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lncRNA deleted in lymphocytic leukaemia 1 (DLEU1) promotes the migration and invasion of human embryonic trophoblast cells

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Summary

To investigate the roles of lncRNA deleted in lymphocytic leukaemia 1 (DLEU1) on migration and invasion of human trophoblast cells. Human chorionic trophoblast cell line HTR8/SVneo was cultured and transfected using lncRNA DLEU1 small interfering RNA. Real-time quantitative polymerase chain reaction was used to detect lncRNA DLEU1 expression. The activity of migration regulatory protein CDC42 was detected by western blot. The downstream miRNA targets of lncRNA and mRNAs targeted by corresponding miRNAs were respectively predicted using bioinformatics analyses. Compared with the control group, the expression of lncRNA DLEU1 in the small interfering RNA group was significantly decreased ($P < 0.05$). There was no significant change in cell proliferation capacity for transfected cells (lncRNA DLEU1 siRNA-1, $P = 0.537$; lncRNA DLEU1 siRNA-2, $P = 0.384$), but cell migration (lncRNA DLEU1 siRNA-1, $P = 0.025$; lncRNA DLEU1 siRNA-2, $P = 0.019$) and invasion (lncRNA DLEU1 siRNA-1, $P = 0.0327$; lncRNA DLEU1 siRNA-2, $P = 0.021$) was significantly reduced. CDC42 activity in the lncRNA DLEU1 knockdown group decreased and the phosphorylation of cofilin increased. Therefore, downregulation of lncRNA DLEU1 suppressed the migration and invasion of human trophoblast cells.

Introduction

Embryo implantation into uterine endometrium is a requisite process for the establishment of successful pregnancy, which involves trophoblast cell adhesion to the endometrium (Godbole *et al.*, 2017; Ashary *et al.*, 2018). The dysregulated migration and invasiveness of trophoblastic cells will affect the capacity for embryo implantation, leading to the loss of a pregnancy. Estimates predict that approximately two-thirds of pregnancy failure is caused by aberrant trophoblast cell migration (Labarrere *et al.*, 2017). Therefore, it is imperative to explore potential molecular mechanisms of migration and invasiveness in human trophoblast cells.

Long non-coding RNA (lncRNA) with a length >200 nucleotides is a subclass of RNA transcripts that do not code for proteins but regulate gene expression (Munschauer *et al.*, 2018). Many studies have revealed that lncRNA is involved in numerous pathological and physiological processes of various diseases (Bubenik and Swanson, 2018; Ritter *et al.*, 2019). Moreover, existing studies have indicated that lncRNA plays important roles in the migratory and invasive processes of cancer cells. For example, Wang and colleagues argued that lncRNA UCA1 increased the proliferation and migration of gastric cancer cells, but restrained cell apoptosis by sponging multiple miRNA targets (Wang *et al.*, 2019b). Zhao and colleagues found that lncRNA PVT1 sponged miR-448 to modulate SERBP1 expression, therefore enhancing the proliferative and migratory ability of pancreatic cancer cells (Zhao *et al.*, 2018). Many reports have shown that lncRNA deleted in lymphocytic leukaemia 1 (DLEU1) was associated with the metastatic process of multiple participants in multiple reproduction-related tumours. Liu and colleagues found that downregulation of lncRNA DLEU1 dramatically restrained the migratory and invasive ability of colorectal cancer cells *in vitro* and *in vivo* (Liu T *et al.*, 2018). Wang and colleagues investigated the roles of lncRNA DLEU1 in breast cancer (BC) cell metastasis and observed that lncRNA DLEU1 was upregulated in BC and that its overexpression increased the migration and invasion of BC cells through sponging miRNA-300 (Wang *et al.*, 2019a). However, the possible mechanisms of lncRNA DLEU1 in human trophoblast cell migration and invasion GC development have not been understood.

In this study, we constructed an HTR8/SVneo cell system with downregulation of lncRNA DLEU1 and investigated the underlying roles of lncRNA DLEU1 in the migration and invasion of trophoblastic cells; this approach will offer new insights into understanding invasion of human placenta.

