

# The YY1-HOTAIR-MMP2 Signaling Axis Controls Trophoblast Invasion at the Maternal-Fetal Interface

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We aimed to determine the effect of YY1 expression on the expression profile of long noncoding RNAs (lncRNAs) in trophoblasts, and we studied the involvement of certain lncRNAs and YY1 in the pathogenesis of recurrent miscarriage (RM). RT2 lncRNA PCR arrays revealed that YY1 overexpression in trophoblasts significantly promoted the expression of the HOX transcript antisense RNA HOTAIR and demonstrated that HOTAIR expression was significantly lower in the RM trophoblasts than in control trophoblasts. Ectopic HOTAIR overexpression and knockdown experiments revealed that it was a novel target of YY1. Bioinformatics analysis identified two YY1-binding sites in the HOTAIR promoter region, and chromatin immunoprecipitation (ChIP) analysis verified that YY1 binds directly to its promoter region. Interestingly, HOTAIR overexpression enhanced trophoblast invasion in an ex vivo explant culture model, while its knockdown repressed these effects. Furthermore, liquid chromatography-tandem mass spectrometry (LC-MS/MS) label-free quantitative proteomics screening revealed that HOTAIR overexpression activated phosphatidylinositol 3-kinase-protein kinase B (PI3K-AKT) signaling in trophoblasts. In an ex vivo explant culture model, HOTAIR overexpression effectively elevated matrix metalloproteinase 2 (MMP2) expression via the PI3K-AKT signaling pathway, enhancing trophoblast migration and invasion. These findings reveal a new regulatory pathway in which YY1 activates PI3K-AKT signaling via HOTAIR, promoting MMP2 expression, suggesting that HOTAIR is a potential therapeutic target for RM.

## INTRODUCTION

Embryo implantation is a highly regulated event that is critical for the establishment of pregnancy. Immediately after implantation, trophoblast cells, which form the outermost epithelial layer of the blastocyst, give rise to diverse trophoblast cell types.<sup>1</sup> Currently, two major trophoblast cell lineages have been identified during the early stages of human placental development: villous

and extravillous trophoblasts (EVTs).<sup>2,3</sup> EVT, which are derived from trophoblasts by epithelial-mesenchymal transition, form cell columns and are highly invasive in nature. The EVT then migrates from the attached embryo and invades the uterine epithelium and uterine spiral arteries to establish the maternal-fetal linkage.<sup>4,5</sup> Invasive EVT in the endometrial stroma and inner third of the myometrium are essential both for the development of the definitive maternal-fetal circulation and for pregnancy success in humans.<sup>6</sup> Impaired EVT migration and invasion commonly lead to failure to establish the maternal-fetal connection and are associated with preeclampsia, fetal growth restriction, and early and late recurrent miscarriage (RM).<sup>7</sup>

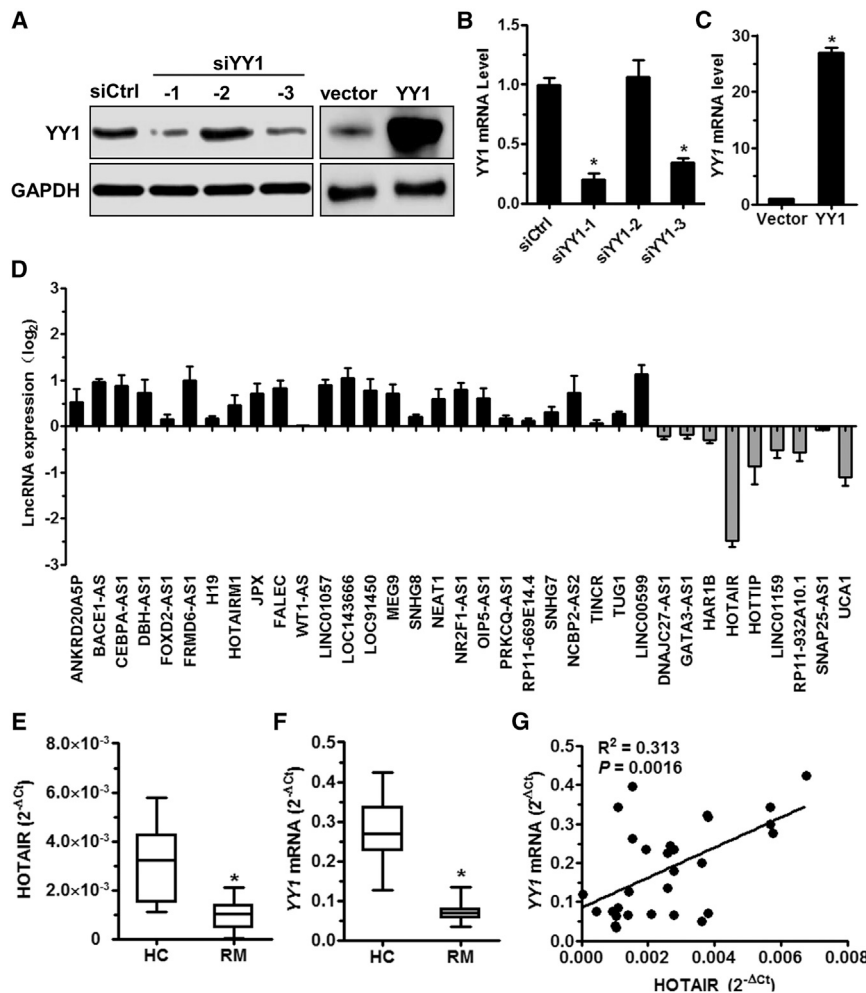
RM, defined as two or more consecutive pregnancy losses or three or more consecutive spontaneous abortions before 20 weeks of gestation, affects ~3% of pregnant women.<sup>8</sup> Couples who undergo recurrent pregnancy loss undergo tremendous psychological distress. The global prevalence of RM has exhibited an increasing trend, particularly due to the increasing age of pregnant women, and the availability of assisted reproductive technologies.<sup>9</sup> Moreover, epidemiological studies have revealed that pregnancies after a series of miscarriages have an increased risk of preterm birth, stillbirth, preeclampsia, and fetal growth restriction.<sup>10</sup> Several studies have implicated impaired EVT invasion in RM.<sup>11</sup> In our previous study, we explored the function of YY1 in human villi and trophoblasts during the early stage of pregnancy and preliminary revealed a new regulatory pathway of YY1 and matrix metalloproteinase 2 (MMP2) in trophoblast cells invasion

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**Figure 1. YY1 Represses HOTAIR Expression in Trophoblasts**

(A–C) Western blot analysis and real-time PCR were performed to determine the YY1 expression level in HTR-8 cells transfected with siCtrl, siYY1-1, siYY1-2, siYY1-3, control, or YY1-overexpressing vector after 48 hr. (D) Real-time PCR was performed to determine the levels of long noncoding RNAs (lncRNAs) in trophoblasts transfected with siYY1 or siCtrl for 48 hr. (E and F) *HOTAIR* (E) and *YY1* mRNA (F) expression levels in the villous tissue of RM patients (n = 30) and HCs (n = 21) were determined by qRT-PCR. The relative amount of RNA was calculated using the  $2^{-\Delta Ct}$  method and normalized with internal control gapdh. (G) The *HOTAIR* level in villous tissue from patients (n = 30) was measured using qRT-PCR and correlated with the *YY1* mRNA level in villous tissue from the corresponding patients (n = 30).

(PI3K-AKT) signaling, a major pathway that connects inflammation with cancers, and has been shown to promote cancer cell invasion.<sup>21</sup> Several studies have reported that *HOTAIR* is overexpressed in several types of malignant diseases, including breast cancer, gastric cancer, liver cancer, and sarcoma. Moreover, several in vitro studies have shown that *HOTAIR* promotes cancer cell proliferation, invasion, and metastasis but inhibits apoptosis of cancer cells.<sup>22–24</sup> However, the exact role of *HOTAIR* in human trophoblast migration and invasion remains unknown.

during early pregnancy and indicated that YY1 was involved in the pathogenesis of RM.<sup>12</sup> However, the precise molecular mechanisms for YY1 regulating trophoblast invasion in RM and its relationship to fetoplacental development are largely unknown.

