Silencing H19 regulated proliferation, invasion, and autophagy in the placenta by targeting miR-18a-5p

Lei Zhang | Xinru Deng | Xian Shi | Xiaojing Dong

Abstract
Fetal growth restriction (FGR) is a serious pregnancy complication associated with increased perinatal mortality and morbidity. It may lead to neurodevelopmental impairment and adulthood onset disorders. Recently, long noncoding RNAs (lncRNAs) were found to be associated with the pathogenesis of FGR. Here we report that the lncRNA H19 is significantly decreased in placentae from pregnancies with FGR. Downregulation of H19 leads to reduced proliferation and invasion of extravillous trophoblast cells. This is identified with reduced trophoblast invasion, which has been discovered in FGR. Autophagy is exaggerated in FGR. Downregulation of H19 promotes autophagy via the PI3K/AKT/mTOR and MAPK/ERK/mTOR pathways of extravillous trophoblast cells in FGR. We also found that the expression level of microRNAs miR-18a-5p was negatively correlated with that of H19. H19 can act as an endogenous sponge by directly binding to miR-18a-5p, which targets IRF2. The expression of miR-18a-5p was upregulated, but IRF2 expression was downregulated after the H19 knockdown. In conclusion, our study revealed that H19 downexpressed could inhibit proliferation and invasion, and promote autophagy by targeting miR-18a-5p in HTR8 and JEG3 cells. We propose that aberrant regulation of H19/miR-18a-5p-mediated regulatory pathway may contribute to the molecular mechanism of FGR. We indicated that H19 may be a potential predictive, diagnostic, and therapeutic modality for FGR.

KEYWORDS
autophagy, fetal growth restriction (FGR), H19, invasion, miR-18a-5p, proliferation

1 | INTRODUCTION
Fetal growth restriction (FGR) is defined as the fetal weight below the tenth percentile for gestational age or the birth weight less than 2500 g after 37 weeks of gestation. FGR increases the incidence of fetal distress and neonatal asphyxia/hypoglycemia and is the second leading cause of perinatal death. FGR is also a risk factor of adult hypertension, neurocognitive delay, and metabolic disorders. Furthermore, there is yet a lack of effective therapeutic strategies. Therefore, exploration of the underlying pathogenesis is urgently needed.

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 tumor growth, apoptosis, proliferation, invasion, and autophagy. The IncRNA H19 is transcribed from a maternally expressed imprinted gene that locates on the chromosome 11p15.5, and an upregulation of H19 has been detected in certain cancers (breast, stomach, and bladder). MiRNAs, which act as oncogenes or tumor suppressors, were found to be abnormally expressed in diverse cancers by degrading or repressing translations of the messenger RNA (mRNA) targets. It is known that IncRNAs sometimes function by acting as precursors of miRNAs. IncRNAs can directly bind with particular DNA or RNA strands to participate in the transcriptional and posttranscriptional gene regulation and can act as “miRNA sponge” to inhibit the activity of miRNA, thus affecting the expression of miRNA target genes. The interplay of miRNAs and IncRNAs was reported to exert an important regulatory role in tumors.

Previous data have revealed that H19 may play a part to activate autophagy. It is reported that autophagy is a conserved intracellular self-degrading by lysosome, which is the main degradation response to the stresses such as hypoxia and starvation. Autophagy is prosurvival or prodeath, depending on the types of stressors and cells. The autophagy process is mainly modulated by mTOR-dependent or mTOR-independent pathways, and the mTOR-dependent pathway is the most common route to activate autophagy. It is reported that autophagy is increased in FGR. The expression of autophagy markers, including LC3-II and Beclin-1, is upregulated in placenta from patients with FGR compared with normal pregnant women. Therefore the changes in cell viability, invasion, and autophagy are key to the pathophysiology of FGR.

In this study, the correlation between the H19 expression level and FGR and the underlying mechanisms were explored. The preliminary data demonstrated that an enhancement of autophagy due to the H19/miR-18a-5p pathway was involved in the pathogenesis of FGR. We found that H19 was significantly decreased in human placenta tissues with FGR compared with normal pregnancy. We demonstrate that downexpression of H19 may decrease trophoblast cell HTR8 and choriocarcinoma cell JEG3 proliferation and invasion. It also promotes autophagy by targeting miR-18a-5p. We hypothesized that dysregulation of this newly identified H19/miR-18a-5p-mediated regulatory pathway may contribute to the underlying mechanism of FGR.