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Materials and methods

Reagents

The RevertAid First Strand cDNA synthesis kit and quantitative real-time polymerase chain reaction (qPCR) kit were purchased from Unibiotest Co., Ltd (Wuhan, China). Cell division cycle 42 (*CDC42*) Activation Assay kit was purchased from New East Biosciences Inc. (Malvern, USA). Lipofectamine 2000 Transfection Reagent, TRIzol reagent, Dulbecco's modified Eagle's medium (DMEM) and trypsin were all obtained from ThermoFisher Scientific Inc. (Rockford, IL, USA).

Cell transfection and RNA extraction

HTR8/SVneo cells were cultured in a six-well plate. Lipofectamine 2000 reagent and lncRNA DLEU1 small interfering RNA (siRNA) were mixed at a ratio of 5:1. After incubation for 30 min, the mixture was added into adherent HTR8/SVneo cells, which were incubated for 6 h. Subsequently, the initial culture medium was replaced with fresh medium and the plate was put in an incubator containing 0.5% CO_2 in air at 35°C for 48 h. Total RNA was isolated from transfected HTR8/SVneo cells using the TRIzol RNA Extraction kit (Thermo, USA) following standard instructions from the manufacturer and then RNA concentration was measured. Finally, RNA templates were inversely transcribed to cDNA and directly stored at −80°C for following analyses. Sequences for lncRNA DLEU1 siRNA-1 and lncRNA DLEU1 siRNA-2, respectively, are 5′-CACUUAAGCCUCGGAACAA-3′ and 5′-CUGGUAGCUAUAAGACGAC-3′.

qPCR assay

First, a cDNA library was constructed using RNAs extracted from HTR8/SVneo cells with or without lncRNA DLEU1 siRNA transfection. Then, lncRNA DLEU1 expression levels were assessed using a qPCR kit (Uni Biotest, Wuhan, China) according to the standard protocol and the RNA bands were imaged using a qPCR system (Bio-Rad, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and U6 acted as the internal references for the expression levels of lncRNA DLEU1. Finally, relative level of lncRNA DLEU1 was computed using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay

Transfected HTR8/SVneo cells were plated onto a 96-well plate at a density of 2×10^5 cells/well after transfection for 48 h and then cultured for 24 h. Next, the supernatant was removed and reagents from the Cell Counting Kit 8 (CCK8; Macklin, Shanghai, China) were added to the wells in line with the standard procedures from the manufacturer. Then, cells were incubated for 10–30 min and a Microplate Reader iMark (Bio-Rad, USA) was utilized to determine absorbance at 450 nm.

Transwell assay

For the transwell analysis, transfected HTR8/SVneo cells were collected after transfection for 48 h, cultured and suspended. Then, the HTR8/SVneo cell suspension (2 ml) was added into the upper transwell chambers and the cell culture medium was added into the lower wells. In addition, cell invasion was also assessed. Notably, cell culture medium and Matrigel were mixed at a ratio of 1:1 and added to the upper transwell chambers. The cell suspension and medium were respectively added to the upper and lower chambers as described above. After this step, the cells were cultured for 48 h and stained using 0.1% crystal violet. Six fields were chosen at random and the cells were photographed and counted.

Western blotting

After cell transfection, activated *CDC42* was isolated using the CDC42 activation assay kit. Briefly, cells were first lysed. Total proteins were obtained and incubated with GST-PBD-coupled agarose magnetic beads. After several washes, the separated proteins were run on a gel and detected by western blotting. Specifically, protein samples were separated using a 10% SDS-PAGE gel (Macklin, Shanghai, China) and then transferred onto polyvinylidene fluoride (PVDF) membranes, which were subsequently blocked by soaking in 5% skimmed milk. Primary antibodies anti-cofilin and anti-Cdc42 were purchased from Santa Cruz Biotechnology. Primary antibodies and horseradish peroxide (HRP)-conjugated second antibody were added and incubated. Target proteins were detected using an electrochemiluminescence kit and the depth of grey intensity of the bands was assessed using ImageJ software.