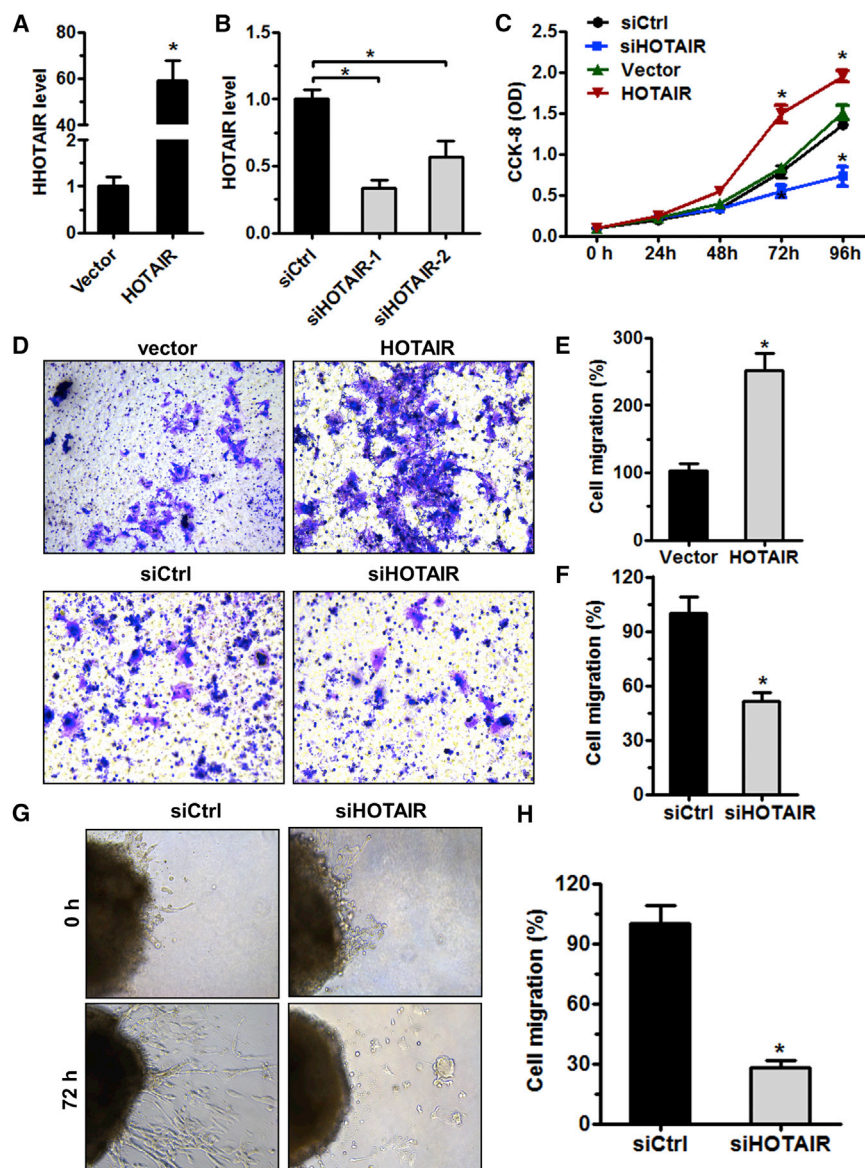
Long noncoding RNAs (lncRNAs) are non-protein-coding RNA molecules ranging from 200 bp to several kilobases in size.<sup>13</sup> Although lncRNAs do not hold protein sequence information, they have been reported to play a critical role in the regulation of diverse cellular processes, such as stem cell pluripotency, development, and cell growth, and consequently affect cell function, associated pathological conditions, and human diseases.<sup>14–16</sup> In recent years, many studies have established a clear link between lncRNA regulation and placental development. The lncRNAs *HOTAIR*, *MEG3*, and *MALAT1* have been suggested to contribute to the behavior of trophoblast cells in preeclampsia.<sup>17,18</sup> In particular, *HOTAIR*, which exerts regulatory transcription activity, can bind PRC2 and the LSD1-CoREST-REST complex and direct to the specific gene sites and cause H3K27 methylation and H3K4 demethylation and gene silencing.<sup>19,20</sup> Interestingly, *HOTAIR* activates phosphatidylinositol 3-kinase-protein kinase B

on the expression profile of lncRNA in trophoblasts. We further explored the relationship between the expression of certain lncRNAs (*HOTAIR*) and *YY1* expression in the pathogenesis of RM

## RESULTS

### YY1 Regulates HOTAIR Expression in Trophoblasts

To explore whether lncRNAs are a downstream target of YY1 during trophoblast invasion, primary trophoblasts obtained from first-trimester placentas of human subjects were transfected with the siYY1 or YY1 overexpression vector. Transfection with siYY1 resulted in downregulation of *YY1* expression, and transfection with the YY1 overexpression vector resulted in its upregulation (Figures 1A–1C). Next, we used RT<sup>2</sup> lncRNA PCR array to compare the expression profiles of 84 lncRNAs in primary human trophoblasts transfected with YY1 siRNA or siRNA controls. The results revealed that 26 lncRNAs were upregulated and 9 were downregulated after *YY1* knockdown in trophoblasts (Figure 1D). Of these, the expression of *HOTAIR* decreased significantly in trophoblasts. We further examined the expression status of *HOTAIR* and *YY1* mRNA in trophoblasts obtained from RM patients and



**Figure 2. HOTAIR Promotes Trophoblast Invasion In Vitro and Trophoblast Outgrowth in Extravillous Explant Cultures In Vivo**

(A and B) qRT-PCR analysis of HOTAIR expression in HTR-8 cells transfected with siCtrl, siHOTAIR-1, siHOTAIR-2, control vector, or HOTAIR-overexpressing vector after 48 hr. (C) HTR-8 cells were transfected with one of the above-mentioned vectors and siRNA. Cell proliferation was measured after at indicated time using the CCK-8 assay. \* $p < 0.05$  compared with siCtrl or control vector. (D–F) HOTAIR overexpression in HTR-8 cells significantly increased cell invasion compared to the vector control cell line (top panels). HOTAIR knockdown reduced cell invasion compared to the scrambled control cell line (original magnification  $\times 200$ ). \* $p < 0.05$  versus siCtrl or control vector. (G) Extravillous explants were obtained from healthy controls at 6–8 weeks of gestation and cultured on Matrigel. Serial pictures of the explants incubated with siHOTAIR or siCtrl were taken under a light microscope after 24 and 72 hr of in vitro culture (original magnification  $\times 100$ ). (H) Statistical assay of the migration distance of villous tips (%). Data are presented as means  $\pm$  SD of three independent experiments.

vector for downregulation or overexpression of HOTAIR, respectively (Figures 2A and 2B). Results of the CCK-8 assay showed that HOTAIR overexpression increased HTR-8 cell proliferation, while its knockdown decreased HTR-8 cell proliferation (Figure 2C). To further explore whether HOTAIR is involved in trophoblast invasion, a Matrigel cell invasion and ex vivo explant culture model was established. The results revealed that HOTAIR overexpression significantly increased the invasive ability of trophoblast cells, whereas its knockdown obviously inhibited trophoblast invasion (Figures 2D–2H). These results suggest that HOTAIR may play an important role in trophoblast invasion, which is essential for the devel-

opment of the definitive maternal-fetal circulation and for pregnancy success in humans.

healthy controls. Consistent with the results of *YY1* mRNA downregulation, HOTAIR was significantly decreased in the trophoblasts from RM patients (Figures 1E and 1F). Linear correlation analysis showed that the HOTAIR level was positively correlated with the *YY1* level in villous tissue (Figure 1G). These findings reveal that *YY1* and HOTAIR are downregulated in the villous tissue of RM patients, suggesting that HOTAIR may be downstream of the *YY1* gene.

