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Supplemental Information

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

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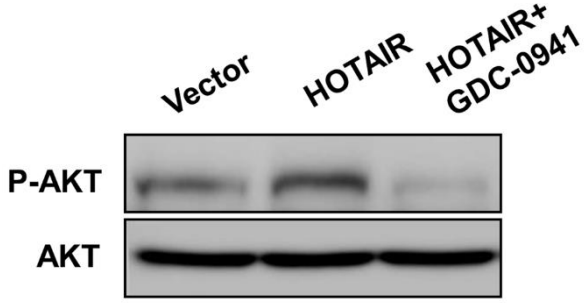
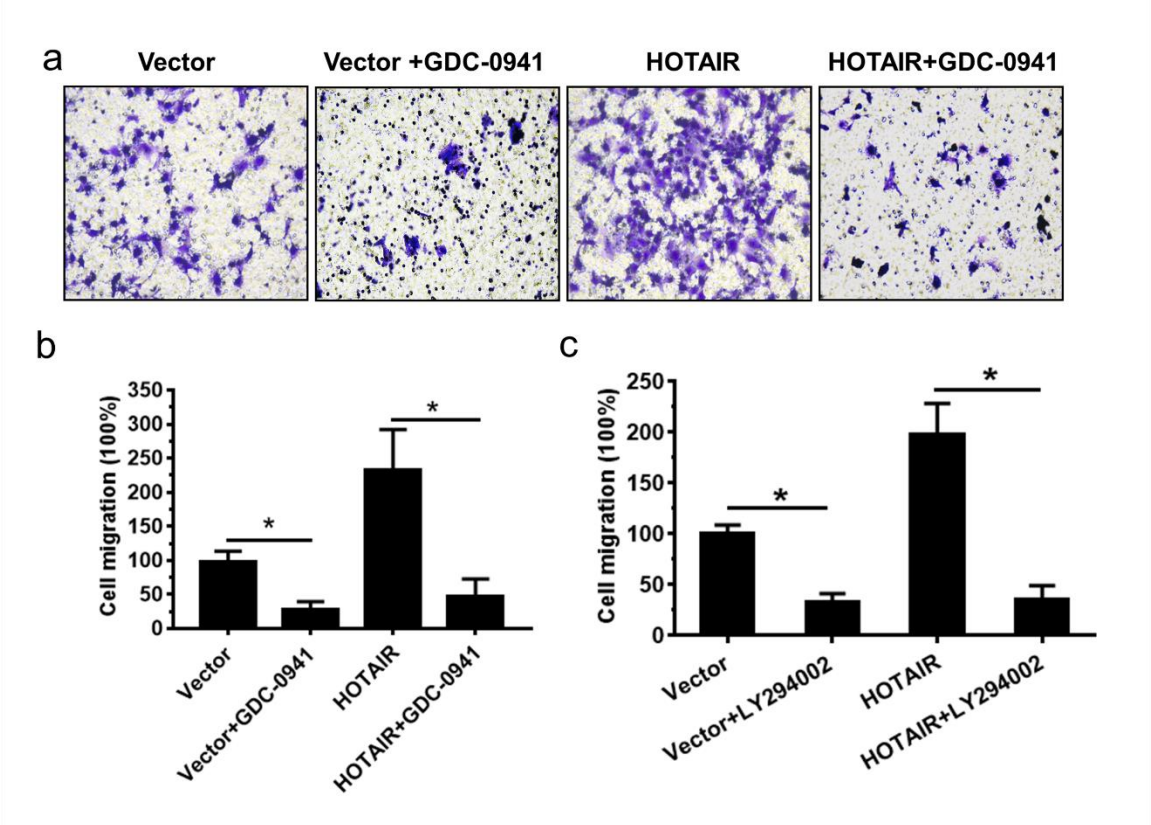
Supplementary Figure and Figure Legends

