Research paper

The enhancer RNA Inc-SLC4A1-1 epigenetically regulates unexplained recurrent pregnancy loss (URPL) by activating CXCL8 and NF-κB pathway

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Background: Enhancer RNAs (eRNAs) are a group of IncRNAs transcribed from enhancers, whose regulatory effects on gene expression are an emerging area of interest. However, the role of eRNAs in regulating trophoblast cells and unexplained recurrent pregnancy loss (URPL) remains elusive.

Methods: We profiled eRNAs in villi from URPL patients and matched controls by RNA-seq. Functions of URPL-related eRNAs were further investigated in vitro.

Results: We identified Inc-SLC4A1-1, which was transcribed from an active enhancer marked with H3K27ac and H3K4me1 and so-called eRNA, highly expressed in URPL patients. Gain-of-function experiments indicated that Inc-SLC4A1-1 facilitated trophoblast cell migration and apoptosis. Mechanistically, as an eRNA, Inc-SLC4A1-1 was retained in the nuclei and recruited transcription factor NF-κB to bind to CXCL8, resulting in increased H3K27ac in the CXCL8 promoter and subsequent elevation of CXCL8 expression. Activation of CXCL8 exacerbated inflammatory reactions in trophoblast cells by inducing TNF-α and IL-1β, which could be blocked by an antagonist of Inc-SLC4A1-1.

Interpretation: These findings indicate that an eRNA, Inc-SLC4A1-1, alters trophoblast function via activation of immune responses and by regulating the NF-κB/CXCL8 axis. Our study provides new insights in understanding IncRNA/eRNA function in pathological pregnancy, potentially informing on therapeutic strategies for URPL.

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1. Introduction

Recurrent pregnancy loss (RPL) is viewed as a distinct disorder of pregnancy loss and it is estimated that 5% of women experience two clinical miscarriages and approximately 1% experience three or more losses [1–3]. The etiology of RPL is complicated, and known causes include anatomic factors (uterine malformations), genetic factors (chromosome inversion, deletion, duplication, etc.), and immune and endocrine factors (luteal insufficiency, hypothyroidism, etc.). Although increasing risk factors have been identified to be related to RPL, the reasons for approximately half of these cases still remain unexplained (unexplained recurrent pregnancy loss, URPL) [4–6].

Long noncoding RNAs (lncRNAs) are a class of noncoding RNAs that are >200 bp in length and are transcribed from exons, introns and gene deserts [7]. Recent studies have demonstrated that lncRNA functions in a cell type-specific or tissue-specific manner, indicating that lncRNA may be the driver of cell specific response [8,9]. Consequently, lncRNA is involved in a variety of biological processes, including genomic imprinting, cell proliferation and differentiation, and cellular developmental processes [10–12]. Several studies have shown that lncRNA-related endocrine and immune pathways may be the major causes of URPL, but the underlying mechanisms remain incompletely understood [13,14].

A small portion of lncRNA is transcribed from the enhancer regions, is nucleus-retained, and has gene regulatory function; thus, it is part of
the so-called enhancer-derived lncRNAs, or eRNAs [15–17]. eRNAs can recruit transcription factors, transcriptional co-activators, and chromatin remodelers in cis or in trans to regulate gene transcription and biological process [18,19]. Unlike common lncRNAs, eRNAs exhibit similar transcription rates but produce fewer stable transcripts [20]. To date, the regulatory roles of eRNAs in URPL, especially in trophoblast cells, have not been explored.

During normal pregnancy, trophoblast cells can maintain maternal immune tolerance through generating ligands to active receptors in maternal immune cells [21]. Those activated lymphocytes take up residence at the maternal-fetal interface and build an appropriate uterine microenvironment to promote fetal growth [22]. In the early stages of pregnancy, trophoblast cells invade the decidualized endometrium and remodel uterine spiral arteries to increase maternal blood flow to the placenta villi, which is essential for the exchange of waste and nutrients between maternal and fetal blood [23]. The failure of transitions from progenitors to different trophoblast cells or compromised trophoblast function will cause significant adverse pregnancy outcomes, including URPL [24]. Previous studies had identified several non-coding RNAs that could regulate trophoblast cell functions, including lncRNAs H19, HOXA11-AS and RPAIN [25–27]. However, the molecular mechanisms by which the eRNAs regulate trophoblast cell functions and URPL remain elusive.