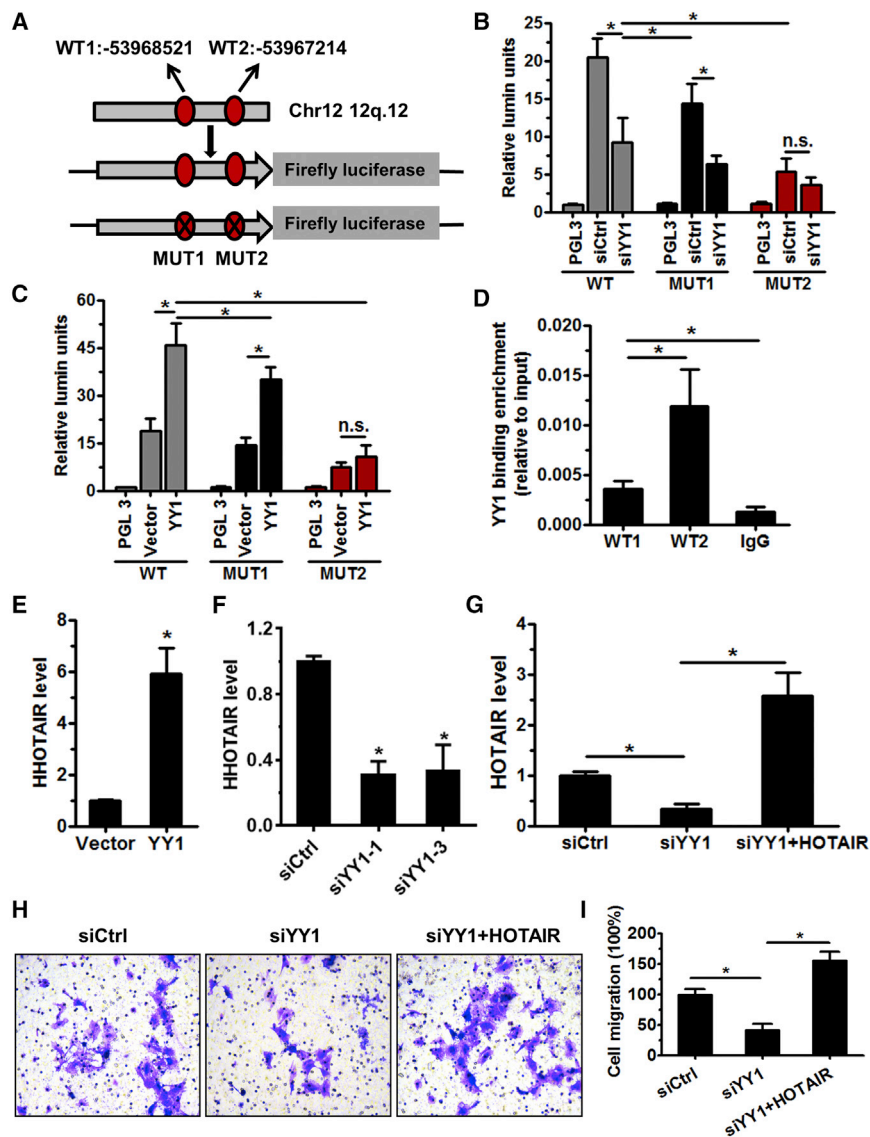
#### HOTAIR Promotes Trophoblast Invasion In Vitro and in an Ex Vivo Explant Culture Model

We then investigated whether HOTAIR is involved in trophoblast proliferation and migration. HTR-8/SVneo (HTR-8) cells were transfected with siHOTAIR-1, siHOTAIR-2 or a HOTAIR-overexpressing

development of the definitive maternal-fetal circulation and for pregnancy success in humans.

#### YY1 Is a Transcriptional Activator for HOTAIR in Trophoblasts

To explore whether *YY1* is a transcriptional factor of HOTAIR, two *YY1* binding sites were predicted to be the putative promoters of HOTAIR by using TRANSFAC software. These two binding sites were selected for further luciferase reporter screening (Figure 3A). The *YY1* binding sites (referred to as wild-type 1 [WT1] and WT2) were located in the conserved region upstream of HOTAIR. Next, WT or two mutant (MUT) (containing *YY1* WT1 or WT2 binding sites mutated individually) reporter plasmids were transfected into HTR-8 cells along with either siYY1 or *YY1* expression plasmids. As expected, results of the luciferase assay revealed that



**Figure 3. YY1 is a Transcriptional Activator for HOTAIR in Trophoblasts**

(A) The YY1 binding site in the promoter region of HOTAIR (top). Construction of the wild-type HOTAIR promoter reporter (WT HOTAIR reporter) and the YY1-binding-site-deleted mutant reporter (mut HOTAIR reporter) (bottom). (B and C) HTR-8 cells were transfected with pGL3-HOTAIR-luciferase reporter (YY1 WT) or with two pGL3-HOTAIR-155-luciferase reporters with mutation in YY1 MUT1/MUT2 sites along with siCtrl or siYY and vector or YY1 overexpression plasmid. Luciferase activity was normalized to the activity of Renilla luciferase. (D) A chromatin immunoprecipitation (ChIP) assay was performed using YY1-specific antibodies for transcriptionally active regions of DNA. The purified DNA was amplified by qRT-PCR. (E and F) qRT-PCR analysis of HOTAIR expression in HTR-8 cells transfected with siCtrl or siYY1-1, siYY1-3, and vector or YY1 overexpression plasmid. (G) qRT-PCR analysis of HOTAIR expression in HTR-8 cells that were transfected with siCtrl or siYY1 or siYY1 + YY1 overexpression plasmid for 24 hr (original magnification  $\times 200$ ). The cells' ability to invade was assessed using ImagePro 6.0 software.

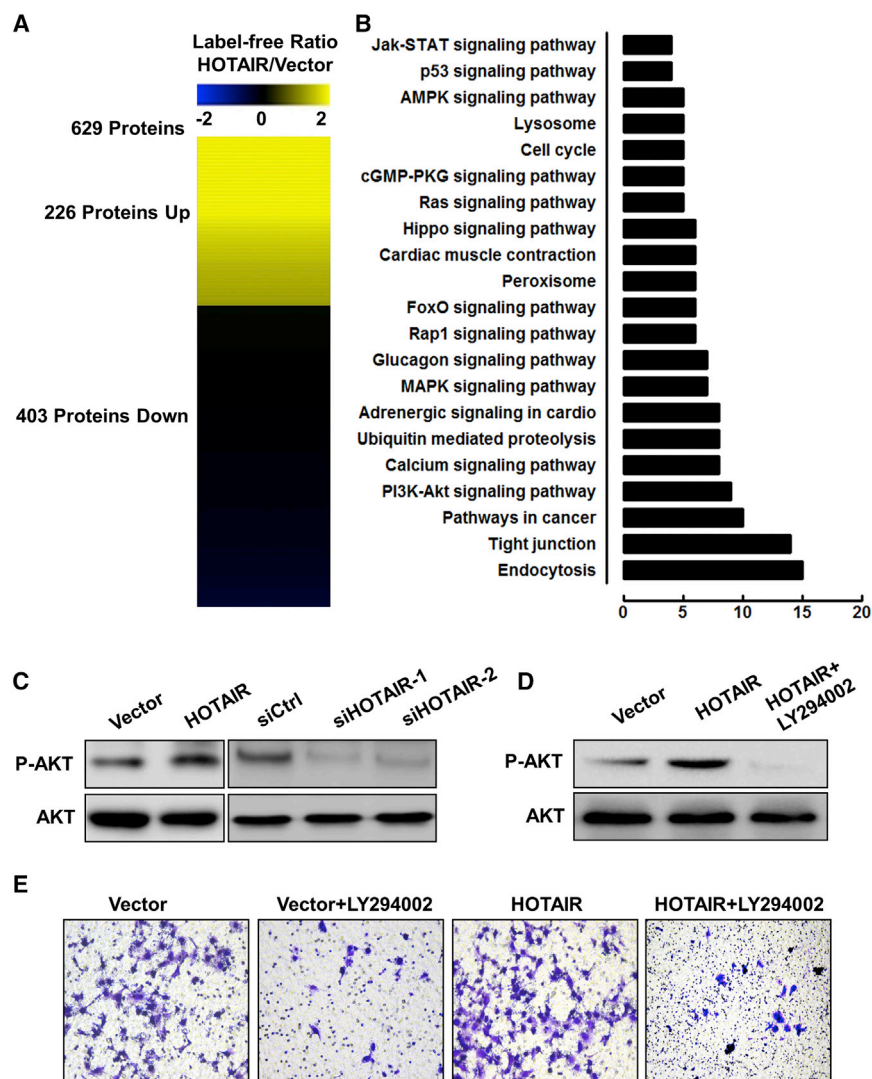
level decreased by YY1 knockdown (Figure 3G). Knockdown of YY1 obviously decreased the invasive ability of HTR-8 cells, while overexpression of HOTAIR reversed the effects caused by YY1 knockdown on trophoblast invasion (Figures 3H and 3I). Taken together, these results support the notion that YY1 is a direct transcriptional activator for HOTAIR.