Prediction analysis of lncRNA DLEU1targets

The sequence of lncRNA DLEU1 was retrieved from the PubMed search engine; potential miRNAs targets of lncRNA DLEU1 were predicted using the miRDB database. Next, miRNAs targets with score >90 were retained and used for downstream target prediction based on the TargetScan online tool.

Statistical analyses

Data were processed using SPSS 20.0 statistic software (IBM, Chicago, IL, USA). and expressed as the mean ± standard deviation. Student's *t*-test and one-way analysis of variance were used respectively for comparison analyses between two groups and among multiple groups. Tukey's test was used for the ex post analysis. A P-value < 0.05 indicated statistical significance.

Results

Knockdown of lncRNA DLEU1 in HTR8/SVneo cells

To investigate the potential influences of lncRNA DLEU1 on trophoblast cell behaviour, we used siRNA technology to silence lncRNA DLEU1 expression. After HTR8/SVneo cells were transfected by lncRNA DLEU1 siRNA, lncRNA DLEU1 levels were evaluated using RT-qPCR. As displayed in Fig. 1, there was an obvious decrease in lncRNA DLEU1 expression in transfected cells compared with the control group (lncRNA DLEU1 siRNA-1, $P = 0.0375$; lncRNA DLEU1 siRNA-2, $P = 0.0368$).

Knockdown of lncRNA DLEU1 did not affect the cell proliferation but decreased the migratory and invasive ability

After transfection for 48 h using lncRNA DLEU1 siRNA, we found that the relative activity of HTR8/SVneo cells did not change (lncRNA DLEU1 siRNA-1, $P = 0.620$; lncRNA DLEU1 siRNA-2, $P = 0.227$; Fig. 2), which suggested that downregulation of lncRNA DLEU1 siRNA did not affect the proliferative ability of HTR8/SVneo. In addition, transwell analysis revealed that knockdown of lncRNA DLEU1 dramatically lowered the cell migratory ability

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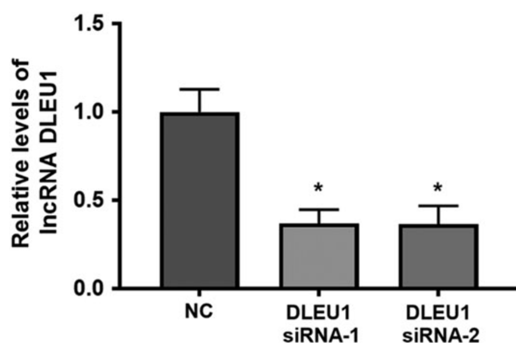


Figure 1. Relative expression of lncRNA DLEU1 in HTR8/SVneo cells transfected with lncRNA DLEU1 siRNA. Human chorionic trophoblast cell line HTR8/SVneo was transfected by lncRNA DLEU1 siRNA. Real-time quantitative polymerase chain reaction was used to detect lncRNA DLEU1 expression. siRNA: small interfering RNA. $^{*}P < 0.05$.

