are located in the nucleus, and their most common mechanism of action is via recruitment of epigenetic modifier proteins to DNA[4]. lncRNAs reported in the cytoplasm, can act as sponges for microRNAs at the posttranscriptional level and therefore protect the

target mRNAs from repression by microRNAs[8]. Besides, some lncRNAs could modulate expression of genes that are positioned in the vicinity of their transcription sites in a cis-acting manner[9,10]. Although few lncRNAs are regarded as important biological molecules and are associated with FGR disease development, the role of many lncRNAs in FGR is not well documented.

In recent years, genome-wide association studies (GWASs) of birth weight have reported a large number of genetic loci related to birth weight, such as CCNL1 and LEKR1, providing a reliable basis for the study of the molecular mechanism of FGR[11]. Interestingly, in our previous study, we identified the birth weight-related genes CCNL1 (Cyclin L1) was highly related with FGR progression, (data not shown here). LINC00881, a lincRNA adjacent to CCNL1 in position has attracted our close attention. The LINC00881 have already been described as specific of the cardiomyocyte-lineage[12]. Interestingly, a Genome-wide scan has highlighted association for the LINC00881 in determining fetal growth [13,14]. During our verification of the hypothesis, we found that LINC00881 cannot regulate CCNL1, but it can regulate many biological functions of FGR. In the clinical tissues, we found that LINC00881 was significantly decreased in placentae from pregnancies with FGR. Furthermore, we applied LINC00881 lentivirus and siRNA mixture mediated gain-or loss-of-function experiments and rescue experiments to explore the role and possible mechanism of lncRNA LINC00881 in modulating FGR function.

2. Materials And Methods

2.1. Clinical tissue samples

Placenta tissues were sampled from the department of Gynecology and Obstetrics, Nanjing Maternity and Child Health Care Hospital. Placentas from 40 participants were included in the study, including 20 pregnant women with FGR and 20 normal pregnant women. FGR is defined as a fetus that has failed to achieve its genetically determined growth potential and is defined as EFW less than the 10th centile with respect to the reference value for current pregnancy age. This study was approved by the Ethics Committee of the Nanjing Maternity and Child Health Hospital. Written informed consent was obtained from all patients before using. All placenta tissues frozen in liquid nitrogen were then stored at−80℃.

2.2. RNA isolation and quantitative reverse transcription PCR (qRT-PCR) assay

Total RNAs were extracted from cells and tissues by traditional methods. cDNA was synthesized using the reverse transcription kit (Vazyme, China) in a 20 μl reaction containing 1000ng of total RNA. qRT-PCR reactions were carried out triplicate with 384-well plates on ViiA7 real-time PCR System-Life Tech (Applied Biosystems, USA) with a standard absolute quantification thermal cycling program. The threshold cycle (Ct) values of each sample were used in the post-PCR data analysis. Comparative quantification was performed according to the $2^{-\Delta Ct}$ method and GAPDH was used as internal reference of mRNA for normalization. The sequences of the primers were as follows: LINC00881, Forward primer 5'-AGGTGCTGTATTGGCTCTTGAC-3'; Reverse primer 5'-GCTTGCTGGCTTGTTTCTGC-3'；FSCN1, Forward primer 5'- CCTTCCGTACCCACACG-3'; Reverse primer 5'- CATTGGACGCCCTCAGT-3'. ACTB, Forward primer

5'- TCTCCCAAGTCCACACAGG-3'; Reverse primer 5'- GGCACGAAGGCTCATCA-3'. PRDX6, Forward primer 5'- GAGCTGTTCAAGGGCAAGA-3'; Reverse primer 5'-TCGCCAGTCACAAAGGC -3'. ANXA1, Forward primer 5'-AAATGCCTCACAGCTATCG-3'; Reverse primer 5'-CCTTATGGCGAGTTCCAA-3'. PRDX1, Forward primer 5'- GAAACAAGGAGGACTGGGA-3'; Reverse primer 5'- CCCTGAACGAGATGCCT-3'. CALR, Forward primer 5'-CCTGCCGTCTACTTCAAGGAG-3'; Reverse primer 5'- GAACTTGCCGGAACTGAGAAC-3'. GAPDH, Forward primer 5'- GAACGGGAAGCTCACTGG-3'; Reverse primer 5'- GCCTGCTTCACCACCTTCT-3'.

2.3. Cell culture and transfection

The HTR8/SVneo trophoblast cell is the storage cell line of our laboratory, which was derived from placental trophoblast. The HTR8/SVneo trophoblast cells were cultured in complete growth medium: DMEM basic(Gibco, USA), supplemented with 10% fetal bovine serum(10% FBS) (Gibco, USA) under the condition of 37 °C and 5% CO_2. Three Small interfering RNAs (siRNAs) sequences of FSCN1 are as follows: NC, Sense5'-3' UUCUCCGAACGUGUCACGUTT; Antisence 5'-3'ACGUGACACGUUCGGAGAATT. hs-FSCN1-si1, Sense5'-3' GCAAGAAUGCCAGCUGCUATT; Antisence 5'-3' UAGCAGCUGGCAUUCUUGCTT. hs-FSCN1-si2, Sense5'-3' GCAAGUUUGUGACCUCCAATT; Antisence 5'-3' UUGGAGGUCACAAACUUGCTT. hs-FSCN1-si3, Sense5'-3' GCUCCAGCUAUGACGUCUUTT; Antisence 5'-3' AAGACGUCAUAGCUGGAGCTT. Overexpression plasmid of FSCN1, overexpression lentiviruses and negative control of LINC00881 were synthesized from HANBIO (Shanghai, China). Smart Silencer and negative control were synthesized from RIBOBIO (Guangzhou, China). For preparation, HTR8/SVneo trophoblast cells were seeded in six-well plates overnight until growing to 60-70%. Afterwards, the operation was carried out according to the manufacturer's protocol of Lipofectamine 3000(Invitrogen, USA). Transfected cells were divided into LINC00881□NC, LINC00881□OE, LINC00881-SiNC, LINC00881-Si, LINC00881-OE+NC-FSCN1, LINC00881-OE+OV-FSCN1 NC, Si1, Si2, Si3, NC-FSCN1, OV-FSCN1.