**HOTAIR Promotes Trophoblast Invasion by Activating the PI3K-AKT Signaling Pathway**

To elucidate the molecular mechanism of the effect of HOTAIR on trophoblast invasion, HTR-8 cells were transfected with HOTAIR

overexpression plasmid for 48 hr. We used label-free protein quantification methods to identify proteins dysregulated by HOTAIR overexpression in HTR-8 cells (Table S1). A total of 629 proteins were found to be dysregulated after HOTAIR overexpression. Of these, 226 were upregulated and 403 were downregulated (Figure 4A; Table S2), the majority of which had a fold-change value (HOTAIR/vector) between 0.5 and 1.5 after HOTAIR overexpression. We then classified the HOTAIR-regulated proteins using the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway to obtain an overview of the signaling pathway distribution. KEGG pathway analysis revealed that the HOTAIR-regulated proteins were involved in a variety of signaling pathways, including endocytosis, cancer, and mitogen-activated protein kinase (MAPK) pathways, and particularly the PI3K-AKT pathway (Figure 4B). Further, we used western blot analysis to analyze the

WT reporter plasmid activity was potentially reduced by YY1 knockdown, but it was enhanced by YY1 overexpression in HTR-8 cells. In particular, luciferase activity of MUT1 reporter plasmid was significantly decreased (compared to the control) by YY1 knockdown, but it was enhanced by YY1 overexpression in HTR-8 cells. Conversely, MUT-2 reporter plasmid activity was not obviously affected by YY1 knockdown or overexpression (Figures 3B and 3C). This result indicates that WT1 is a possible YY1 binding site. This result was further verified using chromatin immunoprecipitation (ChIP) assay in HTR-8 cells. We found that the promoter of the HOTAIR fragment was effectively enriched by anti-YY1 antibodies compared with the immunoglobulin G (IgG) control (Figure 3D). Moreover, YY1 overexpression was found to increase HOTAIR expression in HTR-8 cells, while its knockdown significantly decreased HOTAIR expression (Figures 3E and 3F). HOTAIR overexpression rescues the HOTAIR



**Figure 4. HOTAIR Promotes Trophoblast Invasion by Activating the PI3K-AKT Signaling Pathway**

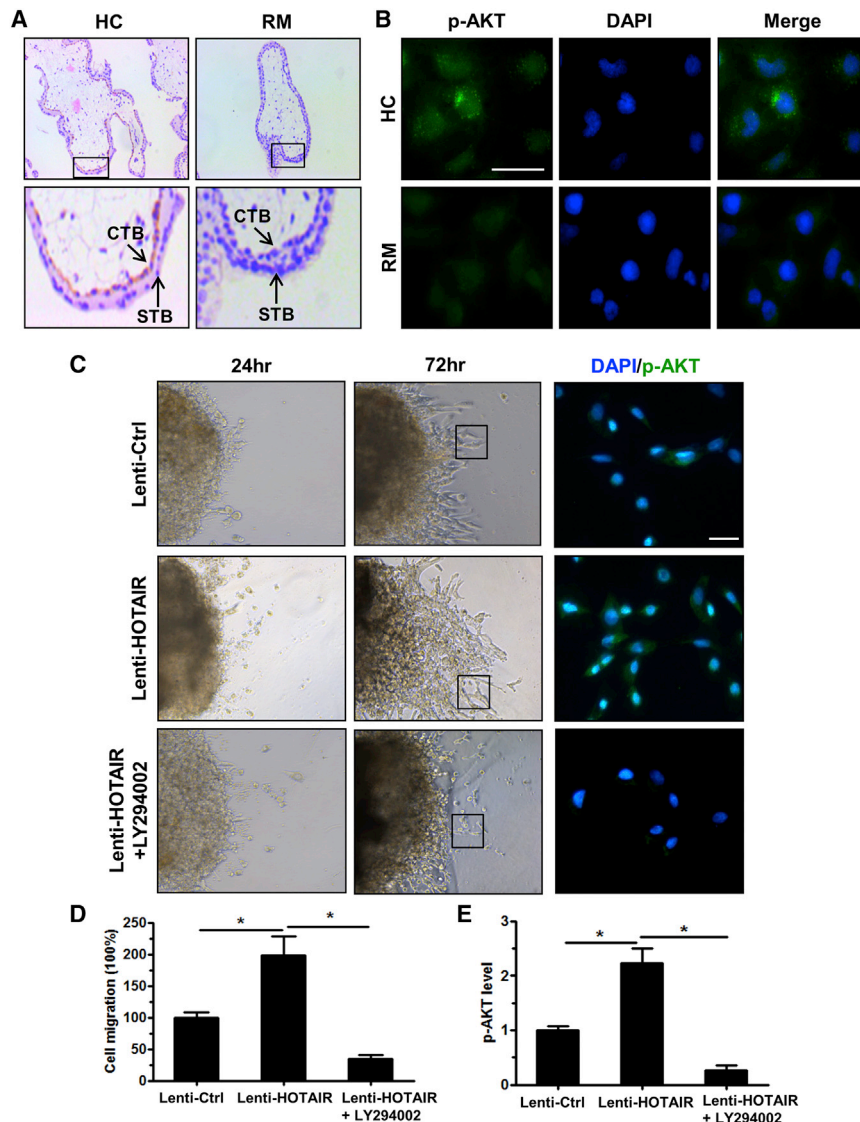
(A) Heatmap showing proteins with differential expression after HOTAIR overexpression. (B) KEGG pathway maps showed classification of differentially expressed signaling pathway after HOTAIR overexpression. (C) western blot analysis of p-AKT ser473 and AKT expression in HTR-8 cells transfected with siCtrl, siHOTAIR-1, siHOTAIR-2, and control vector, or HOTAIR-overexpressing vector after 48 hr. (D) HTR-8 cells were transfected with control vector or HOTAIR-overexpressing vector after 48 hr, and the HOTAIR-overexpressing vector group was then treated with LY294002 for 2 hr. p-AKT ser473 and AKT expression levels were determined by western blot analysis. (E) HTR-8 cells were transfected with control vector or HOTAIR-overexpressing vector for 48 hr, and the control vector or HOTAIR-overexpressing vector group was then treated with LY294002 for 2 hr. The invasive ability of the cells was assessed by crystal violet staining (original magnification  $\times 200$ ).

#### AKT Phosphorylation Is Involved in the Pathogenesis of RM

Immunohistochemical analysis was performed to address the role of p-AKT in RM. To this end, paraffin-embedded first-trimester chorionic villous tissue and rabbit IgG to human p-AKT were used. The p-AKT signal that we detected in cytotrophoblasts of chorionic villi tissues was stronger in normal tissue than in tissue obtained from RM patients (Figure 5A). In contrast, no positive p-AKT signal was found in syncytiotrophoblasts of normal controls and RM patients. These findings were confirmed by immunofluorescence, which showed that p-AKT expression was much higher in trophoblasts isolated from healthy controls than in those isolated from RM patients (Figures 5B

and S3). To further confirm the role of p-AKT in trophoblast invasion and migration in vivo, explants were freshly obtained from one placenta and separated into three groups. Explants were treated with lenti-ctrl, lenti-HOTAIR, or lenti-HOTAIR + LY294002. There was no significant difference between the groups at 24 hr of culture, when explants were anchored onto the Matrigel and started to exhibit outgrowth. At 72 hr of in vitro culture, lenti-HOTAIR group explants showed significantly greater migration distance compared with the lenti-ctrl group. However, the effect of HOTAIR on cell migration was reversed in the lenti-HOTAIR + LY294002 group. Interestingly, a whole-mount immunofluorescence assay showed that the p-AKT level was higher in the EVT explants of the lenti-HOTAIR group than in those of the lenti-ctrl group. Similar to that observed in the previous experiment, the effect of HOTAIR on p-AKT expression was reversed in the lenti-HOTAIR + LY294002 treatment group (Figures 5C–5E). This result is consistent with our finding that elevated HOTAIR levels are required to activate the PI3K-AKT pathway,

phosphorylation level of AKT in HTR-8 cells transfected with siHOTAIR-1,-2 or HOTAIR overexpression vectors. As shown in Figure 4C, HOTAIR overexpression enhanced AKT phosphorylation at Ser473, while HOTAIR knockdown significantly decreased AKT phosphorylation. Furthermore, HTR-8 cells were transfected with vector or HOTAIR overexpression plasmid for 48 hr, followed by the AKT phosphorylation inhibitor LY294002 and GDC-0941. We observed a decrease in the phosphorylation of AKT in HTR-8 cells (Figures 4D and S1). The results of the Matrigel invasion assay revealed a significantly higher number of invaded cells in HOTAIR-overexpressing HTR-8 cells compared with the control. Additionally, treating HTR-8 cells with the HOTAIR overexpression vector plus LY294002 or GDC-0941 reversed the effect of HOTAIR overexpression on cell invasion (Figures 4E and S2). These results indicate that HOTAIR promotes trophoblast invasion by activating the PI3K-AKT pathway, suggesting that HOTAIR is a critical regulator in RM.