2 | MATERIALS AND METHODS

2.1 | Tissue specimens

A total of 20 pairs of placenta tissues were sampled from the Second Affiliated Hospital of Chongqing Medical University. The tissue specimens were aliquoted and then stored at −80°C (for protein extraction) or in RNA storage fluid (for RNA extraction). The use of human tissues was ethically approved by the Institutional Review Board.

2.2 | Cell culture and transfection

Human trophoblast cells HTR8 and choriocarcinoma cells JEG3 (Beijing Beina Chuanglian Biotechnology Institute, Beijing, China) were cultured in RPMI-1640 medium (Corning Inc., New York state, NY) containing 12% fetal bovine serum (FBS; PAN, Bavaria, Germany) at 37°C and 5% CO₂ concentration. Small interfering RNAs (siRNAs) for H19 and negative control, and miR-18a-5p mimics and inhibitor were synthesized from GenePharma Co Ltd (Shanghai, China). The siRNAs, miR-18a-5p mimics, and inhibitors were transfected into HTR8 and JEG3 cells using a transfection kit from GenePharma Co Ltd (Shanghai, China). The sequences were H19 siRNA, 5′-CCCGUCCCUUCCUGAAUUUAT-TUAAAUUCAGAAGGGACGGGT-3′; miR-18a-5p mimics, 5′-UAAGGUCAUCAGUGCAGAUAUCUGCAUCAGAUCGCCACCUAAU-3′; or miR-18a-5p inhibitor, 5′-CUAUCUGACAUAGUCACCUUU-3′.

2.3 | Cellular invasion

A transwell invasion assay was performed using Boyden’s chamber. Cells were transfected and subsequently dissociated, and the transfected cells were resuspended in 200 μL serum-free MEM medium and placed in the upper chamber with an 8-μm pore, 6.5-mm polycarbonate filter (Corning Inc). The cells were planted in the upper chamber of Matrigel-coated (Corning Inc) with serum-free MEM medium and incubated for 24 hours. The insert was placed in a well with medium containing 12% FBS. The cells that did not migrate through the pores were removed by a wet cotton swab after 24 hours. The lower membrane cells were fixed in 4% paraformaldehyde for 30 minutes and stained with 0.5% crystal violet (Beyotime, Jiangsu, China). A total of five randomly selected fields were observed under a microscope, and the cell number was counted (magnification, ×200).
2.4 | 5-Ethynyl-2′-deoxyuridine-based proliferation assay

Cell proliferation was measured by the EdU DNA Cell Proliferation kit (C10310; Guangzhou RiboBio Co, Ltd, China).

2.5 | Cell viability assay

Cell viability was determined by a WST-8 assay (Hanbio Biotechnology Co Ltd, Shanghai, China). Cells were plated in a 96-well plate, and siRNA was transfected after 24 hours. The absorbance was measured with a multifunctional microplate reader (Thermo Fisher Scientific) at 450 nm at 24, 48, and 72 hours.

2.6 | RNA isolation and real-time polymerase chain reaction

Total RNA was extracted from cells and tissues using the High-purity Total RNA Rapid Extraction kit (RP1201; BioTeke Corporation, Beijing, China; CA). cDNA was synthesized using the All-in-One First-Strand cDNA Synthesis Kit (GeneCopoeia, Guangzhou, China) or the microRNAs qPCR Kit (SYBR Green method) performed using the All-Taq polymerase kit (GeneCopoeia). Gpoeia) or the microRNAs qPCR Kit (SYBR Green method) performed using the All-Taq polymerase kit (GeneCopoeia). The primers for H19, β-actin were 5′-CCACGGAGTCGCACACTATG-3′ and 5′-GAGCTGCTAGCACCATTCTTC-3′ for H19, and 5′-ACTAAAGGTCACTAGTCAGATA-3′ for miR-18a-5p. The real-time polymerase chain reaction (PCR) was performed using the All-in-One qPCR Mix Kit (GeneCopoeia) or the microRNAs qPCR Kit (SYBR Green method) kit (GeneCopoeia). Gpβ-actin or U6 was the reference (Bio-Rad, CA).