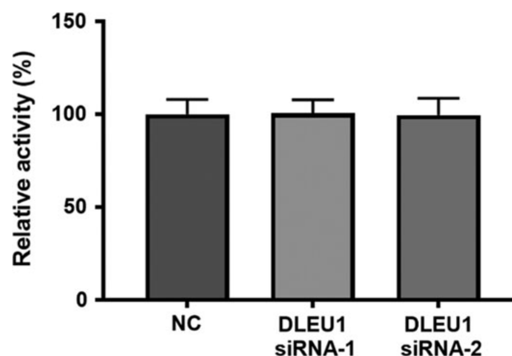
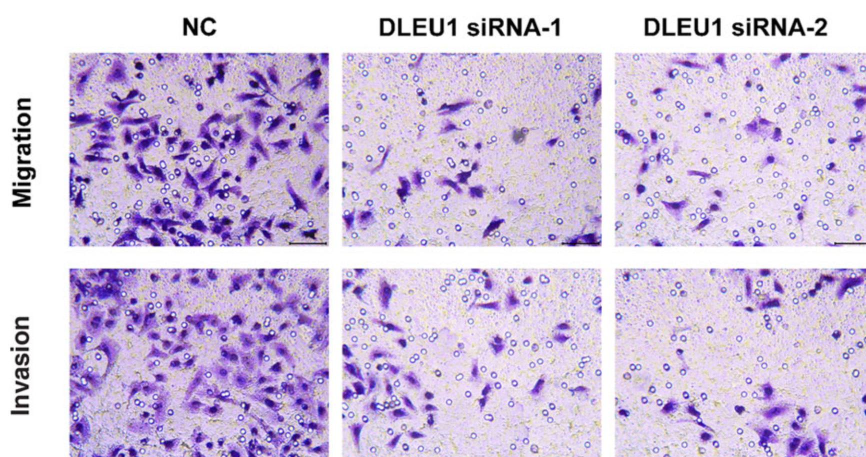


Figure 2. Relative activity of HTR8/SVneo cells. Human chorionic trophoblast cell line HTR8/SVneo was transfected by lncRNA DLEU1 siRNA and Cell Counting Kit 8 assay was performed to assess the cell proliferative ability after transfection for 48 h. siRNA: small interfering RNA.



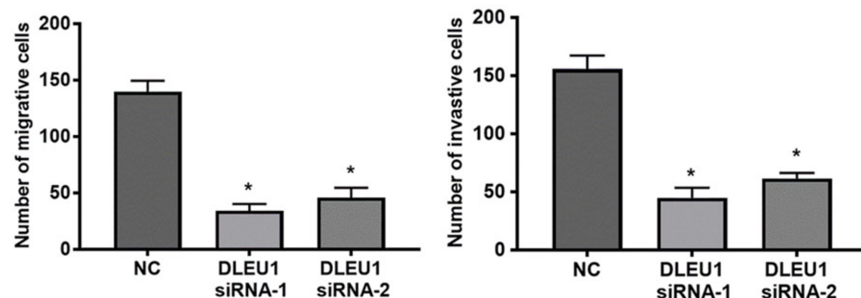


Figure 3. Downregulation of lncRNA DLEU1 inhibits the migration and invasion of HTR8/SVneo cells. Human chorionic trophoblast cell line HTR8/SVneo was transfected by lncRNA DLEU1 siRNA and Transwell assay was conducted to evaluate the cell migratory and invasive ability after transfection for 48 h. siRNA: small interfering RNA. $^{*}P < 0.05$.

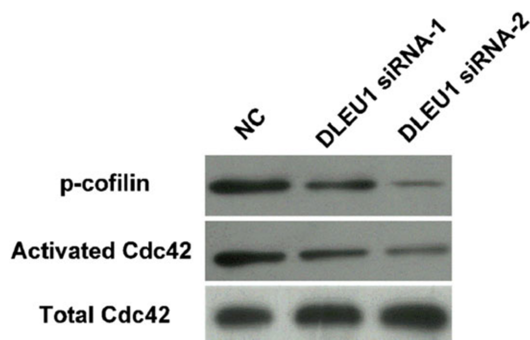


Figure 4. Expression of CDC42 and cofilin were detected using western blotting. Human chorionic trophoblast cell line HTR8/SVneo was transfected by lncRNA DLEU1 siRNA and western blotting assay was performed to evaluate the expression of CDC42 and cofilin. siRNA: small interfering RNA.

(lncRNA DLEU1 siRNA-1, $P = 0.0217$; lncRNA DLEU1 siRNA-2, $P = 0.0151$; Fig. 3). Similarly, the number of invasive cells was also decreased markedly in transfected HTR8/SVneo cells by lncRNA DLEU1 siRNA-1 compared with the controls (lncRNA DLEU1 siRNA-1, $P = 0.0315$; lncRNA DLEU1 siRNA-2, $P = 0.0402$; Fig. 3).