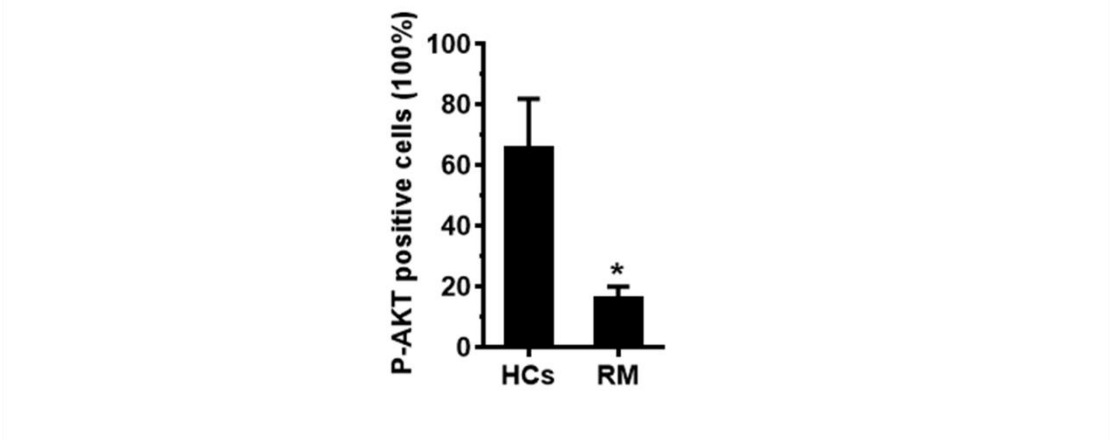


Supplementary Figure S1. HTR-8 cells were transfected with control vector or HOTAIR-overexpressing vector after 48 h, and the HOTAIR-overexpressing vector group was pretreated with GDC-0941 for 2 h. p-AKT ser473 and AKT expression levels were determined by Western blot analysis.

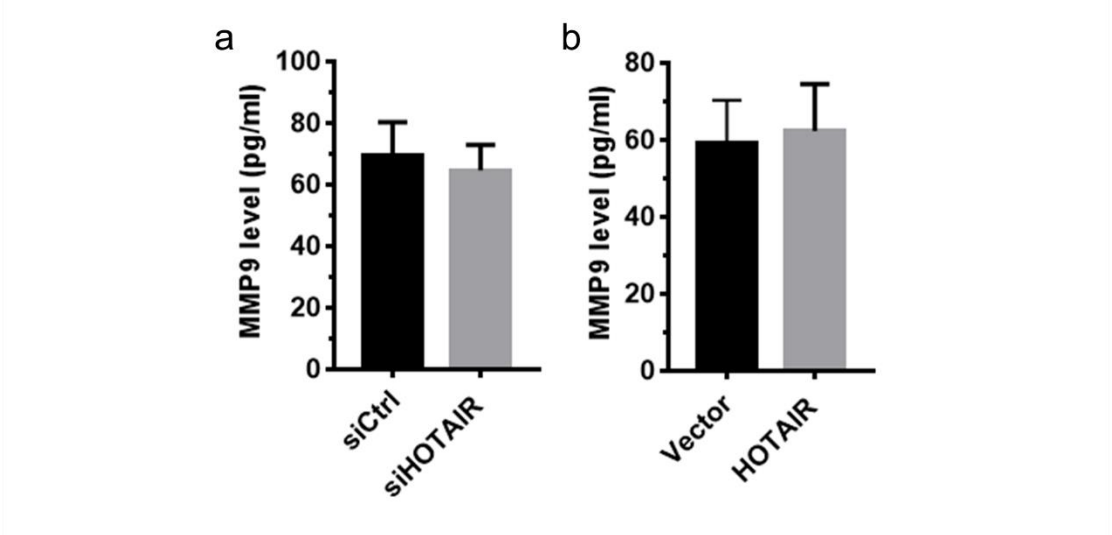


Supplementary Figure S2. (a and b) HTR-8 cells were transfected with control vector or HOTAIR- overexpressing vector, and the control vector or HOTAIR-overexpressing vector

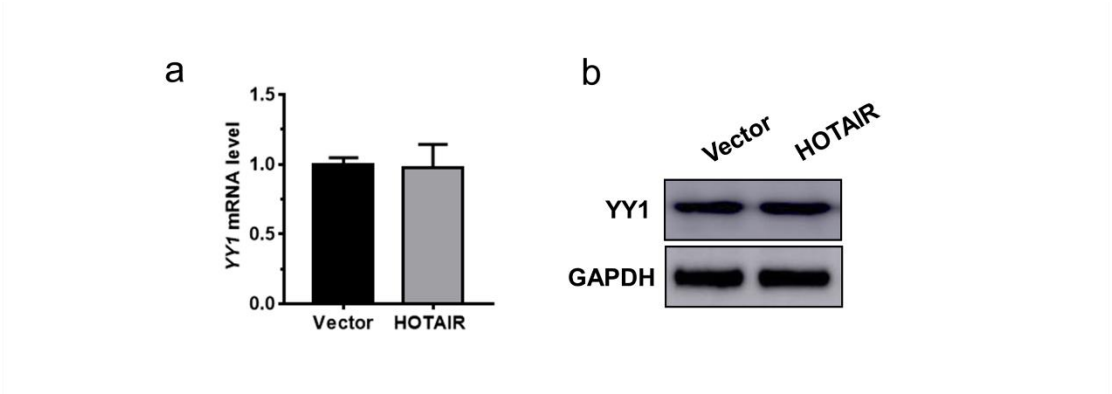
group was pretreated with GDC-0941 for 2 h. After 48 h, The invasive ability of the cells was assessed by crystal violet staining. Statistical assay of number of the cell migration (%). (c) HTR-8 cells were transfected with control vector or HOTAIR- overexpressing vector, and the HOTAIR-overexpressing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