2.4. Cell proliferation, Cell migration and invasion assay

The Cell Counting Kit-8 (CCK8) assay (Dojindo, Japan) was applied to test the cell proliferation as manufacturer's protocol. Briefly, the transfected cells were plated in 96-well plates at a density of 3×10^3 cells per well and cultured at 37 °C with 5% CO_2 for 0, 24, 48 and 72 h. 10μl per well of CCK-8 was added to the cells and the 96-well plates were incubated for another 2h. The absorbance was measured by a multifunctional microplate reader at 450 nm. Cell migration assays were performed using the Transwell system (Corning, USA). Cells (3×10^4) were added to the upper well in 200μl of culture medium without FBS. Next, 600 μl of medium (with 10% FBS) was added to the lower chamber. The protocol for the cell invasion assay was the same as the procedure for the migration assay, except that the upper side of the polycarbonate membrane was coated with a thin layer of Matrigel (BD Biosciences, USA). 60 μl Matrigel, 1:7 diluted in serum-free DMEM, was coated onto the upper chamber and incubated at 37°C for 2 h. The cells that migrated (after 48h) and the cells that invaded (after 96h) through the membrane were fixed in methanol and stained with 0.5% crystal violet (Beyotime, China). The cells that did not migrate through the pores were removed from the upper surface of the membrane by scraping with a cotton swab. The mean number of cells was calculated from counts in five random fields under a microscope.

2.5. Cell apoptosis and cycle assay

Cell apoptosis rate was evaluated using the AnnexinV-PE/7AAD Apoptosis Detection kit (Vazyme, China) according to the instructions from the manufacturer. Cells were collected, washed with PBS, and resuspended in 500 binding buffer. Then 5μl Annexin V-PE and 5μl 7-AAD staining solution were added to the buffer and incubated at room temperature for 15 min in the dark. Cells were analyzed by flowcytometry (Beckman Coulter, USA) within 1 h. Cell cycle analysis was evaluated using the cell cycle analysis kit (Beyotime, China) according to the instructions from the manufacturer. The transfected cells were collected, washed with cold PBS, and fixed with 70% ethanol at 4 ℃ for more than 12 h. The fixed cells were washed with cold PBS (Gibco, USA) and then stained with propidium iodide for 60 min at 37°C in the dark. Cells were analyzed by flowcytometry within 24 h. The percentage of cells in the G1 phase, the Sphase, and the G2 phase was analyzed.

2.6. RNA pull-down and mass spectrometry assay

The synthesis of LINC00881 was carried out with MAXIscript® Kit (Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. The interaction between LINC00881 and proteins were examined using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, Inc.) following the instructions of the manufacturer. The RNA-binding protein complexes were washed, eluted, and could be analyzed by Mass spectrometry (MS) (5600-plus, AB SCIEX, USA) and/or western blot (WB) analysis.

2.7. RNA immunoprecipitation (RIP) assay

The RIP assay was conducted by the RNA-Binding Protein Immunoprecipitation Kit (EMD Millipore, USA) according to the instructions of manufacturer. Briefly, the cells were lysed and the resulted lysis solutions were subsequently incubated with antibody against FSCN1 or isotype control IgG. The RNA-protein complexes were immunoprecipitated with protein A agarose beads, and the RNA was extracted and purified. LINC00881 could be quantified by qPCR.

2.8. Western blot analysis

Total protein was extracted using the RIPA lysis buffer phosphatase inhibitor (Beyotime, China), and the concentration was measured with the BCA assay (Beyotime, China). Proteins (40μg) were separated with SDS-PAGE and then transferred to a PVDF membrane (Millipore, USA). The membrane was blocked with 5% skim milk (Biofroxx, Germany) for 2 h and incubated with primary antibody overnight at 4℃, including cyclin L1(Santa Cruz, USA), monoclonal anti-Fascin (ab126772, Abcam, Britain) and monoclonal anti-GAPDH (ab8245, Abcam, Britain). The membranes were then washed with TBST (three times, 10 minutes/time), incubated with the HRP-conjugated goat anti-rabbit IgG (7074P2,Cell Signaling Technology (CST), USA) and HRP-conjugated horse anti-mouse IgG (7076P2,Cell Signaling Technology (CST), USA). Proteins were visualized with the ECL system (Millipore, USA) using the automatic chemiluminescence image analysis system (Tanon, China). Bands on Western blot gels were quantified using ImageJ. These values were then normalized to GAPDH level.

2.9. Statistical analysis

All data were presented as mean ± sd. Student's two-tailed t-test was used to analyze the differences between groups. Values were considered statistically significant at $P < 0.05$.

3. Results

3.1. The biology and expression characteristics of LINC00881

LINC00881 is an intergenic lncRNA located on chromosome 3q in the human genome, having a total length of 791bp (Fig. 1A). Coding Potential Calculator analyses were used to distinguish noncoding RNAs from protein coding RNAs based on the sequence features. LINC00881 had no reliable protein-coding open reading frame, with a coding potential score of 0.39 (Fig. 1B). LINC00881 is highly conserved in Chimp, Gorilla, Orangutan, Gibbon, Rhesus and so on. Sequences of LINC00881 locus between human and mouse genomes have high identity (Fig. 1C). The vitro transcription assay suggested that the LINC00881 transcript was polyadenylated and transcribed from a single exon; 791bp in length (Fig. 1D). To explore the possible role of LINC00881 in human FGR, we investigated the expression profile of LINC00881 in clinical samples. The qRT-PCR results showed that LINC00881 expression levels were significantly downregulated in the 20 pregnant women with FGR tissues as compared to the 20 healthy placental tissues (Fig. 1E). The clinical characteristics of the study patients include the females with the control group (n=20) and FGR (n=20) are shown in table1. There were no significant differences between the two groups in aspects of maternal age, body mass index (BMI) and gravidity. The group of FGR had a significantly lower birth weight than those whose mothers were in the control group. These results preliminarily suggested that LINC00881 may be associated with FGR progression.

Table1 Clinical characteristics of study patients.