In this study, we profiled lncRNAs in villi from URPL patients and matched normal controls by RNA-seq. Our results indicated that Inc-SLA4A1-1, which was characterized as an eRNA, was upregulated with increased H3K27ac modification in unexplained recurrent pregnancy loss (URPL) patients. This upregulation altered trophoblast cells migration and apoptosis. Mechanistically, we demonstrated that eRNA Inc-SLA4A1-1 interacted with NF-kB to promote the expression of CXCL8 and activate the inflammatory response, which might affect trophoblast cell functions and eventually lead to URPL. Our work provides new insights in understanding the etiology of URPL.

2. Materials and methods

2.1. Participating cohorts

This study was approved by the Institutional Ethics Committee of Nanjing Medical University. The study cohort included women with confirmed URPL with 2 or more consecutive pregnancy losses before 20 weeks of undetermined etiology. We excluded patients with abnormal karyotype, infection, endocrine disorders, thyroid dysfunction or abnormal uterine anatomy of URPL. The control group consisted of randomly selected women who underwent legal termination of an apparently normal early pregnancy at the same hospital during the same period, without medical reasons, history of pregnancy loss or any other pregnancy complication. A questionnaire was used to collect clinical and characteristic information, such as personal information, lifestyle factors and medical history. All women enrolled in the study provided signed informed consent. Clinical characteristics of URPL patients and controls are shown in Supplementary Material, Table S1. As expected, there was no significant differences between two groups with respect to baseline characteristic factors, including age, body mass index (BMI), gestational age and childbirth frequency. Villi samples were isolated from products of conception (POC) under a dissecting microscope at the time of dilation and curettage and stored at −80 °C immediately after being washed thoroughly. All activities involved in this study were done under full compliance with government policies and the Helsinki Declaration. All experiment protocols were approved by the Institutional Review Board (IRB) of Nanjing Medical University (NMU) prior to the study (IRB No. NMU (2016)132).

2.2. RNA sequencing and analysis

Total RNA was extracted using RNaseq Kits (Qiagen, Duesseldorf, Germany) and treated with DNase I (Life Technologies, Gaithersburg, USA) according to standard protocols. Tissue RNA-seq (3 URPL patients and 3 controls) was done in Genesky using TruSeq Stranded Total RNA kits (Genesky, Shanghai, China). Cell RNA-seq (3 replicates each group) was done in Ribobio (Ribobio, Guangzhou, China). Briefly, intact RNA was fragmented, end repaired, adapter ligated and PCR amplified following the Illumina protocol. Libraries were sequenced by Illumins Hiseq 2000 (Illumina, San Diego, USA). The sequenced reads were aligned to the human reference genome (H19) using TopHat v1.4.1. Differential gene expression (DEG) analysis was performed with cuffdiff and DESeq (tissue RNA-seq), or DEseq and DESeq (cell RNA-seq). LncRNAs/genes with fold change >2 or <−2 and FDR P value <0.05 were selected as DEGs. The data reported in this study have been uploaded in the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo) (Tissue RNA-seq: GSE No. 121950, cell RNA-seq: GSE No. 121951).

2.3. Quantitative PCR (qPCR)

Total RNA was extracted from villi or cells with Trizol (Invitrogen, Carlsbad, USA); nuclear and cytoplasmic RNA was prepared using a PARIS™ Kit (Invitrogen Carlsbad, USA). We used 20 URPL patients and 20 controls in the cohort for the validation. First strand cDNA was synthesized (Vazyme, Nanjing, China) and quantitative PCR was performed. All reactions were performed in triplicate with SYBR Green master mix (Vazyme, Nanjing, China) under the following conditions: 5 min at 95 °C for initial denaturation, followed by 40 cycles of segments of 95 °C for 30 s and 60 °C for 30 s in the ABI Prism 7900HT/FAST (Applied
2.4. In vitro experiments

The HTR-8/SVneo cell line was cultured in 1640 medium with 10% FBS (Gibco, Carlsbad, USA). The JEG-3 cell line was cultured in MEM medium (Gibco, Carlsbad, USA) with 10% FBS (Gibco, Carlsbad, USA), 100 U/ml penicillin (Gibco, Carlsbad, USA), and 100 μg/ml streptomycin (Gibco, Carlsbad, USA) at 37 °C under 5% CO₂. Cells were co-transfected with pcDNA-IncRNAs or pcDNA-control using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) for overexpression of IncRNAs. The relative expression of transfected IncRNAs after 24 h was detected by qRT-PCR.