Knockdown of lncRNA DLEU1 inhibits the activity of CDC42

Our findings demonstrated that downregulation of lncRNA DLEU1 markedly suppressed CDC42 activity according to western blotting analysis (Fig. 4). Interestingly, the activity of cofilin, a downstream protein of CDC42, was dramatically enhanced (Fig. 4).

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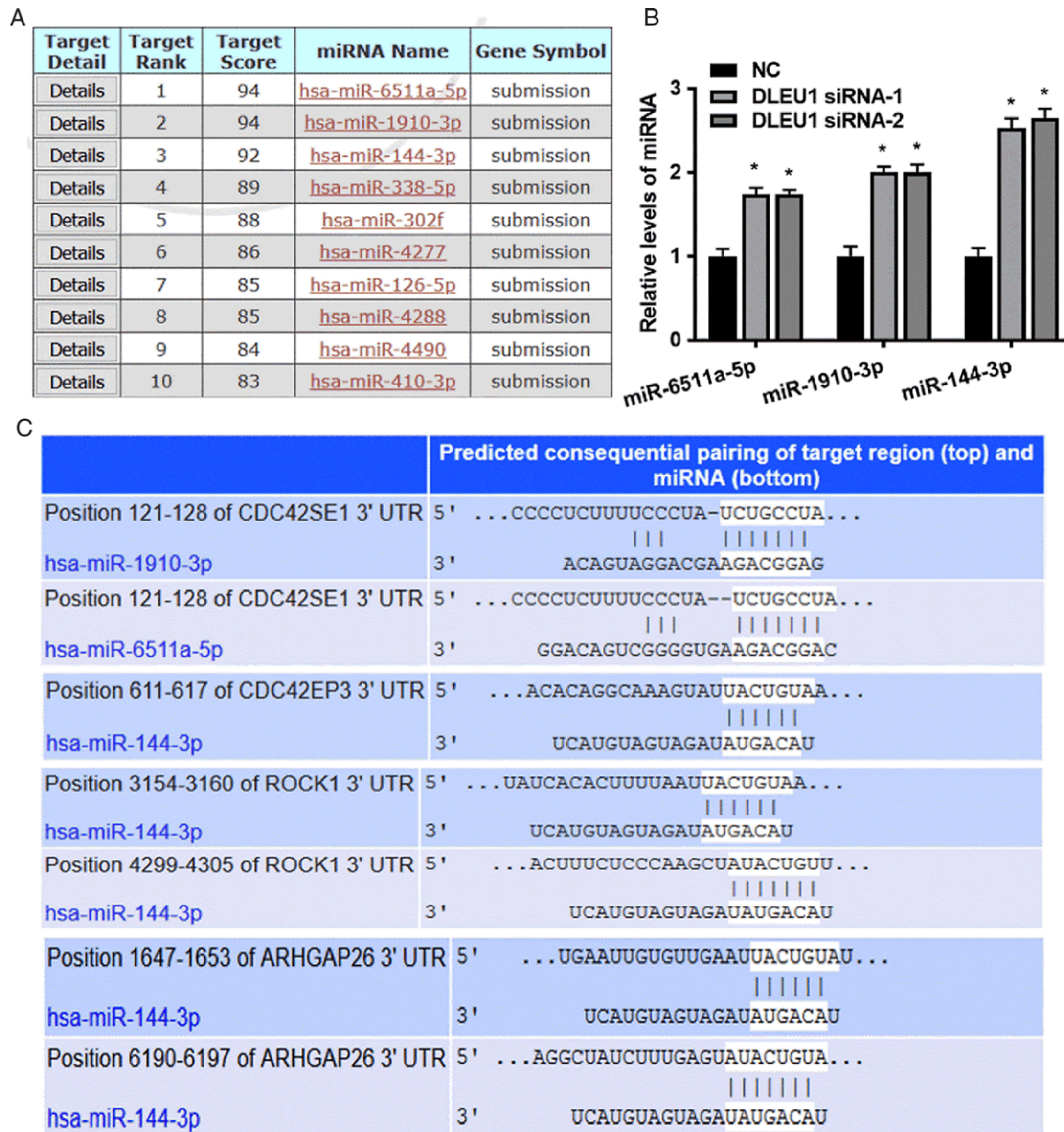