	Control (n=20)	FGR (n=20)	P-value
Maternal age (years)	29.90±3.54	27.90±3.43	0.08
Maternal BMI (kg/m^2)	25.89±2.94	26.49±4.66	0.63
Gravidity	1.95±1.32	1.65±0.99	0.36
Birth weight (g)	3294.50±300.97	2109.50±251.99	****0.000**

Data are presented as the mean ± SD.**P<0.01

3.2. LINC00881 knockdown inhibits migration and invasion and promotes apoptosis in HTR-8 cells

Considering the dysregulated down expression of LINC00881 in FGR placental tissues, we further explored its function by overexpressing or silencing LINC00881 in HTR-8 cells. By qRT-PCR, the relative overexpression and knockdown efficiency of LINC00881 was confirmed in HTR-8 cells upon transfection with lentivirus and siRNA mixture. The expression of LINC00881 was increased 360-fold (Fig. 2A),

furthermore, silencer of LINC00881 demonstrated the highest efficacy in silencing the expression of LINC00881 at 100 nM when compared to that of the LINC00881-NC group (Fig. 2B). Subsequently, we examined the effect of LINC00881 overexpression or knockdown on the migration, invasion, and apoptosis of HTR-8 cells. CCK8 assay showed that the proliferation ability of HTR-8 cells was significantly enhanced at 24h, 48h and 72h ($P<0.05$) after LINC00881 overexpression (Fig. 2C). While the viability of LINC00881 knockdown cells was significantly inhibited at 24h, 48h and 72h ($P<0.05$) (Fig. 2D). Subsequently, the effect of LINC00881 overexpression or knockdown on the migration and invasion of HTR-8 cells were examined. The results of the transwell assay further revealed that the number of migrated or invaded cells per field was higher in the LINC00881 OE group than in the LINC00881-NC group (Fig. 2E), but was lower in the LINC00881-Si group than in the LINC00881-SiNC group (Fig. 2F). To further explore other effects on HTR-8 cells performed by LINC00881, we detected the effect of LINC00881 on the cell cycle and apoptosis. Flow cytometry analysis results revealed that the percentage of early apoptotic cells was higher in the LINC00881-Si group than in the LINC00881-SiNC group (Fig. 2G). However, whether LINC00881 is knockdown or overexpressed, it has no obvious effect on the cell cycle distribution (Fig. S1). Overall, these results indicate that LINC00881 knockdown inhibits cell proliferation, migration and invasion and promotes apoptosis in HTR-8 cells. However, overexpression of LINC00881 produces the opposite effects.

3.3. LINC00881 interacts with FSCN1

LncRNAs could modulate expression of genes that are positioned in the vicinity of their transcription sites in a cis-acting manner. We empirically searched the transcription position in the UCSC genome browser and found that LINC00881 transcription sites located upstream of the CCNL1 gene. To further verify the relationship between LINC00881 and CCNL1, we detected the expression of CCNL1 with the overexpression and knockdown of LINC00881, respectively. Both qRT-PCR and Western blot revealed that overexpression or knockdown of LINC00881 boosted no obvious expression on CCNL1 (Fig. S2). To further investigate how LINC00881 affected the trophoblast function, we initiated this study by screening LINC00881-interacting proteins (Fig. 3A). The profile of the RNA-pull down combined with Mass Spectrometry (MS) experiments identified 62 endogenous potential candidates for LINC00881-interacting proteins. Combining the information of 863 FGR-related proteins in the Open Targets Platform database (https://platform.opentargets.org/), and the downstream target proteins of the LINC00881 pulldown experiment, we further obtained 6 intersection proteins, namely CALR, ACTB, PRDX6, ANXA1, PRDX1, and FSCN1 (Fig. 3B). qRT-PCR results showed that FSCN1 were downregulated in LINC00881-overexpressing cells compared with matched normal cells, while FSCN1 were upregulated after LINC00881 was knockdown. At the same time, the mRNA expression of ANAX1 had statistical decrease and the mRNA expression of PRDX1 had statistical upregulate following LINC00881 overexpression. PRDX6 had no difference after LINC00881 was overexpressed while was slightly downregulated after LINC00881 konck down. However, ANAX1 PRDX1 had no statistical change while LINC00881 was downregulated. However, ACTB and CALR had no obvious change whether LINC00881 is knockdown or overexpressed (Fig. 3C).

Consistent with PCR results, western blot showed that FSCN1 expression was decreased in LINC00881-overexpressing cells, while was increased in LINC00881-knockdown cells (Fig. 3D). The above experiment initially reveals that FSCN1 has aroused our interest.. Furthermore, we identified their relationship using a RIP assay. The results of the RIP assay indicated that the IP group has a significant enrichment trend relative to the IgG group, and it can be preliminarily judged that LINC00881 can regulate FSCN1 expression (Fig. 3E). Recently results also indicated for the first time that the downregulation of fascin is involved in the pathogenesis of early recurrent miscarriage[15] These data suggest that FSCN1 may be the negative regulatory gene of LINC00881.

3.4. Inhibition of FSCN1 promotes migration, invasion and suppresses apoptosis of HTR-8 cells.

To evaluate the potential role of FSCN1 in FGR, we analyzed the effect of FSCN1 knockdown on proliferation, migration, invasion and apoptosis of HTR-8 cells. Firstly, knockdown efficiency of FSCN1 was verified by qPCR and Western Blot (Fig. 4A-4B). We examined the effect of FSCN1 on cell proliferation. The CCK-8 assay was used to detect the growth activity of the transfected FSCN1- knock down cells in 72 h. At 24, 48 and 72 h, knockdown of FSCN1 promoted cell proliferation（Fig. 4C）. At the same time, transwell assay results revealed that the migration or invasion of cells per field was evidently higher in the FSCN1 knockdown group than in the NC group (Fig. 4D). Flow cytometry analysis indicated that the percentage of cells undergoing early apoptosis was lower in the FSCN1-Si2 knockdown group than in the NC group, while no significant changes in the other two groups (Fig. 4E). Overall, these results suggest that the knock down of FSCN1 suppresses apoptosis and promotes the migration and invasion of HTR-8 cells.

3.5. Overexpression of FSCN1 suppresses migration and invasion of HTR-8 cells.

The effects of FSCN1 gain-of-function on HTR-8 cells were also performed. Overexpression FSCN1 plasmid were transfected into the HTR-8/SVneo trophoblast and the empty plasmid were transfected as a control. Firstly, 60-fold overexpression of FSCN1 mRNA was confirmed by qPCR（Fig. 5A）and about 2-fold by Western Blot（Fig.5B）. Then, CCK8 assay showed that the proliferation ability of HTR-8/SVneo cells was significantly inhibited at 24h, 48h and 72h ($P<0.05$) after FSCN1 was overexpressed (Fig. 5C). Transwell assay showed that overexpression of FSCN1 also significantly suppressed HTR-8/SVneo cell migration and invasion ($P<0.05$) (Fig. 5D).

3.6. FSCN1 overexpression rescues the effect of LINC00881 overexpression in HTR-8 cells

The above results had been preliminarily revealed that LINC00881 participated in HTR-8 cells function affect possibly by regulating FSCN1, we evaluated whether FSCN1 overexpression could rescue the effect of LINC00881 overexpression in HTR-8 cells. We constructed HTR-8 cell line stably overexpressing LINC00881 and co-transfected with FSCN1 plasmid. Firstly, western blot showed that overexpression of FSCN1 significantly rescued the LINC00881-OE mediated decreases in FSCN1 expression (Fig. 6A). CCK8 assay further showed that the proliferation ability of HTR-8/SVneo cells was decreased in the LINC00881-OE+OV-FSCN1 group compared to that in the LINC00881-OE+ NC-FSCN1 group in 24 h (Fig. 6B).