After 24 h transfection, cells were harvested for cell proliferation, apoptosis, cell cycle and invasion analysis. For cell proliferation analysis, approximately 3000 cells were seeded in each well of a 96-well plate. After transfection, cells were treated with 10 μl Cell Counting Kit-8 solution in each well and cultured for 0.5–1 h before measuring absorbance at 450 nm. For invasion analysis, 500 μl medium containing 10% fetal bovine serum was added per well in a 24-well plate. Transwell chamber inserts were put into the wells with 100 μl cell suspension mixed with serum free medium. The cells were immobilized with Polyoxymethylene and photographed after 24 h. For cell apoptosis analysis, cell suspensions were prepared and analyzed immediately by a FACs Calibur Flow Cytometer (BD Medical Technology, Lake Franklin, USA) 24 h after transfection. For cell cycle analysis, single cell suspensions were immobilized with 70% ethanol, stained with PI and analyzed using a FACs Calibur Flow Cytometer (BD Medical Technology, Lake Franklin, USA) 24 h after transfection.

2.5. Western blot analysis

Western blots were performed using antibodies against P65 (1:1000, RRID: AB_10859369) (Cell Signaling Technology, Beverly, USA), CXCL8 (1:10, RRID: AB_444617) (Abcam, Cambridge, UK) and GAPDH (1:1000, RRID: AB:2715590) (Beyotime Biotechnology, Shanghai, China) following standard protocols. Briefly, 80 μg protein was applied to SDS-PAGE and transferred to PVDF membrane. Non-specific binding of antibody was blocked by 5% skim milk for 1 h. After incubation with primary antibody overnight, the membrane was washed and incubated with HRP-conjugated secondary antibody for 1 h. GAPDH was used as an internal control. The blots were visualized using the chemiluminescent detection method (Pierce, Thermo Scientific, Wallingford, USA). Three repeats were set up for each treatment. The intensity of the western blot bands was quantified by ImageJ.

2.6. ChIP-qPCR

The ChIP methodology was described previously [28]. Briefly, cells were fixed with 1% formaldehyde for 10 min at room temperature. Cross-linking was terminated by addition of 0.125 M glycerine. Cells were lysed and sonicated using a Bioruptor (Diagenode, Denvile, USA) to generate short fragments for immunoprecipitation. The chromatin was immunoprecipitated by incubating overnight with 1 μg of H3K4me1 (RRID: AB_310614) (Millipore, Bedford, USA) and H3K27ac (RRID: AB_2118291) (Abcam, Cambridge, UK) antibody. After incubating with either protein A or G (EMD Millipore, Bedford, USA) beads for 2 h, the samples were washed with low salt buffers, then high salt buffers, then LiCl buffers and finally TE buffer. The protein–DNA complexes were reverse crosslinked at 65 °C overnight with Proteinase K. Three repeats were set up for each treatment. All the primer sequences used for ChIP-qPCR are listed in Supplementary Material, Table S3.

2.7. RIP-qPCR

Cells were fixed with formaldehyde at a final concentration of 1%. Cells were lysed with 500 μl lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, and RNase inhibitor) and incubated overnight at 4 °C with 50 μl of Protein G Dynabeads that were prewashed and premixed with 5 μl antibodies P50/P105 (RRID: AB:33056), P65 (RRID: AB:2118291), AP-1 (RRID: AB:253112) and c-Jun (RRID: AB:2,130,165) (Cell Signaling Technology, Beverly, USA) or IgG control (Millipore, Bedford, USA). After washing three times, RNA was extracted by Trizol and reverse transcribed for qPCR analysis. Three repeats were set up for each treatment. All the primer sequences used for RIP-qPCR are listed in Supplementary Material, Table S4.