**Figure 5. P-AKT Promotes Trophoblast Outgrowth in Extravillous Explant Cultures**

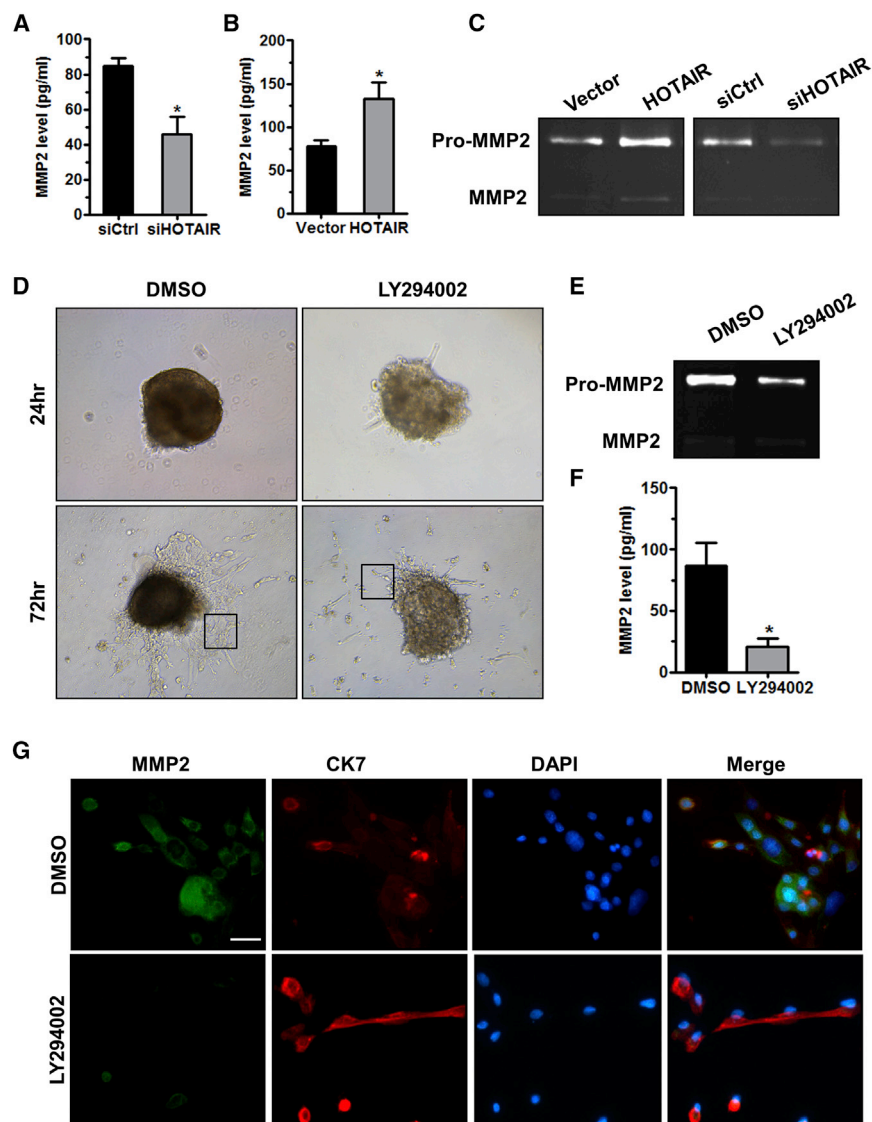
(A) Single staining of maternal villous cytotrophoblasts and syncytiotrophoblasts from RM patients (n = 15) and healthy controls (n = 15) using rabbit IgG anti-human-p-AKT ser473 antibodies developed with a streptavidin biotin+ horseradish peroxidase (HRP) labeling kit. The sections were counterstained with hematoxylin (top panels: original magnification  $\times 40$ ). (B) Representative immunofluorescence of YY1 in primary trophoblasts from first-trimester decidual tissue (6–10 weeks of gestation) of RM patients (n = 12) and healthy controls (n = 12). Fluorescence signals specific to anti-p-AKT antibodies appear green; and the DAPI-stained nuclei appear blue. Scale bars, 25  $\mu$ m. (C) Extravillous explants from healthy controls (6–10 weeks) were maintained in culture on Matrigel. Serial pictures of the explants incubated with lenti-ctrl, lenti-HOTAIR lentivirus, or lenti-HOTAIR lentivirus + LY294002 were taken under a light microscope after 24 and 72 hr of in vitro culture (original magnification  $\times 100$ ). Scale bars, 25  $\mu$ m. (D) Statistical assay of the migration distance of villous tips (%). (E) Fluorescence intensity of the p-AKT signaling was assessed Leica confocal SP8 software.

further supporting the notion that HOTAIR is an important regulator of RM.

**MMP2 Is Upregulated by the YY1-HOTAIR-PI3K-AKT Axis in Trophoblasts**

Gelatinases (MMP2 and MMP9) have been implicated in extracellular matrix (ECM) remodeling in the trophoblast invasion process.<sup>25</sup> To determine whether HOTAIR expression affects MMP2 or MMP9 production by trophoblasts, we estimated MMP2 and MMP9 expression levels in the supernatant of HTR-8 cells with HOTAIR knockdown or overexpression using ELISA. The results of the ELISA revealed that MMP2 expression was significantly higher in the HOTAIR overexpression group, while it was decreased in the knockdown group (Figures 6A and 6B). However, MMP9 levels were similar between the HOTAIR knockdown and overexpression groups

(Figure S4). We used gelatin zymography to measure MMP2 activity in the conditioned medium of the trophoblasts treated with siHOTAIR and HOTAIR-overexpressing vector. HOTAIR overexpression promoted MMP2 activity compared with the control-vector-transfected cells, whereas HOTAIR knockdown inhibited MMP2 activity (Figure 6C). Further, we used western blotting to detect YY1 expression in trophoblasts treated with control vector or HOTAIR-overexpressing vector and found that YY1 levels were similar between the control and HOTAIR overexpression groups (Figure S5). Moreover, explants were freshly obtained from a placental sample and divided into two groups. Explants were then treated with DMSO, LY294002, or GDC-0941. At 24 hr of culture, explants were anchored in Matrigel and started to exhibit outgrowth. No significant difference was observed at this point. At 72 hr of in vitro culture, migration was significantly lower in explants from the LY294002 group than in those from the DMSO-treated group (Figure 6D). These results were also confirmed by gelatin zymography (Figure 6E) and ELISA (Figures 6F and S6). Whole-mount immunofluorescence staining of villous samples showed MMP2 expression in both groups. A trophoblast cell marker, CK7, was used to identify trophoblast cells in the outgrowth area of the villous tip. MMP2 levels were significantly lower in EVT of the LY294002 group than in EVT of the DMSO group (Figure 6G). Taken together, these results indicate that the YY1-HOTAIR-PI3K-AKT-MMP2 axis is of functional importance in the regulation of trophoblast invasion.



**Figure 6. The HOTAIR/PI3K-AKT/MMP2 Axis Is Functionally Important for Regulating Trophoblast Invasion**

(A and B) ELISA analysis of MMP2 expression in HTR-8 cells which were transfected with siCtrl or siHOTAIR (A), and vector or HOTAIR-overexpressing vector (B) for 48 hr. (C) Serum-free culture medium of HTR-8 cells transfected as indicated with siCtrl, siHOTAIR, and vector or HOTAIR overexpression plasmid was collected for gelatin zymography assay. (D) Extravillous explants from healthy controls (6–10 weeks) were maintained in culture on Matrigel. Serial pictures of the explants incubated with DMSO or LY294002 were taken under a light microscope after 24 and 72 hr of culture (original magnification  $\times 100$ ). (E and F) The supernatants were collected for gelatin zymography assay and ELISA. (G) Extravillous explants were cultured on Matrigel for 72 hr. Immunofluorescence staining using anti-MMP2 antibodies showed an obvious decrease in MMP2 protein levels in the LY294002-treated group compared to the DMSO-treated group. Green fluorescence signals indicate bound anti-MMP2 antibodies, red indicates CK7 staining, and the DAPI-stained nuclei are blue. Scale bars, 25  $\mu\text{m}$ .

and human RM specimens. Therefore, these results demonstrate that HOTAIR may play a key role in trophoblast invasion and migration.