Transwell assays showed that overexpression of LINC00881 significantly promoted the migration and invasion capacity of HTR-8 cells compared with that in control cells containing the empty vector, whereas increased FSCN1 expression successfully reversed LINC00881-OE mediated promotion of HTR-8 cell migration and invasion (Fig. 6C-6D). Generally, our data indicate that LINC00881 represses FGR progression through regulated of FSCN1 in vitro.

4. Discussion

LncRNAs play important regulatory roles in tumorigenesis progression recent years[16]. However, little attention has been given to the functions and mechanisms of lncRNAs in FGR. In the present study, we found that the expression of LINC00881 was significantly downregulated in FGR placental tissues compared to that in healthy controls. Functional experiments demonstrated that LINC00881 overexpression could enhance and LINC00881 knockdown could weaken the migration and invasion capabilities of HTR-8 cells. We also found that LINC00881 knockdown increased the apoptosis level of HTR-8 cells. Mechanistically, LINC00881 could potentially regulate trophoblasts function through regulating FSCN1. Further rescue experiments showed that FSCN1 overexpression partially restrained trophoblasts function in LINC00881 -overexpressed HTR-8 cells.

In the human placenta, there are three major trophoblast subpopulations: cytotrophoblasts, extravillous cytotrophoblasts, and syncytiotrophoblasts[17]. Proliferation of trophoblast cells was involved in the placental development[18]. In addition, Inadequate migration and invasion and low viability of trophoblast cells may be are reasons of FGR[19]. Furthermore, apoptosis is thought to be a normal part of villus trophoblast renewal. Excessive apoptosis of trophoblast cells is one of the factors leading to deficient trophoblast invasion[20]. It suggested that placental dysfunction above may be part of the important causes of FGR. HTR-8 is a cell line derived from human extravillous trophoblasts, HTR-8 cells are usually used as a cell model of FGR. In our study, LINC00881 overexpression can promote the proliferation, migration and invasion of HTR-8/SVneo trophoblast, while LINC00881 downregulation reduced the cellular proliferation, invasion and promoted the apoptosis of HTR-8 cells. Hence, our results suggest that LINC00881 plays a vital role in regulating placental trophoblast cell function.

The molecular mechanisms by which lncRNAs exert their biological functions are diverse and complex. Many lncRNAs are now reported to modulate the expression of surrounding genes as cis-acting regulatory elements[4,21]. However, in our study of LINC00881, we unexpectly found that LINC00881 did not affect the expression of CCNL1 at the transcription and translation levels. Recently, some studies focused on the critical roles that RNA binding proteins (RBP) may play in modulating cancer initiation and progression through binding to RNAs and regulating their processing, stability, modification, localization or translation [22] [23,24]. We firstly performed RNA-pull down combined MS to identify the potential targets of LINC00881 in endogenous HTR-8 cells. A number of LINC00881-potential gene, such as FSCN1, ANXA1, ACTB, PRDX6, and PRDX1, have been found to be possible involved in FGR progression. Q-PCR and western blot were further carried out to identify genes FSCN1 regulated by

LINC00881. RIP assay results further proved that FSCN1 is a potential downstream target gene of LINC00881.

FSCN1 is a 55-kd protein and forms tight and stable cytoplasmic bundles with filamentous actin[25,26]. It is expressed at a low level in normal epithelial cells, but at a high level in tumor cell which plays a vital role in cell motility and adhesion [27-29]. Recently, researches have found that fascin was an early recurrent miscarriage (ERM)-associated gene and knockdown of fascin inhibited the proliferation but increased apoptosis of trophoblastic HTR8/SVneo cells [15]. However, in contrast to this research, in our study, overexpression of FSCN1 inhibited the growth, migration and invasion of HTR-8 cells, while knockdown of FSCN1 played the opposite role. The tissues specificity and potential biological mechanisms may partly explain this controversial phenomenon. Since the FSCN1 has been implicated in the development of FGR, we examined whether LINC00881 exerts its function in FGR by regulating FSCN1 signaling pathway. We found that the expression level of FSCN was increased in LINC00881-OE plus OV-FSCN1 cells compared with LINC00881-OE group. Functional rescue experiment assays of CCK8, migration and invasion further proved that LINC00881 exert their biological functions by regulating FSCN1.Thus, our findings suggest that LINC00881 acts via the FSCN1 signaling pathway to regulated FGR in HTR-8 cells. Although the precise molecular mechanism that underlies regulation of FSCN1 is not fully understood, we identified that FSCN1 may be one of the target genes of LINC00881 and LINC00881 may exert its function by negatively regulating on FSCN1. To the best of our knowledge, we were firstly to analyze the functional role of LINC00881 and FSCN1 in FGR, and initially clarified the negative regulatory relationship between LINC00881 and FSCN1. However, our research also has certain limitations. Firstly, the function of LINC00881 in HTR-8 cells does not conclusively demonstrate that LINC00881 regulates the pathogenesis of FGR. In future experiments, the precise molecular mechanism that underlies regulation of LINC00881 is necessary.

In summary, our study firstly demonstrates that LINC00881 expression levels are significantly down-regulated in FGR placental tissues, gain and loss experiments show that LINC00881 can regulate the proliferation, migration, invasion and apoptosis of trophoblast cells, further leading to the disorder of placental function. At the same time, the vitro function of FSCN1 in FGR was reported for the first time. Mechanistically, LINC00881 possibly regulates placental function through targeting FSCN1, which shed new light onto how lncRNAs harmonizes the regulation of downstream gene expression and placental function. These results suggest that downregulation of LINC00881 may aggravate the development of FGR by increasing FSCN1 expression.

Abbreviations

FGR: fetal growth restriction; LncRNAs: long noncoding RNAs; SiRNAs: small interfering RNAs; HTR-8: Human trophoblast cells HTR8; CCNL1: Cyclin L1; qRT-PCR: quantitative reverse transcription PCR; WB Western blot; CCK8: cell counting kit-8; RIP: RNA Immunoprecipitation; GWASs: genome-wide association studies; EFW: estimated fetal weight; MS: Mass Spectrometry; ERM: recurrent miscarriage; BMI: mass index; FBS: fetal bovine serum.