2.8. Elisa

TNF-α, IL-1β and CXCL8 in culture supernatants were analyzed by enzyme-linked immunosorbsent assay (ELISA), following the Elabscience ™ ELISA kit instructions (Elabscience, Wuhan, China). Three repeats were set up for each treatment. Absorbance values were measured using a microplate reader and a two-tailed Student’s t-test was used for statistical analysis.

2.9. Statistical analysis

All statistical analyses were performed using Graphpad Prism 5.0 (GraphPad Software, San Diego, USA). The Kolmogorov-Smirnov test was used to test the assumption of normal distribution. Student’s t-test was used to show differences in continuous variables such as age and body mass index (BMI).

For data that do not conform to the normal distribution, we used the Mann-Whitney U test. Multiple comparisons were subjected to one-way analysis of variance (one-way ANOVA) followed by ad-hoc testing. The significance was set for p < 0.05. All data were expressed as mean ± SEM.

3. Results

3.1. Dysregulation of lncRNAs in URPL

To identify the lncRNAs that are potentially involved in URPL, we profiled the transcriptome by RNA-seq and analyzed the data following the pipeline illustrated in Fig. 1a. Comparing URPL with matched controls, 476 and 250 differentially expressed lncRNAs (DELs) were identified in villus tissue using Cuffdiff and DESeq with 2-fold change and an FDR p value <0.05, respectively. One hundred and thirty-one lncRNAs were selected as confident DELs from the intersection of Cuffdiff and DESeq results (Fig. 1b). KEGG pathway analysis was used to predict the enriched biological pathways of URPL-related DELs. The top 10 up-regulated and top 10 down-regulated pathways are shown in Fig. 1c.d. Most of the pathways were associated with immune function, including Toll-like receptor signaling pathway, T cell receptor signaling pathways and Jak-STAT signaling pathways.

lncRNAs transcribed from enhancer regions are called enhancer RNAs (eRNAs), and have been shown to recruit transcription factors and co-activators to promote gene activation. To identify potential eRNAs among URPL-related DELs, we compared their locations with annotated enhancer regions in VISTA Enhancer Browser (https://enhancer.lbl.gov/) (Fig. 1e). There are approximately 55,000 potential enhancers in the human genome and among the 131 URPL-related DELs (Supplementary Material, Table S5), 38 DELs were found to overlap with enhancer regions (32 up-regulated and 6 down-regulated).

Most of these potential eRNAs have not been studied, and their functions are still largely unknown. We then selected 12 potential eRNAs with the largest fold change for validation in an independent cohort as described in methods. The results were highly consistent except for...
Fig. 1. Dysregulation of IncRNAs in URPL. (a) The screening process pipeline used in our study. (b) Heatmap of differentially expressed IncRNAs between URPL and controls by RNA-seq (n = 3 per group). (c) Top 10 upregulated KEGG pathways. (d) Top 10 downregulated KEGG pathways. (e) Example of eRNA in UCSC genome browser. (f) Validation of differentially expressed IncRNAs in URPL and controls (n = 20 per group). (Two-tailed paired Student’s t-test.) *P < 0.05, **P < 0.01, ***P < 0.001. Data are presented as mean ± SEM. Three repeats were set up for each treatment.
Inc-PAWR-1 ($t = 2.761, p = 0.028$) (Fig. 1f). Seven eRNAs were upregulated, including Inc-ERGIC1-4 ($t = 4.531, p = 0.011$), Inc-MRPS30-5 ($t = 3.717, p = 0.021$), Inc-RCAN1-1 ($t = 6.107, p = 6.117 \times 10^{-5}$), Inc-SLC4A1-1 ($t = 6.078, p = 3.549 \times 10^{-5}$), Inc-TMEM135-8 ($t = 2.092, p = 0.045$), Inc-CES1-1 ($t = 6.078, p = 3.549 \times 10^{-4}$), Inc-TMEM135-8 ($t = 6.107, p = 6.117 \times 10^{-5}$), Inc-DNAJC19-1 ($t = 3.920, p = 0.017$), Inc-PBK-2 ($t = -3.183, p = 0.003$), Inc-SOX4-1 ($t = -4.431, p = 0.013$) and Inc-AC106873.4-1-8 ($t = -4.391, p = 0.013$), were down-regulated (Fig. 1f).