2.7 | Western blot analysis

Total protein was extracted using the cell lysis buffer, and the concentration was measured with the BCA assay (Beyotime). Proteins (40 μg) were separated with SDS-PAGE and then transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was blocked with 8% skim milk (Difco Laboratories, Detroit, MI) for 1 hour and incubated with primary rabbit antibody overnight at 4°C, including monoclonal anti-Pi3 Kinase Class III (D9A5; #4263; Cell Signaling Technology, MA), monoclonal anti-β-actin antibody (1:1000; #4970; Cell Signaling Technology), monoclonal anti-MAPK (1:1000; #4060; Cell Signaling Technology), monoclonal anti-ERK1 (1:1000; #4691; Cell Signaling Technology), monoclonal anti-p-ERK1 (1:1000; #4267; Cell Signaling Technology), monoclonal anti-MAPK (1:1000; #8480; Cell Signaling Technology), monoclonal anti-ERK1 (1:1000; #4695; Cell Signaling Technology), monoclonal anti-p-ERK1 (1/2:1000; #4730; Cell Signaling Technology), monoclonal anti-mTOR (7C10; 1:1000; #2983; Cell Signaling Technology), monoclonal anti-p-mTOR (Ser2448; 1:1000; #5536; Cell Signaling Technology), monoclonal rabbit anti-ULK1 antibody (D8H5; 1:1000; #8054; Cell Signaling Technology), monoclonal anti-Beclin1 (1:1000; #3495; Cell Signaling Technology), monoclonal anti-P62 (1:1000; #5114; Cell Signaling Technology), monoclonal anti-LC3 LC3A/B (D3U4C; 1:1000; #12741; Cell Signaling Technology), or monoclonal anti-β-actin antibody (1:1000; #4970; Cell Signaling Technology). The membranes were then washed with TBST (three times, 10 minutes/time), incubated with the HRP-conjugated secondary antibody at room temperature for 2 hours, and then washed with TBST (three times, 10 minutes/time). Proteins were visualized with the ECL system (Beyotime) using the ChemiDoc XRS system (Bio-Rad).

2.8 | Dual-luciferase reporter gene assay

For the H19 3′-untranslated region (3′-UTR) luciferase reporter assay, wild-type or mutant reporter constructs (termed wt or mut; Gene Pharma Co Ltd, Shanghai, China) and miR-18a-5p control or miR-18-5p mimics, were cotransfected into HTR8 and JEG3 cells using Lipofectamine 2000 (Invitrogen, CA). After 48 hours, luciferase activity was measured using the Dual Luciferase Assay System, with Renilla activity as the reference.

2.9 | Statistical analysis

All statistical analyses were performed using the software GraphPad6.0 (GraphPad Inc, San Diego, CA) and SPSS (version 24.0; Chicago, IL). The Student t test or analysis of variance was used to determine statistical significance. The statistical significance was set at P < 0.05.

3 | RESULTS

3.1 | IncRNA H19 expression level was downregulated in FGR placenta tissues

The placenta tissues of normal and FGR pregnancy was sampled. The PCR demonstrated a lower level of H19 in FGR placenta in comparison with the control placenta (Figure 1; P < 0.0001). The mRNA level of β-actin in each sample was also quantified as an internal standard and used to normalize the levels of H19 from the same sample.

3.2 | Downregulation of H19 suppressed cell proliferation and invasion

Human trophoblast cells HTR8 and choriocarcinoma cells JEG3 were used for silencing trials. PCR showed that the expression level of H19 was decreased in HTR8 and JEG3 cells after siRNA transfection (Figure 2A; P = 0.0006; Figure 2C; P = 0.0001). Cell Counting Kit-8 (CCK-8) and
5-ethynyl-2′-deoxyuridine assays indicated that the percentage of proliferative cells was decreased in H19-silenced cells (Figure 2B; \( P = 0.0090 \); Figure 2E and 2F; \( P = 0.0108 \); Figure 2D; \( P = 0.0474 \); Figure 2I and 2J; \( P = 0.0040 \)). Silencing H19 inhibited the cellular invasion (Figure 2G and 2H; \( P = 0.0161 \); Figure 2K and 2L; \( P = 0.0048 \)).

3.3 H19 regulated autophagy via the PI3K/AKT-mTOR and MAPK/ERK-mTOR pathways

The autophagy biomarker LC3 was determined by Western blot. Knockdown of H19 resulted in an increase in the ratio of LC3-II/LC3-I, indicating enhancement of autophagy (Figure 3A-D).

To explore the mechanisms of H19 in regulating autophagy, t-AKT, p-AKT, PI3K, MAPK, t-ERK1/2, p-ERK1/2, t-mTOR, p-mTOR, ULK1, Beclin1, and P62 were detected. After silencing H19, the level of p-AKT, PI3K, MAPK, p-ERK1/2, p-mTOR, and P62 was decreased, and the...
The level of ULK1 and Beclin1 was increased; however, the level of t-AKT, t-ERK1/2 and t-mTOR was not altered.