Many *in vivo* and *in vitro* studies have demonstrated that the process of invasion and migration of trophoblast cells is associated with complex biochemical interactions involving the destruction of the ECM into the damaged zone, increased cellular adhesiveness, and the increased proliferative ability of the cells.<sup>26,27</sup> MMPs, tissue inhibitors of MMPs (TIMPs), and the ECM and integrins have been reported to be related to the invasive ability of trophoblasts. These include MMP2 and MMP9 (also known as gelatinase A and B), which are capable

of digesting collagen IV, a major component of the basement membrane.<sup>28</sup> In a previous study, MMP2 and MMP9 were found to be strongly localized to the placental bed, primarily in EVT cells, in early pregnancy, and they appeared to regulate trophoblast invasion.<sup>29</sup> MMP2 has been implicated in ECM remodeling during the trophoblast invasion process.<sup>30</sup> Here, we found that HOTAIR promoted trophoblast invasion by activating AKT phosphorylation, which enhanced the expression of MMP2. Further, gene rescue assays and gelatin zymography experiments further confirmed that HOTAIR plays a key role in regulating trophoblast invasion by upregulating MMP2 expression. Moreover, our previous study reported that YY1 is a transcriptional activator of MMP2 and directly regulated its expression.<sup>12</sup> In the current study, however, YY1 was found to activate HOTAIR, which promoted PI3K-AKT signaling activation, thus enhancing MMP2 expression and consequently promoting

## DISCUSSION

It is well known that EVTs display a phenotype strikingly similar to cancer cells with their capacity for proliferation, migration, and establishment of blood supply, making them a suitable model for oncologic comparison. Trophoblast and cancer cell invasion share a series of signal transduction pathways, including the JAK-STAT pathway, focal adhesion kinases (FAKs), G proteins, Rho-associated kinase, MAPKs, PI3K-AKT, and SMAD family proteins.<sup>5</sup> In the present study, we found that p-AKT ser473 was highly expressed in trophoblasts from healthy controls (HCs) compared with RM. We established HOTAIR as an important regulator of trophoblast invasion, acting upstream of the PI3K-AKT signaling pathway (Figure 4). In the present study, multiple lines of evidence support the role of HOTAIR in mediating trophoblasts, including *in vitro* cell migration and invasion assays, *in vivo* extravillous explant cultures experiments,

trophoblast invasion. These results clearly indicate that YY1 regulates trophoblast invasion by directly and indirectly increasing MMP2 expression.

HOTAIR, an lncRNA frequently overexpressed in human cancers, was originally identified in 2007 as an lncRNA located in the HOXC cluster on chromosome 12 that regulates the HOXD gene cluster on chromosome 2. Since then, many studies have established that HOTAIR is associated with cancer cell growth and migration, and modulation of *HOTAIR* evokes pronounced differences in gene expression.<sup>31</sup> However, the underlying mechanism of HOTAIR regulation and its role in trophoblast invasion in the first trimester of pregnancy are not fully understood. In this study, we used gain- and loss-of-function analyses and determined that HOTAIR is an important regulator of trophoblast invasion. The genes downstream of HOTAIR that are regulated by this lncRNA remain to be determined. Recently, Li et al. reported that HOTAIR knockdown decreased PTEN methylation in laryngeal squamous cell carcinoma and enhanced PTEN expression in cells.<sup>32</sup> In contrast, in our study, label-free protein quantification results suggest that HOTAIR overexpression inhibited PTEN expression in HTR-8 cells. Western blotting result also demonstrated that HOTAIR overexpression increased HTR-8 cell-inhibited PTEN protein expression, while its knockdown decreased HTR-8 cell-increased PTEN expression (Figure S7). These findings revealed that HOTAIR activated the PI3K-AKT signaling pathway by inhibiting PTEN expression in trophoblasts, which in turn enhanced MMP2 expression to promote trophoblast invasion. However, the precise molecular mechanism of HOTAIR in regulation PTEN expression in trophoblasts warrants further investigation. Taken together, our results imply that HOTAIR plays an important role in trophoblast invasion.

In conclusion, we found that HOTAIR expression was upregulated in trophoblasts at the maternal-fetal interface. Interestingly, the levels of HOTAIR produced by trophoblasts were notably higher in the normal group than in the RM group, and this increase was associated with lower MMP2 production. Furthermore, HOTAIR overexpression promoted trophoblast invasion and EVT migration in an in vitro extravillous explant model. The correlation between decreased expression of HOTAIR and RM provides a pathological criterion that may be applied for the diagnosis and treatment of RM.

## MATERIALS AND METHODS

### Patient Characteristics

Between July 2015 and September 2016, 31 patients with RM (25–37 years old; mean age,  $31.7 \pm 5.3$  years) who had been treated at the Department of Obstetrics and Gynecology in the International Peace Maternity and Child Health Hospital, China Welfare Institute, Shanghai Jiao Tong University School of Medicine, China, were included in this study. Patients with the following features were excluded: (1) absence of uterine abnormalities or cervical incompetence on pelvic examination and ultrasound, (2) abnormal karyotype analysis of the parents or abortus, (3) comprehensive hormonal status assessment to rule out luteal phase defects, hyperprolactinemia and

hyperandrogenemia, and (4) no symptoms of endocrine or metabolic diseases (e.g., diabetes, hyperthyroidism, and hypothyroidism).

Additionally, 24 women aged 23–36 years (mean age,  $28.9 \pm 7.1$  years) with normal early pregnancies were recruited as healthy controls; all of these women had previous pregnancies without any history of spontaneous abortion, preterm labor, or preeclampsia. All women recruited to the control group had undergone artificial abortions to terminate their unwanted pregnancies at 6–10 weeks of gestation, and villous tissue samples were collected from these patients and stored in liquid nitrogen until analysis. The study protocol was approved by the Medical Ethics Committee of the International Peace Maternity and Child Health Hospital of China Welfare Institute, Shanghai. Written informed consents were obtained from all the participants before enrolment.

### Cell Culture

Primary trophoblasts were isolated by trypsin-DNase I digestion and discontinuous Percoll gradient centrifugation from pooled villi obtained from three to five patients, as previously described.<sup>33</sup> The resultant trophoblast cell culture had a purity of ~95%, which was determined by flow cytometry for cytokeratin 7-positive, HLA-G-positive, and vimentin-negative cells. Purified trophoblasts were seeded in the wells of 12-well plates at a concentration of  $6 \times 10^5$  cells/mL and cultured in DMEM/F12 plus 10% fetal bovine serum (FBS; GIBCO) for further experiments.

The HTR-8/SVneo cell line,<sup>34</sup> which is derived from human invasive EVTs, was a kind gift from Dr. P.K. Lala (University of Western Ontario, London, Ontario, Canada). The cells were cultured in DMEM/F12 plus 10% FBS with penicillin/streptomycin (P/S) antibiotics.

### Detection of lncRNAs in Trophoblasts

Total RNA was extracted from primary trophoblasts using TRIzol reagent according to the manufacturer's instructions. The extracted RNA was subjected to cDNA synthesis using an RT<sup>2</sup> PreAMP cDNA Synthesis Kit (QIAGEN) according to the manufacturer's instructions. The cDNA sample was then processed using the Human RT<sup>2</sup> lncRNA PCR Array Human Cell Development and Differentiation (catalog no. LAHS-003Z, QIAGEN), which contains 84 lncRNA genes known to be related to cell development and differentiation; five housekeeping genes were used for control. Statistical analysis was evaluated using the web-based RT<sup>2</sup> Profiler PCR Array Data Analysis.

### Overexpression of HOTAIR and YY1

The PLVX-IRES-ZsGreen-YY1<sup>12</sup> construct and the control vector were purified using an Endofree Plasmid kit (QIAGEN) and transfected into the cells using Lipofectamine 3000 (Life Technologies). LZRS-HOTAIR was a gift from Howard Chang (Addgene plasmid # 26110).<sup>19</sup>

### Knockdown of YY1 and HOTAIR

YY1 and *HOTAIR* knockdown was performed using specific small interfering RNAs specific for YY1 (siYY1). Unless otherwise



indicated, all oligonucleotides were purchased from GenePharma and transfected into cells at a final concentration of 100 nmol/L using Oligofectamine reagent (Invitrogen).