3.2. URPL-related eRNAs regulated trophoblast cell functions

We used the JEG-3 (derived from a choriocarcinoma) and HTR-8/SVneo (derived from normal first trimester placenta) cell lines to study the eRNA’s function [29]. In an attempt to determine whether URPL-related eRNAs have effects on trophoblast cell function, we overexpressed selected URP-related eRNAs (Inc-ERGIC1-4, Inc-SLC4A1-1 and Inc-TMEM135-8), which showed large differences (fold change $>5$) in both screening and validation stages in HTR-8/SVneo and JEG-3. After transfection, the expression levels of Inc-ERGIC1-4, Inc-SLC4A1-1 and Inc-TMEM135-8 were significantly higher than that in controls (Supplementary Material, Fig. S1a). Cell proliferation was significantly decreased in both HTR-8/SVneo ($t = 11.420, p = 2.422 \times 10^{-5}$) and JEG-3 ($t = 2.579, p = 0.032$) cells after overexpression of Inc-SLC4A1-1, while the other IncRNAs had no effects (Fig. S2a, b). Correspondingly, cell apoptosis was significantly increased in HTR-8/SVneo ($t = 2.282, p = 0.045$) and JEG-3 ($t = 2.883, p = 0.045$) cells with high expression of Inc-SLC4A1-1, revealing that growth inhibition was accompanied by increased apoptosis (Fig. 2a). We also found a significantly increased number of cells migrating through transwell pores after Inc-SLC4A1-1 ($t = 11.520, p = 7.019 \times 10^{-6}$) and Inc-ERGIC1-4 ($t = 11.930, p = 7.361 \times 10^{-6}$) overexpression in both HTR-8/SVneo and JEG-3 cells (Fig. 2b). All three IncRNAs had no effects on cell cycle (Supplementary Material, Fig. S2c, d). Collectively, our results suggested that URPL-related eRNAs inhibited cell proliferation, but promoted apoptosis and migration.

Since Inc-ERGIC1-4 and Inc-TMEM135-8 had mild effects on cell function, subsequent functional study was focused on Inc-SLC4A1-1. Inc-SLC4A1-1 (ENST00000498270) (http://www.lncipedia.org/) locates on Chr.17 with a full length of 460 bp and containing four exons in total. To ascertain the eRNA potency of Inc-SLC4A1-1, we performed ChIP-qPCR at the Inc-SLC4A1-1 transcription locus and detected a robust enrichment of H3K27ac ($t = -4.051, p = 0.015$) (Supplementary Material, Fig. S3a). In addition, subcellular localization analysis showed that Inc-SLC4A1-1 localized in the nucleus rather than in the cytoplasm ($t = 2.941, p = 0.042$), which indicated that Inc-SLC4A1-1 might be an eRNA and have gene regulatory effect within the nucleus (Supplementary Material, Fig. S3b).

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**Fig. 2.** URPL related eRNAs regulated trophoblast cell function. (a) Cell apoptosis analysis in HTR-8/SVneo and JEG-3 cells. (b) Transwell assay in HTR-8/SVneo and JEG-3 cells. (Two-tailed paired Student’s t-test.) $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data are presented as mean ± SEM. Three repeats were set up for each treatment. a: Control, b: Inc-ERGIC1-4, c: Inc-SLC4A1-1, d: Inc-TMEM135-8.
3.3. Lnc-SLC4A1-1 enhanced CXCL8 transcription in trophoblast cells