Declarations

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Conflict of Interest

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

RT, HJ and JW designed the research; RT and SY conducted the research; RT and SY analyzed data and performed the statistical analysis; RT and SY prepared the initial manuscript draft; HJ revised subsequent drafts; HJ and RT funded the experiments; and all authors read and approved the final manuscript.

Ethics approval and consent to participate. The present study was approved by the ethical review committee of Nanjing Maternity and Child Health Care Hospital, Women's Hospital of Nanjing Medical University. Written informed consent was obtained from all enrolled patients.

Consent for publication

Patients agree to participate in this work.

Data Availability Statement

The data sets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Figures

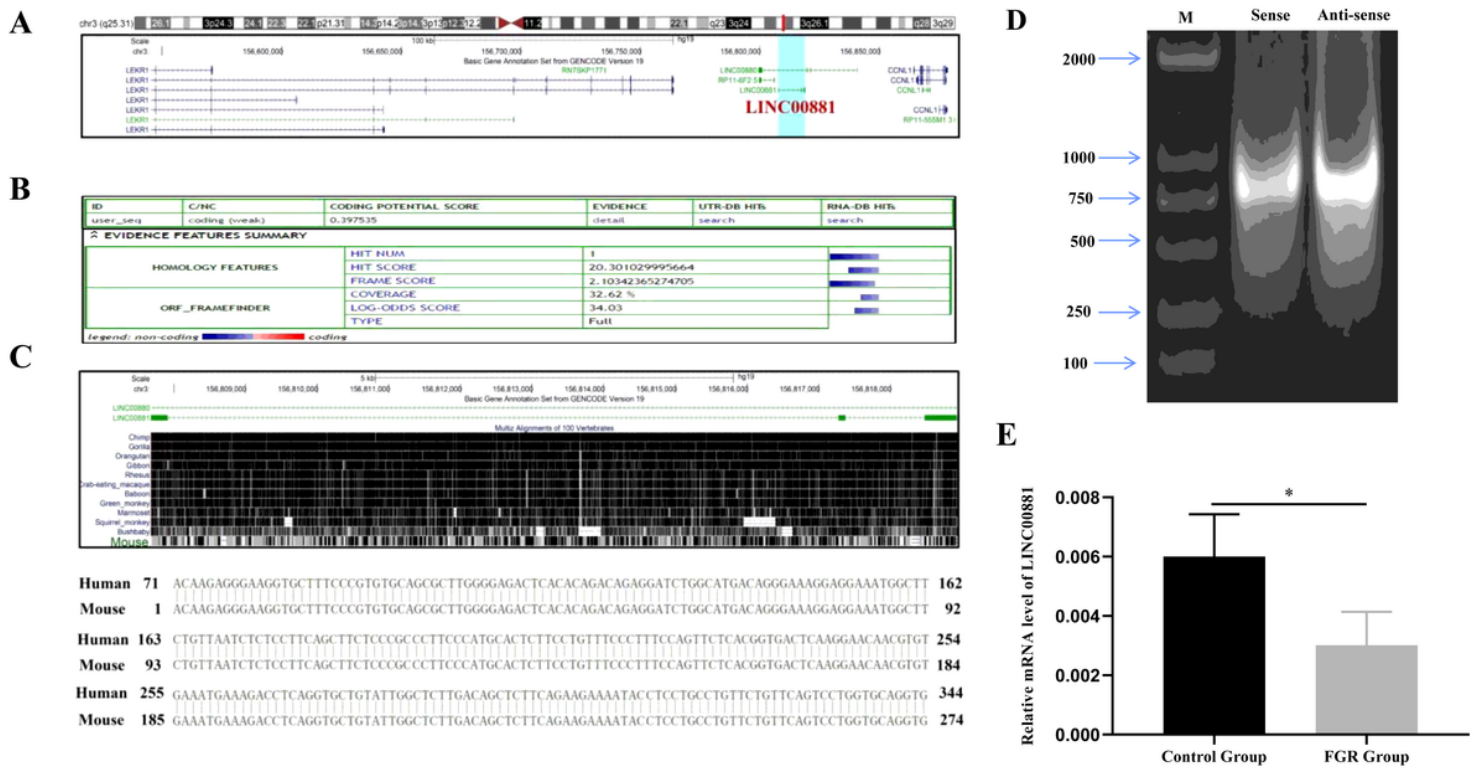

Figure 1

The biology and expression characteristics of LINC00881.**(A)** The Birth weight related gene CCNL1 is adjacent to LINC00881. **(B)** Coding potential calculator analysis of the possibility of the protein encoded by the LINC00881 sequence. **(C)** Conservation analysis of the LINC00881 locus found it to be highly conserved in Chimp, Gorilla, Orangutan, Gibbon, Rhesus. Sequence alignment by DNAMAN (http://www.lynnon.com) of LINC00881 between human and mouse genomes showed high identity. **(D)** In vitro transcription assay of LINC00881 in length. (E) Relative expression of LINC00881 in placental tissues in the FGR (n=20) and control groups (n=20) analyzed by RT-qPCR. Data presented as mean ± standard error of the mean. *P< 0.05.