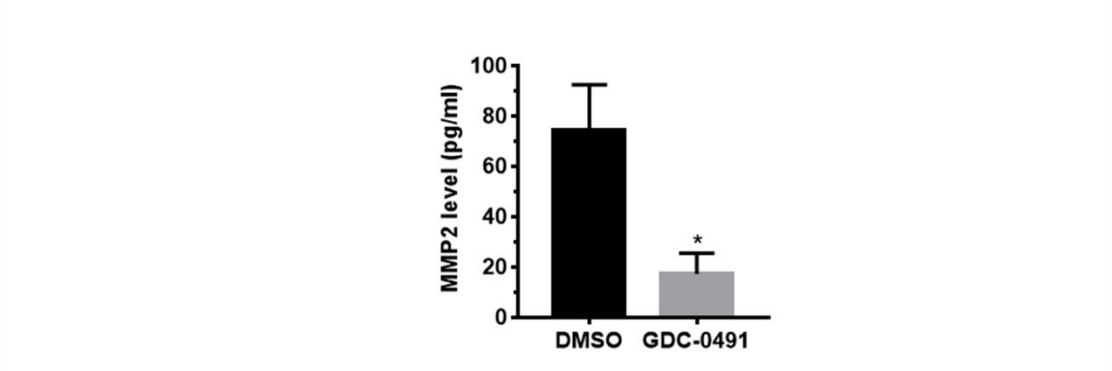
Supplementary S3. The number of p-AKT positive cells and total cell was calculated using ImagePro 6.0 software, and then the percentage of p-AKT positive cells normalized to the number of total cells in trophoblasts of RM and HCs samples was assessed.



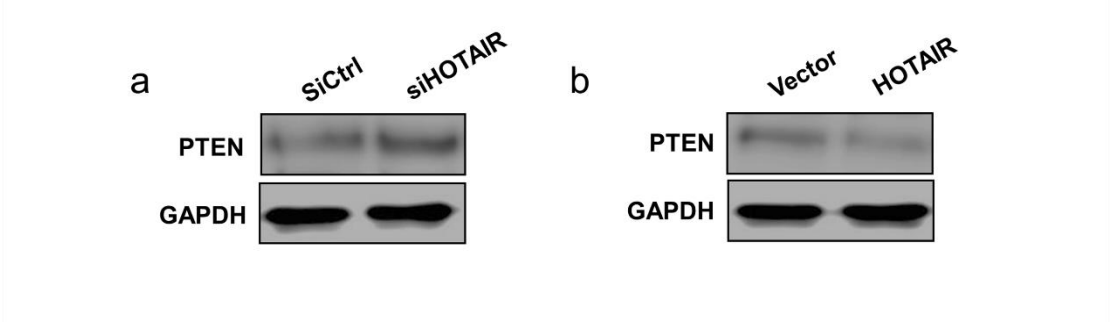
Supplementary Figure S4. (a, b) ELISA analysis of MMP-9 expression in HTR-8 cells which were transfected with siCtrl or siHOTAIR, and vector or HOTAIR-overexpressing vector for 48h.



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, 2×10^3 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′-ACGAGGTGAGTTCTCTCCAAT-GAT-3′; *HOTAIR* F: 5′- CAAACGGGACTTTGCACTCT-3′, *HOTAIR* F: 5′ -CAAA-CGGGACTTTGCACTCT-3′, R: 5′-GCACCCCTTCTGTGTCTACAT-3′; R: 5′-GCACCC-CTTCTGTGTCTACAT-3′; *GAPDH* F: 5′-CACTGGGCTACACTGAGCAC-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[1]. 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 proteimics 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 regent (Pierce) according to the manufacturer's protocol. Approximately 4μ g of protein were used to performed LC-MS/MS label-free experiments according to previously described[2].

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 μm; diameter, 6.5 mm; Corning) were coated with 25 μL of Matrigel™ (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, 1×10^5 cells/200 μL of DMEM were placed into the upper chamber of each insert. The lower chambers were filled with 800 μL of DMEM containing 10% FBS, and the cells were incubated at 37℃ 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 × 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℃ 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 CaCl2, 1 mM ZnCl2, 1% Triton X-100 [pH 7.5]) for 24 h at 37℃. 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).

References

1. Qiu Q, Yangi MY, Tsang BK, et al. 2004 Both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signalling are required in epidermal growth factor-induced human trophoblast migration. *Mol Hum Reprod*.**10**:677-684.
2. Tang X, Meng Q, Gao J, Zhang S, Zhang H, Zhang M.. 2015. Label-free Quantitative Analysis of Changes in Broiler Liver Proteins under Heat Stress using SWATH-MS Technology. *Sci Rep*. **5**:15119.