3.4 | H19 regulated IRF2 through miR-18a-5p

IncRNAs can function as competing endogenous RNAs (ceRNAs) to sponge miRNA. Analyses utilizing the online target sequence prediction database (http://starbase.sysu.edu.cn/) suggested that miR-18a-5p may have potential binding sites to H19, and therefore the expression level of miR-18a-5p mRNA in placenta tissues was determined by qRT-PCR. The level of miR-18a-5p mRNA in FGR placenta tissues was increased, but that of H19 was decreased, compared with control (Figure 4A). The miR-18a-5p level negatively correlated with the H19 level (Figure 4B). miR-18a-5p expression was promoted after H19 downregulation (Figure 4C and 4F). H19 expression was inhibited after transfection with miR-18a-5p mimics (Figure 4D and 4G) and was increased after transfected with the miR-18a-5p inhibitor (Figure 4E and 4H).

To verify whether miR-18a-5p was targeted and directly bound to IncRNA-H19, a luciferase reporter vector containing the predicted wild-type or mutated miR-18a-5p binding sites in H19 was constructed (Figure 4K). As shown in HTR8 (Figure 4I) and JEG3 (Figure 4J), the overexpression of miR-18a-5p remarkably reduced the luciferase intensity in the H19-3′-UTR wild-type. In addition, the luciferase reporter system revealed that miR-18a-5p overexpression suppressed the luciferase activity of pGL3-IRF2-3′-UTR-WT (Figure 4N) in HTR8 (Figure 4L) and JEG3 (Figure 4M). These data indicated that miR-18a-5p suppressed H19 expression by directly binding to its 3′-UTR and that IRF2 was a target of miR-18a-5p.
3.5 | IncRNA H19/miR-18a-5p impacted on cell proliferation and invasion

The effects of IncRNA-H19/miR-18a-5p on cell proliferation and invasion were explored. The cells were transfected with si-H19 or were cotransfected with si-H19 and miR-18a-5p inhibitors. Downexpression of H19 decreased the ability of proliferation and invasion in HTR8 and JEG3 cells. This effect was reversed when cotransfecting si-H19 and miR-18a-5p inhibitor in HTR8 (Figure 5A-C, 5G, and 5H) and JEG3 (Figure 5D-F, 5I, and 5J). Cotransfection also reversed the enhancement of autophagy attributable to knockdown of H19 (Figure 6A and 6B) and JEG3 (Figure 6D and 6E). miR-18a-5p inhibitor increased the level of IRF2 (Figure 6C and 6F). However, the modulating effects of H19 on IRF2 were diminished after cotransfection with the miR-18a-5p inhibitor.
DISCUSSION

In this study, we observed a decreased expression of H19 in the placenta of FGR. We also found that silencing H19 inhibited proliferation and invasion and promoted autophagy of extravillous trophoblast cells by targeting miR-18a-5p. PI3K/AKT/mTOR and MAPK/ERK/mTOR pathways were regulated by H19. Our results indicated that H19 was a potential treatment target for FGR.

Previous studies have reported that H19 was downregulated in papillary thyroid carcinoma compared with adjacent paracancerous tissues. Inhibition of H19 expression can promote the proliferation, migration, and tumor growth. The expression level of H19 was notably higher in tongue squamous cell carcinoma. H19 promotes tongue squamous cell carcinoma invasion through β-catenin/GSK3β/EMT signaling via association with EZH2. Accumulating evidence highlighted that aberrant expression of H19 was closely related to embryogenesis, fetal growth, and tumorigenesis. Proper development of human placenta is essential for the maintenance of normal pregnancy. In this study, the biological function of H19 in HTR8 and JEG3 cells was explored. Downregulation of H19 reduced the cellular proliferation and invasion. Proliferation of trophoblast cells was involved in the placental development, and the invasion of trophoblast cells into the uterine spiral artery was the determining process for the formation of uteroplacental circulation. Inadequate invasion and low viability of trophoblast cells may be a reason of FGR. These results indicated that the downexpression of H19 was associated with FGR. Recently, a growing number of research studies have suggested that the interaction between lncRNA H19 and miRNAs played an effective role in the gene regulation and was involved in various biological processes. lncRNAs can act as ceRNA sponges for miRNAs to regulate the downstream genes to regulate physiological and pathological processes. In human placental trophoblast cell, H19 can produce miR-675 that targets Nodal Modulator 1, and trophoblast cell proliferation was decreased after downexpression of H19. In ovarian cancer cell lines, SKOV-3 and OVCAR3 cells, H19 could directly bind to miR-370-3p and effectively act as its ceRNA. H19 overexpression or a
miR-370-3p knockdown promoted TGF-β-induced EMT. In glioma cells U251 and U87, H19 promotes proliferation and invasion by downregulating miR-152. H19 gene may participate in placenta development by a posttranscriptional mechanism. In our study, the data showed that the expression of miR-18a-5p was negatively correlated with the level of H19 in FGR placenta tissues. Downexpression of H19 increased the activity of miR-18a-5p, followed by a decrease of the downstream target IRF2. Luciferase activity assay indicated that H19 and miR-18a-5p directly regulated each other. H19 served as a molecular sponge for miR-18a-5p to regulate its target IRF2. The suppression in proliferation and invasion in HTR8 and JEG3 cells due to H19 downexpression was reversed when transfected with the miR-18a-5p inhibitor. Moreover, downregulation of H19 suppressed cell proliferation and invasion by negatively regulating miR-18a-5p expression. This study provided important clues to understand the interaction between H19 and miR-18a-5p in FGR.