### Explant Culture

Small 2- to 3-mm tissue sections were obtained from the tips of the first-trimester human placental villi (6–10 weeks), dissected, and explanted in 24-well culture dishes precoated with phenol-red-free Matrigel substrate (Corning). The inserts were placed into 24-well culture dishes (Costar). The explants were cultured in DMEM/F12 media containing 10% FBS. Placental villi, anchored on Matrigel and successfully initiated to outgrow, were used for the subsequent experiments and are referred to as 24 hr samples. EVT sprouting and migration from the distal end of the villous tips were recorded daily for up to 72 hr. The extent of migration was measured at defined positions using ImagePro software. To test the effect of HOTAIR on EVT migration, two wells of culture were treated with 200 nM HOTAIR-specific siRNA or control siRNA. Extravillous explants from HCs were incubated with lenti-ctrl or lenti-HOTAIR lentiviral vectors, and images were obtained after 24 and 72 hr of in vitro culture under a light microscope. All explant experiments with cultured villi were repeated six times. Ten explants were analyzed per experiment for both the HOTAIR siRNA and control groups.

### Statistical Analysis

Data were analyzed using an independent-sample t test for comparison between the two groups, and multi-group comparison was carried out by one-way ANOVA followed by Tukey's post hoc test. Correlation was analyzed using the Spearman's rank correlation test. Data are presented as mean  $\pm$  SD. All p values are two-sided, and a p value of < 0.05 was considered statistically significant. All statistical values were calculated using SPSS 22.0.

Additional materials and methods are provided in [Supplemental Materials and Methods](#).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods, seven figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ymthe.2017.06.028>.

### AUTHOR CONTRIBUTIONS

Designed experiments, F.J.-T., L.Y., J.-X.F.; Performed experiments, Y.Z., J.-X.F., X.-C.L., F.-J.S., X.-L.M., F.W., and S.-M.Z.; Analyzed data, Y.Z., J.F., W.-H.Z., X.-R.L.; Wrote the manuscript, Y.Z., F.J., X.-C.L., Y.L., and F.-J.T.; Obtained funding and are senior authors, X.-C.L., Y.Z., Y.L., J.-X.F., and F.-J.T.

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## **Supplemental Information**

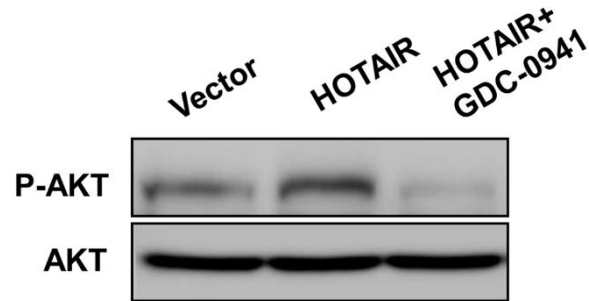
### **The YY1-HOTAIR-MMP2 Signaling Axis**

### **Controls Trophoblast Invasion**

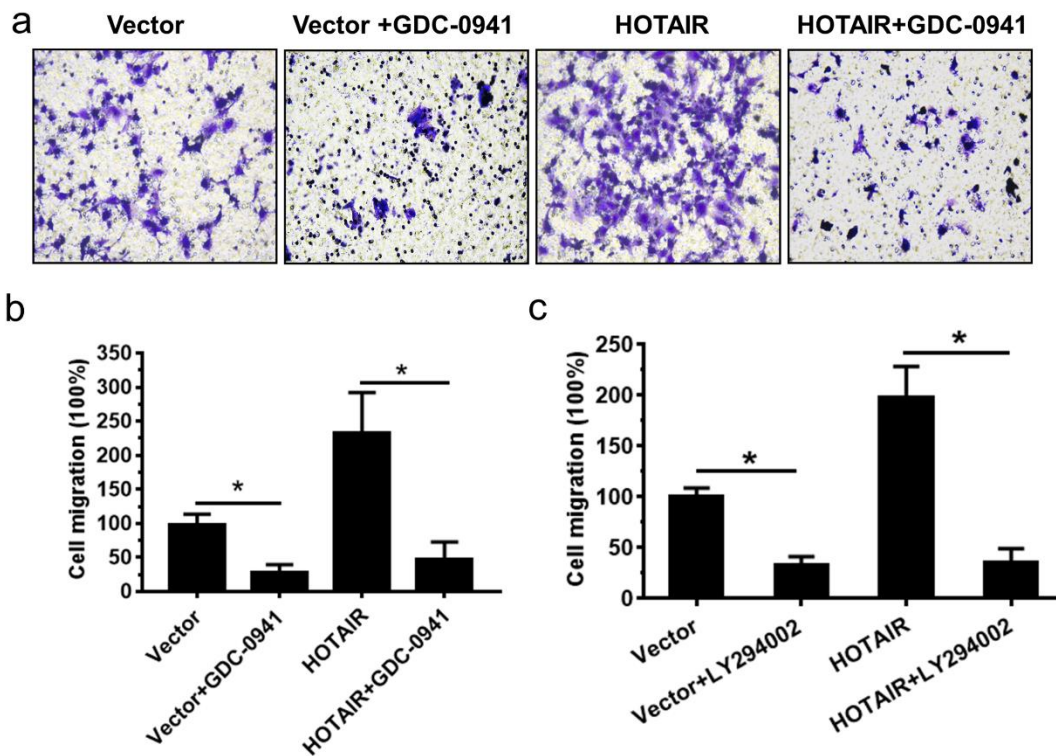
### **at the Maternal-Fetal Interface**

**Yan Zhang, Feng Jin, Xiao-Cui Li, Fu-Jin Shen, Xiao-Ling Ma, Fan Wu, Si-Ming Zhang, Wei-Hong Zeng, Xiao-Rui Liu, Jian-Xia Fan, Yi Lin, and Fu-Ju Tian**

Supplementary Figure and Figure Legends

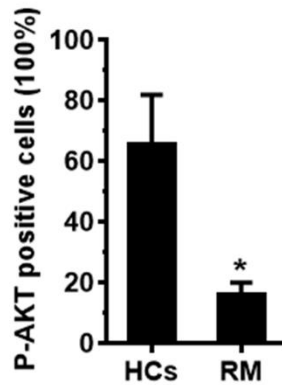


Supplementary Figure S1. HTR-8 cells were transfected with control vector or HOTAIR-overexpressing vector after 48 h, and the HOTAIR-overexpressing vector group was pretreated with GDC-0941 for 2 h. p-AKT ser473 and AKT expression levels were determined by Western blot analysis.

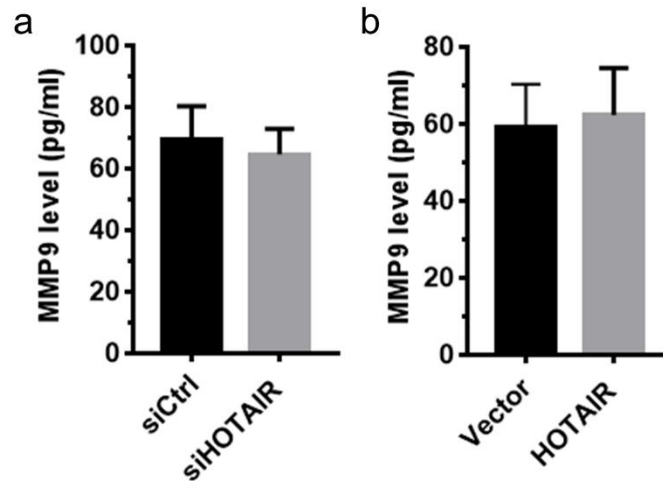


Supplementary Figure S2. (a and b) HTR-8 cells were transfected with control vector or HOTAIR-overexpressing vector, and the control vector or HOTAIR-overexpressing vector

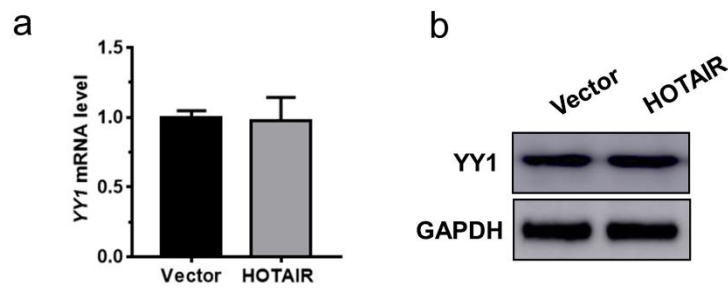
group was pretreated with GDC-0941 for 2 h. After 48 h, The invasive ability of the cells was assessed by crystal violet staining. Statistical assay of number of the cell migration (%). (c) HTR-8 cells were transfected with control vector or HOTAIR- overexpressing vector, and the HOTAIR-overexpressing vector group was pretreated with LY294002 for 2 h. Statistical assay of number of the cell migration (%).