Figure 5. Predictive analyses of miRNAs targets of lncRNA DLEU1 and corresponding mRNA targets prediction. (*A*) Prediction of miRNAs targeted by lncRNA DLEU1 using miRDB database. (*B*) Expression levels of miRNA-6511a-5p, miRNA-1910-3p and miRNA-144-3p in HTR8/SVneo transfected with lncRNA DLEU1 siRNA were detected by real-time quantitative polymerase chain reaction. (*C*) Prediction of mRNAs targeted by miRNA targets of lncRNA DLEU1 using TargetScan online tool. siRNA: small interfering RNA. $^{*}P < 0.05$.

LncRNA DLEU1 interacts with miRNA-6511a-5p, miRNA-1910–3p and miRNA-144–3p

To further explore underlying mechanisms of lncRNA DLEU1 on trophoblast cellular processes, the miRNAs targeted by lncRNA DLEU1 were predicted by bioinformatics analysis based on the miRDB database. Our results suggested that there were close associations between lncRNA DLEU1 and 106 miRNAs. Of these, miRNA-6511a-5p, miRNA-1910–3p and miRNA-144–3p were the top three miRNAs with a score value of > 90 (Fig. 5A). Accordingly, we examined the expression levels of these three miRNAs in transfected HTR8/SVneo cells and discovered that underexpression of lncRNA DLEU1 could clearly elevate their levels (Fig. 5B). Furthermore, we utilized the TargetScan tool to predict underlying mRNAs targets of these three miRNAs. The findings revealed that miRNA-6511a-5p and miRNA-1910-3p strongly interacted with *CDC42SE1*, while miRNA-144–3p was associated preferentially with multiple cytoskeletal protein genes, including *ROCK1*, *CDC42EP3* and *ARHGAP26* (Fig. 5C).

Discussion

Existing studies have suggested that the leading cause of early pregnancy failure was abnormal migration and invasion of placental trophoblastic cells (Graham *et al.*, 1993). Therefore, there is a pressing need to identify novel and promising molecular targets associated with trophoblast dysfunction and which will contribute largely to diagnosis and treatment of pregnancy-associated diseases. Markedly, a growing body of evidence has demonstrated that lncRNAs exert pivotal regulatory roles in cell migration and invasion (Tang *et al.*, 2019; Xu *et al.*, 2019). Numerous researchers have concentrated on elaborating the pathogenetic mechanisms of lncRNAs in tumour progression (Ma *et al.*, 2019; Hosseini *et al.*, 2017;

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Esposito *et al.*, 2019). However, potential influences of lncRNA on the migration and invasiveness of trophoblastic cells have not been clarified completely. Here, this study explores underlying regulatory roles of lncRNA DLEU1 on trophoblastic cell behaviour such as proliferation, invasion and migration.