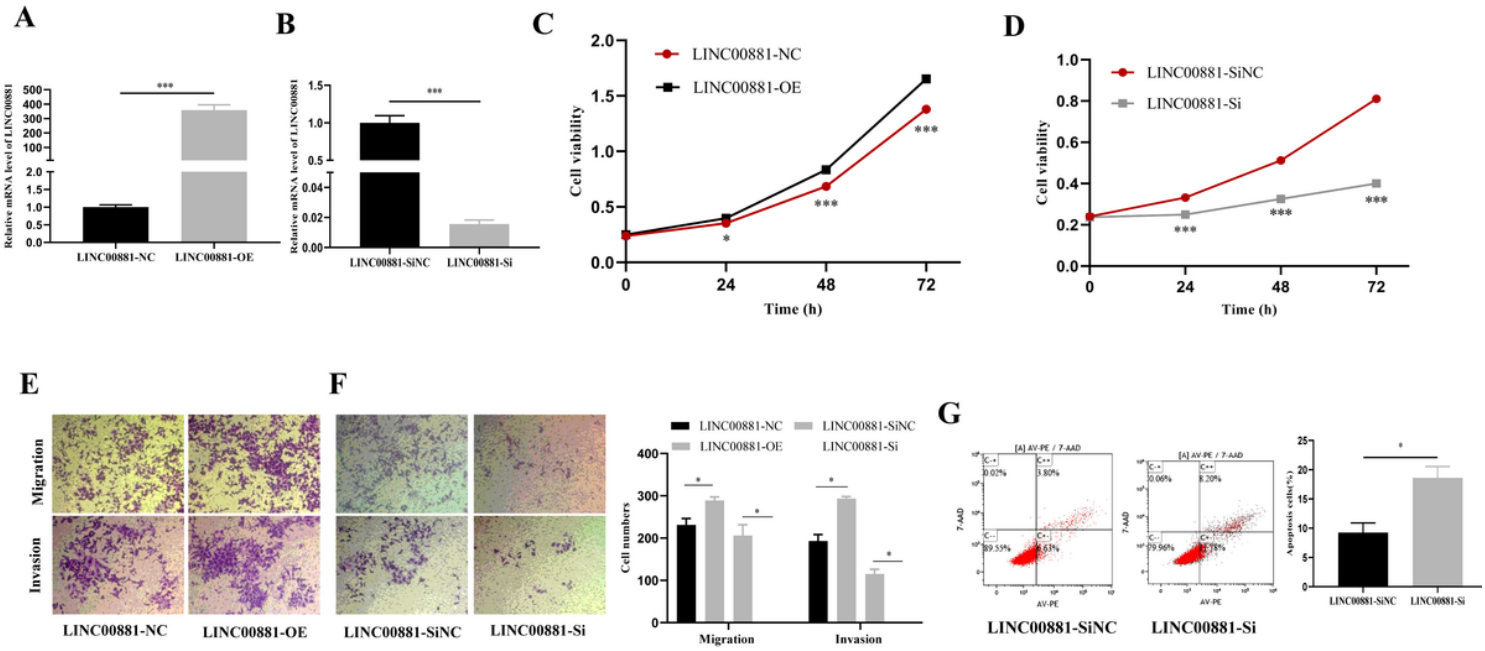

Figure 2

Effects of overexpression and knockdown LINC00881 on the function of HTR-8 cells. **(A-B)** The transfection efficiency of LINC00881 in HTR-8 cells transfected with overexpression lentivirus and smart silencer separately. RNAs were extracted and analyzed by RT-qPCR. **(C-D)** The proliferation ability of cells after overexpression and knockdown LINC00881 applied by CCK8; **(E-F)** The migration and invasion ability of cells after overexpression and knockdown LINC00881 applied by transwell assay (magnification, 200×). **(G)** The percentages of of cell apoptosis after knockdown LINC00881 applied by flow cytometry analysis. Data presented as mean ± standard error of the mean. *P< 0.05；**P< 0.01; ***P< 0.001.

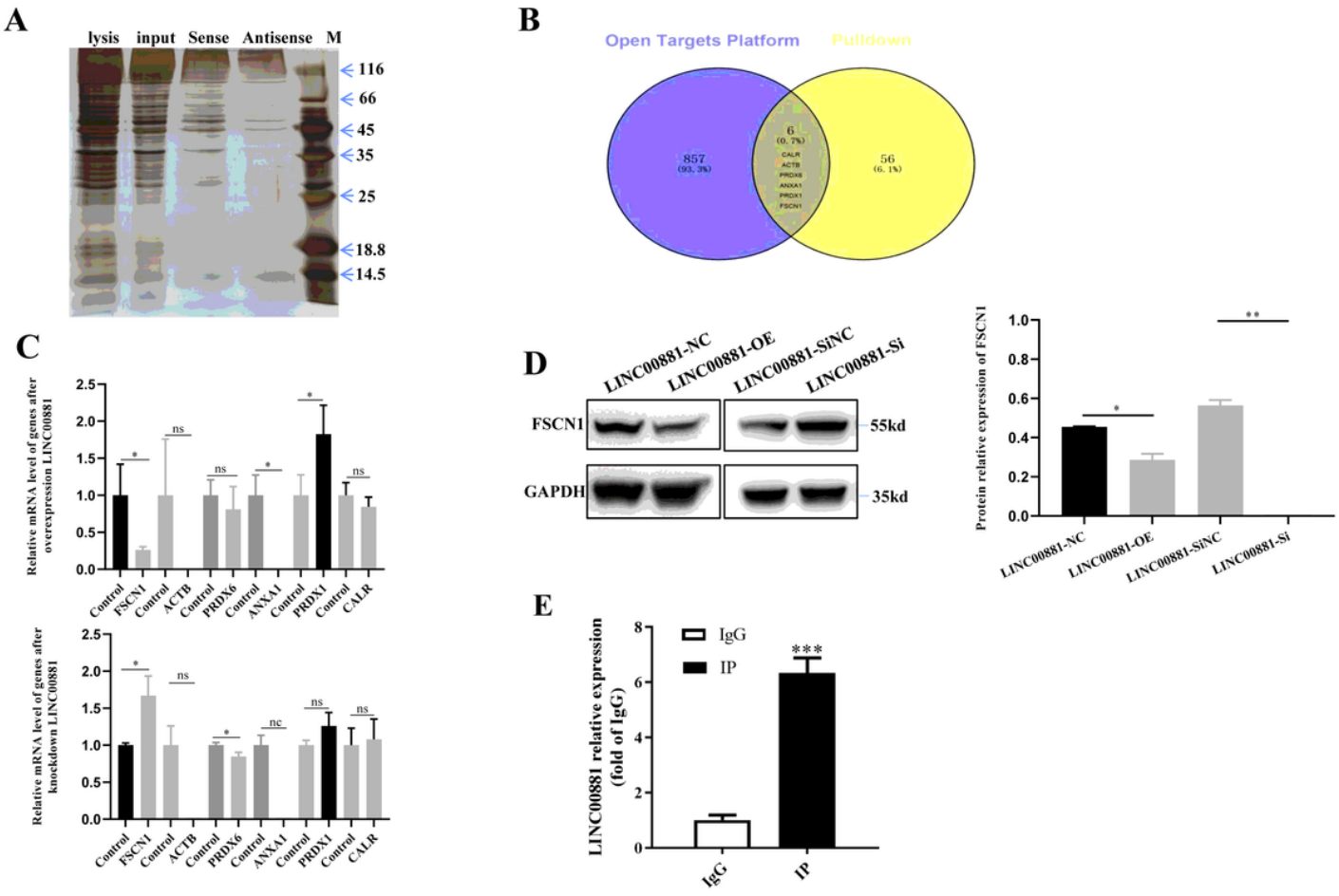

Figure 3

(A) The silver-stained PAGE gel that showed the separated proteins pulled down by LINC00881. **(B)** The intersection of FGR-related proteins in the Open Targets Platform database and the downstream target proteins of the LINC00881 pulldown experiment. **(C)** Relative mRNA expression of genes after overexpression and knockdown LINC00881 analyzed by RT-qPCR. **(D)** Relative protein expression of FSCN1 after overexpression and knockdown LINC00881 analyzed by Western Blot. **(E)** LINC00881 could interact with FSCN1 in HTR-8 cells.