Lnc-SLC4A1-1 was transcribed from an enhancer near the SLC4A1 gene. Thus, we first tested whether lnc-SLC4A1-1 played a cis regulatory role in regulating SLC4A1. However, after overexpression of lnc-SLC4A1-1, no cis-regulatory effect of lnc-SLC4A1-1 on SLC4A1 was found ($t = 0.716, p = 0.514$) (Supplementary Material, Fig. S3c). To further evaluate the function of lnc-SLC4A1-1 in trophoblast cells, we performed RNA-seq in HTR-8/SVneo cells overexpressing lnc-SLC4A1-1. Using a fold change $>2$ and FDR $P$ value $<0.05$ as a cut off, we identified 30 genes that were upregulated and 4 genes that were downregulated upon overexpression of lnc-SLC4A1-1 (Fig. 3a, b). Among those 34 genes, MT2A, ANGPTL4, HSPA1B, DUSP1, PLIN2, NDRG1, KRT34, CXCL8 and HSPA1A were identified by both DESeq and DEGseq. A cytokine-cytokine receptor interaction pathway, JAK−STAT signaling pathway and NF-kappa B signaling pathway were enriched in the KEGG analysis. These findings suggested that lnc-SLC4A1-1 regulated the expression of cytokine and inflammation-related genes (Fig. 3c).

In addition, we used qRT-PCR to validate the differentially expressed genes identified in RNA-seq. Compared with the control, we found that lnc-SLC4A1-1 significantly promoted the expression levels of MT2A ($t = -4.016, p = 0.016$), ANGPTL4 ($t = -3.031, p = 0.039$), DUSP1 ($t =$...
4.238*10^{−4}}, P65 was highly enriched near CXCL8 (t = −2.598, p = 0.010) and IL1β (Z = −2.353, p = 0.024) and IL1β (Z = −2.598, p = 0.010) were induced after overexpression of Inc-SLC4A1-1 (Fig. 4d). This response indicated the activation of inflammation in HTR-8 SVneo and JEG-3 cells after overexpression of Inc-SLC4A1-1. In addition, when Inc-SLC4A1-1 was knocked down by siRNA or when Inc-SLC4A1-1 was overexpressed but we simultaneously added inhibitors of the NF-κB pathway, the induction of CXCL8, TNF-α and IL1β was attenuated (Fig. 4d). Overall, our data indicated that Inc-SLC4A1-1 recruited NF-κB to upregulate CXCL8 and induced the release of TNF-α and IL1β following activation of the inflammation response in trophoblast cells, which might be involved in the URPL (Fig. 5).

4. Discussion

URPL is a common pathological problem in pregnancy and the etiology is complicated. In this study, we sought to identify URPL-associated lncRNAs as potential biomarkers for diagnosis and therapy. For the first time, we showed Inc-SLC4A1-1, which is an eRNA, to be dysregulated in the villi of URPL-derived trophoblast cells. Additionally, we explored the potential mechanisms by which Inc-SLC4A1-1 binding to NF-κB enhanced the expression level of CXCL8, leading to the release of inflammatory factors TNF-α and IL-1β, which might contribute to URPL.

Recently, numerous studies suggested that certain lncRNAs, which were transcribed from active enhancer regions and are referred to as eRNAs, could interact with transcription factors to regulate gene expression [31–34]. It is estimated that ~55,000 possible enhancers exist in the human genome. This number is greater than the number of protein-coding genes, indicating the complexity of the roles of enhancers in gene regulation [35]. Enhancer regions usually harbor different histone marks, for example, poised enhancers enriched with H3K4me1, while activated enhancers are enriched with H3K27ac and H3K4me3. It is estimated that ~55,000 possible enhancers exist in the human genome. This number is greater than the number of protein-coding genes, indicating the complexity of the roles of enhancers in gene regulation [35]. Enhancer regions usually harbor different histone marks, for example, poised enhancers enriched with H3K4me1, while activated enhancers are enriched with H3K27ac and H3K4me3.
transcribed from active enhancers generally exert regulatory effects in two modes: one in which eRNAs recruit transcription machinery to local promoters (cis); the other in which eRNA-induced enhancer-promoter looping activates gene transcription (trans) [38]. eRNAs were reported to interact with CBP, which is a transcription coactivator at enhancers and modifies local histone acetylation, to regulate the target genes [39]. Additionally, in breast cancer cells, estrogen stimulation could activate the ERα and enhance the transcription of eRNAs. Those estrogen dependent eRNAs exerted important roles in forming enhancer-promoter looping to active target genes [31,34].