The siRNA technique was utilized to knockdown lncRNA DLEU1 expression in trophoblasts HTR8/SVneo. RT-qPCR assay confirmed the downregulation of lncRNA DLEU1 in transfected HTR8/SVneo cells. Moreover, we discovered that lncRNA DLEU1 silencing did not influence cell proliferation but dramatically suppressed the migratory and invasive ability of HTR8/SVneo cells, suggesting that lncRNA DLEU1 may play critical roles in placentation. Similarly, Gao and colleagues reported that underexpression of lncRNA DLEU1 could inhibit migration and invasion of pancreatic ductal adenocarcinoma cells (Gao *et al.*, 2019). Accumulating evidence has indicated that cell migratory and invasive processes are strongly correlated with significant cytoskeleton remodelling (Fife *et al.*, 2014). CDC42 is a key regulatory protein for the dynamic structures of the cytoskeleton (Vestre *et al.*, 2019). Several recent studies have highlighted that CDC42 is implicated with trophoblast cell migration (Nicola *et al.*, 2008; Liu Y *et al.*, 2018; Fritz *et al.*, 2019). We hypothesized that lncRNA DLEU1 functioned probably by regulating CDC42. The CDC42 activation assay implied that lncRNA DLEU1 knockdown clearly reduced CDC42 activity and the phosphorylation of cofilin, a downstream protein of CDC42. Other research indicated that cofilin was related to cell pseudopodia growth and that its phosphorylated form was inactivated, which could not cleave actin filaments and thereby promoted the growth of pseudopodia (Frotscher *et al.*, 2017). We inferred that lncRNA DLEU1 participated in regulating migration and invasiveness of HTR8/SVneo cells, possibly via the CDC42–cofilin axis.

To further illuminate the regulatory mechanisms of cell migration and invasion with involvement of lncRNA DLEU1, the corresponding miRNAs targets were predicted based on the miRDB database. We noted that three were from the miRNA 106 family targeted by lncRNA DLEU1. Moreover, hsa-miR-6511a-5p, hsa-miR-1910-3p and hsa-miR-144-3p all showed strong correlation with lncRNA DLEU1. RT-qPCR results revealed that downregulation of lncRNA DLEU1 dramatically enhanced expression levels of these three miRNA targets. Interestingly, hsa-miR-6511a-5p and hsa-miR-1910-3p all closely interacted with CDC42SE1. Jin and colleagues pointed out that CDC42SE1 not only targeted CDC42, but also suppressed CDC42 expression, and that the miR-202-5p/CDC42SE1 axis could regulate primordial germ cell migration by protecting CDC42 expression of CDC42 (Jin *et al.*, 2019). Additionally, hsa-miR-144–3p was linked with three genes (*ROCK1*, *CDC42EP3* and *ARHGAP26*). Numerous reports have demonstrated that these genes have key regulatory roles in migration and metastasis of cancer cells. For example, Wang and colleagues argued that miR-144–3p/ROCK1/ROCK2 could mediate proliferative reversal and metastatic processes of osteosarcoma (Wang *et al.*, 2017). More notably, *ARHGAP26* is a key regulatory factor for the Rho family, and can convert small G protein RhoA into inactive forms (Wang *et al.*, 2013). *ROCK1* is reported to be a significant downstream target for RhoA (Yang *et al.*, 2017). Jiang and colleagues emphasized that the *RhoA*/*ROCK1*/*ARHGAP26* axis participates in invasiveness of ectopic endometrium (Jiang *et al.*, 2018). Farrugia and colleagues suggested that CDC42 could modulate CDC42EP3 and was involved in the regulation of cytoskeletal dynamics (Farrugia and Calvo, 2017). These findings implied that lncRNA DLEU1 regulated the migration and invasiveness of trophoblast cells by a complicated competing endogenous RNA regulatory network. However, addition experiments in follow-up studies need to be performed to confirm our results.

In conclusion, our results suggested that downregulation of lncRNA DLEU1 expression did not change HTR8/SVneo cell activity, but dramatically decreased migratory and invasive ability. Moreover, bioinformatics analysis suggested that lncRNA DLEU1 strongly interacted with hsa-miR-6511a-5p, hsa-miR-1910-3p and hsa-miR-144-3p. In addition, there were close interactions between two miRNAs (hsa-miR-6511a-5p and hsa-miR-1910-3p) and CDC42SE1, while hsa-miR-144-3p was associated with three genes (*ROCK1*, *CDC42EP3* and *ARHGAP26*). However, detailed mechanisms of these downstream effectors involving migration and invasiveness of trophoblast cells need to be investigated in subsequent analyses.

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Conflict of Interest. All authors declare that they have no conflict of interests in the article.

Ethical Standards. Our study complies with ethical standards on the use of stem cells.

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