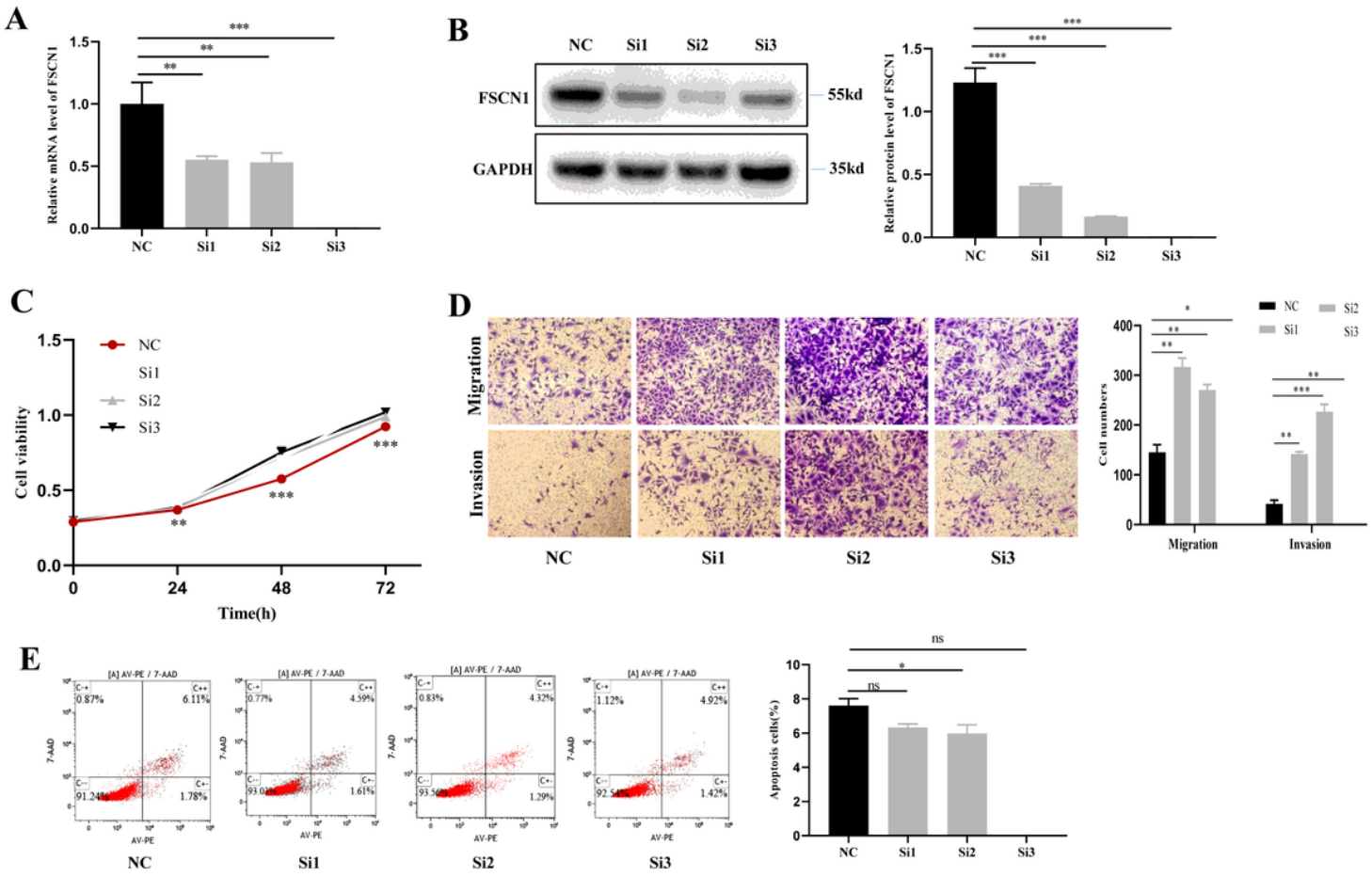

Figure 4

(A) Effects of knockdown FSCN1 on the function of HTR-8 cells. The transfection efficiency of FSCN1 in HTR-8 cells transfected with siRNAs. RNAs were extracted and analyzed by RT-qPCR. **(B)**The transfection efficiency of FSCN1 in HTR-8 cells transfected with siRNAs. Proteins were extracted and analyzed by Western Blot. **(C)** The proliferation ability of cells after knockdown FSCN1 applied by CCK8. **(D)** The migration and invasion ability of cells after knockdown FSCN1 applied by transwell assay (magnification, 200×). **(E)** The percentages of of cell apoptosis after knockdown FSCN1 applied by flow cytometry analysis. Data presented as mean ± standard error of the mean. *P< 0.05，ns, P＞0.05.