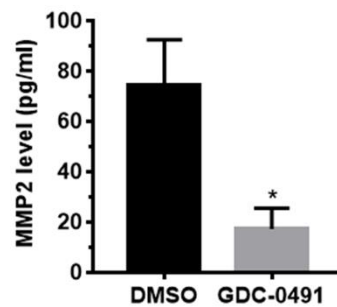
Supplementary S3. The number of p-AKT positive cells and total cell was calculated using ImagePro 6.0 software, and then the percentage of p-AKT positive cells normalized to the number of total cells in trophoblasts of RM and HCs samples was assessed.



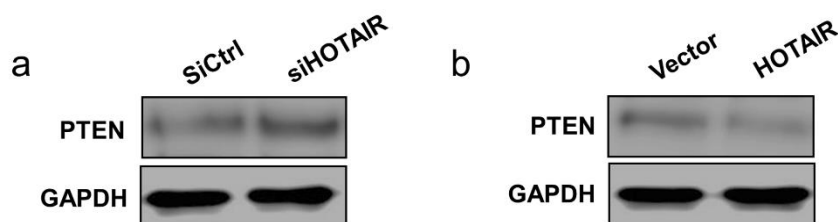
Supplementary Figure S4. (a, b) ELISA analysis of MMP-9 expression in HTR-8 cells which were transfected with siCtrl or siHOTAIR, and vector or HOTAIR-overexpressing vector for 48h.



Supplementary Figure S5. (a, b) Western blot and QRT-PCR analysis and real-time PCR were performed to determine the YY1 protein expression level in HTR-8 cells transfected with control vector or HOTAIR-overexpressing vector after 48 h.



Supplementary Figure S6. Extravillous explants from healthy controls (6-10 weeks) were maintained in culture on Matrigel. the explants incubated with DMSO or GDC-0491 for 24h. The supernatants were collected for ELISA experiment.



Supplementary Figure S7. Western blotting experiments were performed to determine the PTEN protein expression level in HTR-8 cells transfected with siCtrl, siHOTAIR, control vector, or HOTAIR-overexpressing vector after 48 h.

**Supplementary Table S1:** Complete list of the identified proteins in libel free experiments.

**Supplementary Table S2:** Complete list of the differentially expressed proteins in Libel free experiments.

## **Supplementary Materials and Methods**

### *Cell Proliferation Assays*

For cell proliferation studies,  $2 \times 10^3$  HTR-8 cells were inoculated in each well of a 96-well plate. After 24, 48, 72 and 96 h, cell viability was determined using the Cell Counting Kit-8 (CCK8) assay using a commercially available kit (Sigma, St. Louis, MO, USA). Absorbance was measured at 450 nm using a Spectra Max 190 microplate reader (BIO-RAD; Hercules, CA, USA).

### *Quantitative Real-time PCR*

Total RNA was extracted from cultured cells or primary cells using the TRIzol reagent (Life Technologies, Grand Island, NY), according to the manufacturer's instructions, and used to generate cDNA with a PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Shiga, Japan) using random or oligo-dT primers. Realtime-PCR (qRT-PCR) was performed using SYBR Green kit (Takara Bio). For *in vitro* experiments, relative expression was calculated using the  $2^{-\Delta\Delta Ct}$  method and normalized to the internal control gene *GAPDH* (human). For clinical data, relative expression was calculated using the  $2^{-\Delta Ct}$  method and normalized against *GAPDH* values. The primers were as follows: *YY1* F: 5'-AGAATAAGAAGTGGGAGCAGAAGC-3', R: 5'-ACGAGGTGAGTTCTCTCCAATGAT-3'; *HOTAIR* F: 5'-CAAACGGGACTTTGCACTCT-3', *HOTAIR* F: 5'-CAAA-CGGGACTTTGCACTCT-3', R: 5'-GCACCCCTTCTGTGTCTACAT-3'; R: 5'-GCACCC-CTTCTGTGTCTACAT-3'; *GAPDH* F: 5'-CACTGGGCTACTGAGCAC-3', R: 5'-AGTGGTCGTTGAGGGCAAT-3'.

### *Western Blotting*

Antibodies recognizing YY1 (Abcam, ab109237, Cambridge, UK), Akt (CST, #4685) and phospho-Akt (Ser473) (CST, #4060) were used to determine protein levels using western blot. GAPDH (Abcam, ab181602) was detected as loading control.

### *Nuclear Protein Extraction and Chromatin Immunoprecipitation (ChIP)*

Nuclear protein extracts were prepared from trophoblasts using a Nuclear Extraction Kit (Pierce, Rockford, USA), according to the manufacturer's protocol. ChIP was performed using a chromatin immunoprecipitation assay kit (Millipore, 17-371, Billerica, Massachusetts, USA), according to the manufacturer's protocol, using 4 µg of antibodies against YY1 (Santa Cruz, SC-1703, Texas, USA).

### *Immunohistochemistry*

Immunohistochemical staining was performed as previously described<sup>1</sup>. Human villous tissues were labeled with rabbit anti-phospho-Akt (Ser473) (dilution 1:400, CST, #4060) and AKT antibodies (dilution 1:200, CST, #4685).

### *LC-MS/MS Label-free proteomics experiments*

HTR-8 cells were transfected with vector or HOTAIR overexpression plasmid, and cells were harvested 48 hours after transfection, and cells were washed three times with ice-cold PBS. Protein extracts of HTR-8 cells were prepared using protein Extraction reagent (Pierce) according to the manufacturer's protocol. Approximately 4 µg of protein were used to perform LC-MS/MS label-free experiments according to previously described<sup>2</sup>.

### *Quantification of MMP2 and MMP9 expressions*

To evaluate the expressions of MMP2 and MMP9 in response to HOTAIR knockdown, HTR-8 cells were cultured in six well plates, and treated with siCtrl or siHOTAIR1 siRNA for



48h. After centrifugation, the supernatants were collected and analyzed using enzyme linked immunosorbent assay (ELISA) detection kits for MMP2 and MMP9, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

#### *Invasion Assay*

We evaluated the invasive ability of trophoblasts objectively across the extracellular matrix (ECM) using the Transwell Matrigel invasion assay, as previously described for trophoblasts [1]. In brief, cell culture inserts (pore size, 8  $\mu\text{m}$ ; diameter, 6.5 mm; Corning) were coated with 25  $\mu\text{L}$  of Matrigel<sup>TM</sup> (Corning, New York, USA) and placed in a 24-well plate. Two sets of invasion assays were performed: HTR-8 cells were transfected with siCtrl, siHOTAIR, control vector, or the HOTAIR overexpression vector and cultured for 48 h. Then,  $1 \times 10^5$  cells/200  $\mu\text{L}$  of DMEM were placed into the upper chamber of each insert. The lower chambers were filled with 800  $\mu\text{L}$  of DMEM containing 10% FBS, and the cells were incubated at 37  $^{\circ}\text{C}$  for 48 h. The inserts were removed, washed in ice-cold PBS, and the non-invading cells, together with the ECM, were removed from the upper surface of the filter by wiping with a cotton bud. The cells on the lower surface of the inserts were fixed in 4% paraformaldehyde, stained with crystal violet, and observed using an inverted phase-contrast microscope (Leica). The number of cells that had invaded the lower surface was counted at a magnification of  $\times 200$ . To eliminate individual variability, the results were assessed by two independent researchers, and the invasive index was calculated as the proportion of the invading cells in each experimental group expressed relative to the appropriate control cells. Each experiment was performed in duplicate and the experiments were independently repeated three times.

### *Gelatin Zymography*

Gelatinolytic activity was analyzed using 10% (w/v) polyacrylamide gels containing 0.5 mg/mL gelatin (Sigma, St. Louis, MO). Briefly, conditioned medium was diluted in 4× sample buffer (8% sodium dodecyl sulfate [SDS, w/v], 0.04% bromophenol blue [w/v], and 0.25 M Tris) and incubated at 37 °C for 30 min. Equal amounts of protein were then subjected to substrate-gel electrophoresis. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 (v/v) and 50 mM Tris-HCl (pH 7.5) for 30 min at room temperature to remove SDS, and incubated in a calcium assay buffer (50 mM Tris, 10 mM CaCl<sub>2</sub>, 1 mM ZnCl<sub>2</sub>, 1% Triton X-100 [pH 7.5]) for 24 h at 37 °C. The gel was stained with Coomassie brilliant blue R250 (Sigma, St. Louis, MO) in 50% methanol and 10% acetic acid and destained in 10% acetic acid to reveal zones with gelatinase activity. The images were captured with a gel imaging system (Tanon 3500R, Shanghai, China).

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