Autophagy has been generally reported to play a key role in development, aging, neurological disorders, liver and muscle disorders, infection, immunity, and cancer. The Basal level of autophagy is responsible for the maintaining of cellular homeostasis; however, excessive autophagy may induce autophagic cell death. IncRNA is known to regulate autophagy through diverse mechanisms. In this regard, IncRNA-H19 on autophagy in trophoblast cells may affect the progression of FGR. Previous data demonstrated that autophagy was enhanced in FGR placenta. In the placenta of FGR, autophagic vacuoles containing lysosomal enzymes and semi-digested cell organelles were more prominent, indicating that placental autophagy might be associated with pathophysiology of FGR. Beclin-1 is a major component for autophagic initiation and participates in the formation of autophagosomes. P62 is the best-known autophagic substrate, which binds to ubiquitinated proteins.

**FIGURE 6** IncRNA H19/miR-18a-5p regulate IRF2 and autophagy. A,B,D,E, In downexpressed H19 group, the expression of IRF2 and the ratio of LC3-II/LC3-I were significantly increased in protein level. Whereas the expression of IRF2 and the ratio of LC3-II/LC3-I were reversed in the cotransfecting group. C,F, The expression of IRF2 in mRNA level were measured by qRT-PCR. *P < 0.05; **P < 0.01; ***P < 0.001. IncRNA, long noncoding RNA; mRNA, messenger RNA.
and is consumed during autophagosomal degradation.\textsuperscript{39,40} ULK1 is the downstream of mTOR and upstream of Beclin1.\textsuperscript{41} Autophagosome formation is involved in the conversion of the soluble form of LC3-I to the autophagic vesicle-associated form LC3-II.\textsuperscript{42} Thus, the increased ratio of LC3B-II/LC3B-I and expression of Beclin-1, as well as decreased p62 expression after H19 downexpressed, proved that silencing H19 promoted autophagy. There are several signal pathways underlying the regulation of cell autophagy including the PI3K/Akt/mTOR, Bcl2/beclin1, MAPK/Erk1/2, and AMPK signaling pathways.\textsuperscript{43,44} The PI3K, Akt, MAPK, and Erk pathways may regulate autophagy.\textsuperscript{45,46} The PI3K/AKT and mTOR pathways are essential signaling cascades that are involved in cell proliferation.\textsuperscript{47} The trophoblast invasion is decreased as the phosphorylation of mTOR is prevented.\textsuperscript{48} The ERK/MAPK pathway is an evolutionarily conserved signaling pathway that responds to signals from cell surface receptors to promote cell growth, proliferation, and survival.\textsuperscript{49} In our study, the enhancement of autophagy was observed after silencing H19. H19 downexpression promoted autophagy via the PI3K/AKT/mTOR and MAPK/ERK/mTOR pathways. Cotransfection with miR-18a-5p inhibitor reverses the enhancement of autophagy, which is attributable to knockdown of H19. The more validated the autophagy is in association with FGR may unveil novel strategies for disease therapy.

In conclusion, H19 was downregulated in FGR placenta. H19 regulated cellular proliferation, invasion, and autophagy through targeting miR-18a-5p. H19 served as a molecular sponge for miR-18a-5p to regulate IRF2. An aberrant H19/miR-18a-5p inhibitor reverses the enhancement of autophagy in villous trophoblast from normal term pregnancies and FA and identifying autophagy is in association with FGR may unveil novel strategies for disease therapy.

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ORCID

Xiaojing Dong \( \text{http://orcid.org/0000-0002-0358-0042} \)

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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