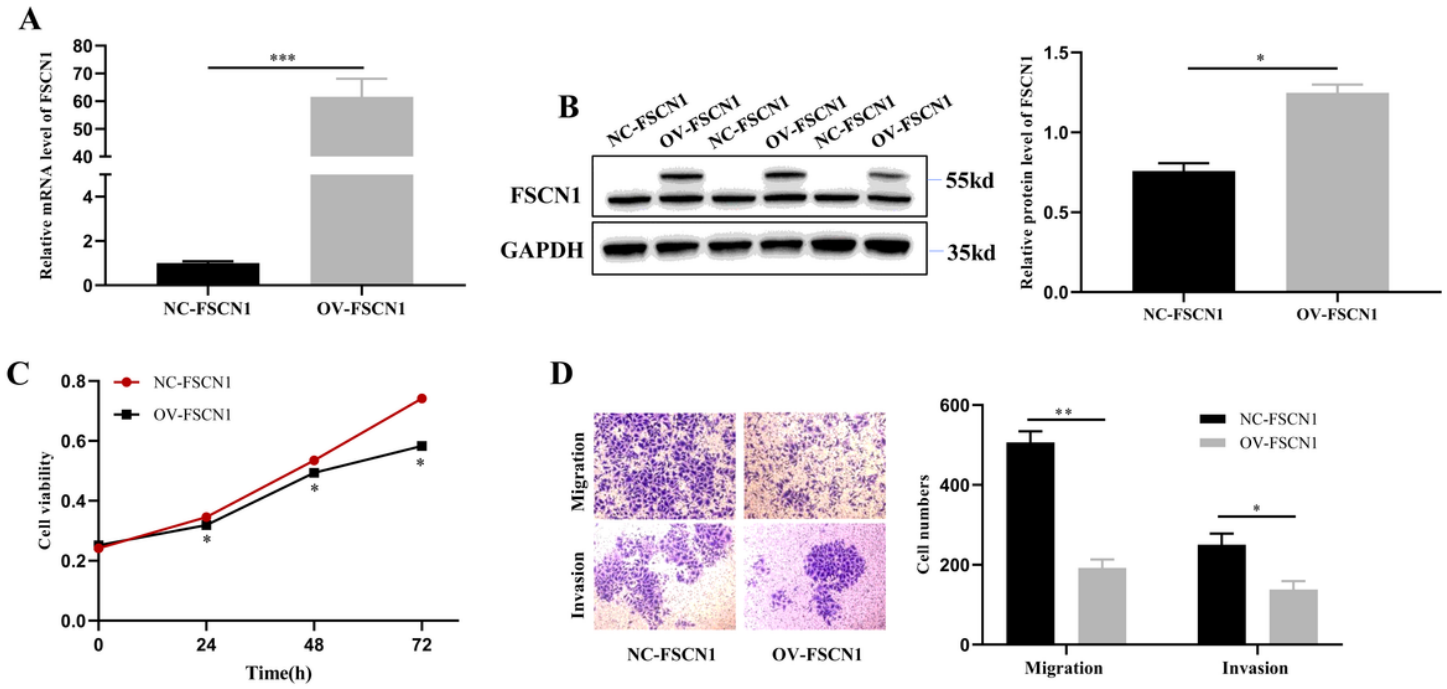

Figure 5

Effects of overexpression FSCN1 on the function of HTR-8 cells. **(A)**The transfection efficiency of FSCN1 in HTR-8/SVneo trophoblast cells transfected with overexpression plasmid. RNAs were extracted and analyzed by RT-qPCR. **(B)** The transfection efficiency of FSCN1 in HTR-8 cells transfected with overexpression plasmid. Proteins were extracted and analyzed by Western Blot. **(C)** The proliferation ability of cells after overexpression FSCN1 applied by CCK8. **(D)** The migration and invasion ability of cells after overexpression FSCN1 applied by transwell assay (magnification, 200×). Data presented as mean ± standard error of the mean. *P< 0.05；**P< 0.01；**P< 0.001.

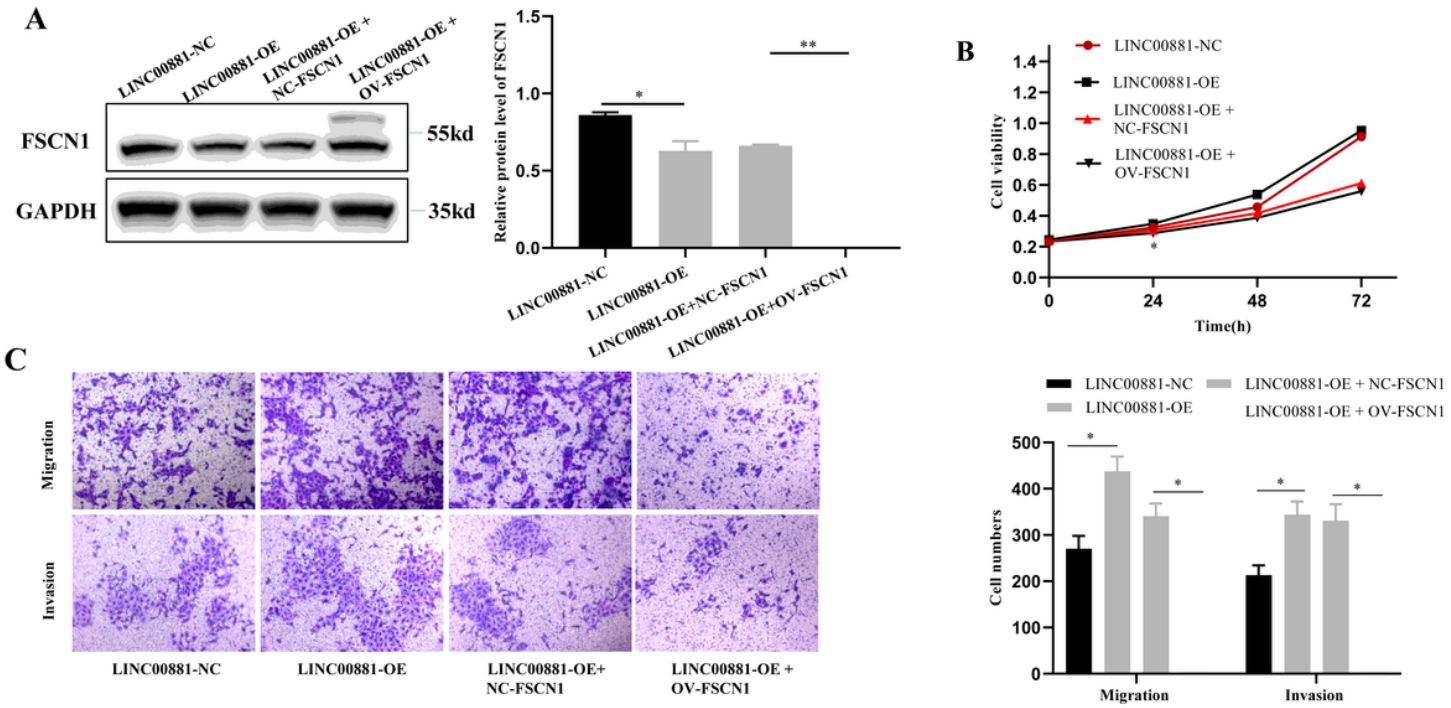

Figure 6

The effect of LINC00881 overexpression could be rescued by FSCN1 overexpression plasmid in HTR-8/SVneo trophoblast cells. HTR-8 cells were transfected with LINC00881-NC, LINC00881-OE, LINC00881-OE+ NC-FSCN1, LINC00881-OE+OV-FSCN1. **(A)** The overexpression of FSCN1 in the four groups was verifed by Western Blot. **(B)** The proliferation ability of cells in four groups. **(C)** The migration and invasion capacities by transwell assay in four groups (magnification, 200×). Data presented as mean ± standard error of the mean. *P< 0.05□**P< 0.01.

Supplementary Files

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