In this study, we found Inc-SLC4A1-1 had a trans regulatory effect on its target gene. Inc-SLC4A1-1 was transcribed from the region containing H3K27ac and H3K4me1 and was retained in the nuclei. Instead of activating SLC4A1 nearby, Inc-SLC4A1-1 upregulated the expression level of CXCL8 in trans, which induced downstream release of cytokines. Although limited DEGs were observed after Inc-SLC4A1-1 was overexpressed, we noted an alteration of trophoblast cell apoptosis and migration. Rescue experiments revealed that the regulatory role of Inc-SLC4A1-1 was mediated, at least in part, via recruiting transcription factor NF-κB.

CXCL8, also known as interleukin-8 (IL-8), plays an important role in inflammation-related diseases including chronic obstructive pulmonary disease, asthma and atherosclerosis [40,41]. CXCL8 regulates inflammatory responses in lung, stomach, pancreas, liver and some cancerous tissues [40,42]. Blocking CXCL8 and CXCL8 receptor with antagonists could reduce the inflammatory response and improve the symptoms of inflammation-related diseases [43]. In our study, CXCL8 also induced inflammatory factors, such as TNF-α and IL-1β, which further exacerbate the inflammatory response. Using CXCL8 antagonists can attenuate the TNF-α and IL-1β levels even when Inc-SLC4A1-1 was overexpressed in trophoblast cells. Inflammation and inflammatory cytokines during pregnancy were associated with trophoblast function [44]. Elevated concentrations of inflammatory cytokines in the trophoblast are associated with preterm delivery (PTD), labor induction, and premature rupture of the membrane [45–47]. For instance, Sherief et al. reported relationships between inflammatory cytokines and PTD, showing significantly increased levels of IL-2, IFNγ, and IL-12 in trophoblast cells from PTD compared with controls [45]. The release of inflammatory cytokines can activate inflammatory pathways, resulting in increased apoptosis and decreased proliferation of trophoblast cells [48,49]. Researchers found that microRNA-145-5p was stimulated by the inflammatory cytokine TNF-α, and overexpression of microR-145-5p significantly impaired the migration and invasion of HTR-8/SVneo cells.

eRNA Inc-SLC4A1-1 was found to recruit NF-κB, indicating it participated in the activation of the NF-κB signaling pathway. The NF-κB signaling pathway has been shown to regulate the expression of downstream inflammatory cytokines [50–52]. The activation of NF-κB was also observed in some pathological pregnancy [53]: increase of NF-κB level in the placenta was related to the downregulation of anti-apoptotic factor Bcl-2 and the increase of caspase-3, suggesting that the apoptosis of trophoblast cells depends on the NF-κB pathway [54]; another study reported that p53’s role in the regulation of trophoblast proliferation and apoptosis was mediated by NF-κB [55].

Although we validated Inc-SLC4A1-1’s function in vitro, there were still some limitations in our study. First of all, the sample size used for sequencing was relatively small and we didn’t perform functional enhancer screening for all candidate eRNAs. Secondly, we didn’t have the spatiotemporal expression data of lnc-SLC4A-1, thus it was difficult to determine the exact role of Inc-SLC4A-1 during URPL. Thirdly, we didn’t use the in vivo model and further study is warranted to fully characterize the causative relationship between Inc-SLC4A-1 and URPL.

In summary, our findings suggest that eRNA Inc-SLC4A1-1 recruits NF-κB and binds to the promoter of CXCL8, which leads to upregulation of CXCL8. Increased CXCL8 exacerbates inflammatory reaction by inducing TNF-α and IL-1β and affects trophoblast function, which may impact the immune response and eventually lead to URPL.

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Declaration of interests

All the authors have no conflict of interests.

Authors’ contributions

C.L. and X.W. directed the study, obtained financial support and were responsible for study design. Z.H. performed overall project management along with G.D. and X.H. and drafted the initial manuscript. Y.Q. performed statistical analysis. L.H. and Y.Z. were responsible for sample collection and processing. G.D. were responsible for population screening and verification. X.H. and M.Y. were responsible for functional analysis in HTR-8/SVneo and Jeg-3 cells. X.H., B.X. and Y.Y. conceived of the study, and participated in its design and coordination and helped to...
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