Supplemental Information

Elevated Tristetraprolin Impairs Trophoblast Invasion in Women with Recurrent Miscarriage by Destabilization of HOTAIR

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Supplementary Materials and Methods

Cell Proliferation Assays

For cell proliferation studies, HTR-8 cells were transfected with siCtrl, shTTP #2, 3 or vector, TTP express vector for 24 h, and then the cells were digested using trypsin. 2.5 × 10^3 HTR-8 cells were plated in each well of a 96-well plate. After 24, 48, 72 and 96 h, the cell viability was determined using the Cell Counting Kit-8 (CCK8) assay using a commercially available kit (Dojindo Molecular Technologies, Inc). 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^{−ΔΔCt} method and normalized to the internal control gene GAPDH (human). For clinical data, relative expression was calculated using the 2^{−ΔCt} method and normalized against GAPDH values. The primers were as follows: TTP F: 5’- CAAGACTGAGCTATGTCGGA-CCT-3’, R: 5’-ATGATGAAAGTGCGAGAGAG-3’; HOTAIR F: 5’-CAAAACGGG-ACTTTGCACCTCT-3’, R: 5’-GCACCCCTTCTGTGTCTACAT -3’; GAPDH F: 5’-CACTGGGCTACACTGAGC-3’, R: 5’-AGTGGTCGTTGAGGGCAA -T-3’.
Immunofluorescence

Primary trophoblasts were isolated from villi tissue of normal controls and RM patients and cultured on poly-L-lysine–coated coverslips in 24-well plates for 24 hours, and then washed three times with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde-PBS for 10 minutes, and stained with IgG (mouse Isotype Controls; ab37355) or TTP antibody [mouse anti-TTP monoclonal antibody (1:100; Santa Cruz Biotechnology)] overnight at 4°C. The next day, secondary fluorescent Alexa Fluor 488 donkey anti-mouse IgG (H+L) (Life Technologies) were used after the plates had been washed three times with PBS. Nuclei were counterstained using 4,6-diamidino-2-phenylindole (DAPI; Abcam). Coverslips were mounted on the glass slides and the slides were visualized using a Leica microscope (Wetzlar, Germany). For the chorionic tissues, rabbit anti-TTP polyclonal antibody (1:50; Santa Cruz Biotechnology) or CK7 (Rabbit anti-CK7 monoclonal (1:100; abcam, ab181598) was used as primary antibody.

Wound Healing Assay

HTR-8 cells were transfected with shctrl, shRNA, empty vector, or the TTP-expressing plasmid. After 36 hours, the cells reached approximately 85% confluency. Then, a sterile 1-mL pipette tip was used to scrape a cross in the center of the monolayer, and the cells were rinsed three times with ice-cold PBS and cultured in serum-free medium. The cells were allowed to migrate for 16 hours, and the gaps were observed and photographed. Light and fluorescent microscopic images were captured using a Leica DMI 3000B microscope. The sizes of the gaps were measured.
from the photomicrographs.

*Migration and 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. 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 and primary trophoblasts were transfected with shCtrl, shTTP, control vector, or the TTP expression vector and cultured for 48 h. Then, $1 \times 10^5$ 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°C for 48 hr. 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.

*Nuclear Protein Extraction and Western Blot Analysis*
Nuclear protein extracts were prepared from trophoblasts using the Nuclear Extraction Kit (Pierce, Rockford, IL) according to the manufacturer’s protocol. Antibodies against TTP (dilution 1:500; Santa Cruz Biotechnology) and hnRNPA1 (Abcam, ab177152) were used to perform Western blot analysis using standard techniques. Antibodies against GAPDH (Abcam, ab181602) or Lamin B (dilution 1:2000; Santa Cruz, Dallas, TX) were used as loading controls.

Immunohistochemistry

Immunohistochemical staining was performed as previously described¹. Human villous tissues were labeled with rabbit IgG (dilution 1:100, abcam, ab172730) or anti-TTP (dilution 1:100, Santa Cruz Biotechnology, sc-14030).

References:

1. Qiu Q, Yang M, Tsang BK, Gruslin A. Both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signalling are required in epidermal growth factor-induced human trophoblast migration. Mol Hum Reprod. 2004;10(9):677-84.