Journal of Pathology
*J Pathol* 2016; **239:** 36–47
Published online 27 March 2016 in Wiley Online Library
(wileyonlinelibrary.com) DOI: 10.1002/path.4694

ORIGINAL PAPER

# The YY1/MMP2 axis promotes trophoblast invasion at the maternal–fetal interface

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## Abstract

**YY1 is a sequence-specific DNA-binding transcription factor that has many important biological roles. However, its function in trophoblasts at the maternal–fetal interface remains to be elucidated. In this study, we used an mRNA microarray and reverse transcription qPCR and compared the *YY1* mRNA expression level in trophoblasts between patients with recurrent miscarriage (RM) and healthy control subjects. Our results revealed that *YY1* mRNA expression was significantly lower in the trophoblasts of the RM group compared with the healthy control group. Furthermore, immunofluorescence and immunohistochemical data showed that YY1 was highly expressed in human placental villi during early pregnancy, especially in cytotrophoblast cells and invasive extravillous trophoblasts, and it was expressed at a much lower level in the placental villi of term pregnancy. YY1 overexpression enhanced, and knockdown repressed, the invasion and proliferation of trophoblasts. Antibody array screening revealed that YY1 significantly promoted MMP2 expression in trophoblasts. Bioinformatics analysis identified three YY1-binding sites in the MMP2 promoter region, and chromatin immunoprecipitation analysis verified that YY1 binds directly to its promoter region. Importantly, inhibition of YY1 by siRNA clearly decreased trophoblast invasion in an *ex vivo* explant culture model. Overall, our findings revealed a new regulatory pathway of YY1/MMP2 in trophoblast cell invasion during early pregnancy and indicated that YY1 may be involved in the pathogenesis of RM.**
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**Keywords:** YY1; MMP2; trophoblast invasion; recurrent miscarriage; maternal–fetal interface

Received 2 September 2015; Revised 22 December 2015; Accepted 20 January 2016

*No conflicts of interest were declared*

## Introduction

Human pregnancy is a complex biological process that requires synchrony between the developing embryo and the receptive uterine endometrium [1]. Successful implantation of the embryo depends on embryo hatching, trophoblast development, and proper maternal–fetal cross talk and immune regulation [2]. Currently, two major trophoblast cell lineages have been identified during the early stages of human placental development: villous trophoblasts and extravillous trophoblasts (EVTs). Villous cytotrophoblasts (CTBs) are trophoblast progenitor cells and follow one of two differentiation pathways to form syncytiotrophoblasts (STBs) or EVTs [3]. EVTs, which are derived from trophoblasts by epithelial–mesenchymal transition, form cell columns and have a highly invasive character, causing them to migrate away from the attached embryo and invade the uterine epithelium and uterine spiral arteries to establish maternal–fetal linkage. Poor EVT migration and invasion often result in failure to establish the maternal–fetal connection and are associated with pre-eclampsia, fetal growth restriction, and early and late recurrent miscarriage (RM) [4]. RM is associated with a high level of psychological distress; therefore, comprehensive molecular studies are required to understand the causes of RM and to develop potential molecular targets.

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Trophoblast invasion is regulated temporally and spatially in an autocrine or paracrine manner by trophoblast and uterine factors at the maternal–fetal interface [5]. This regulation guarantees that the invasion of trophoblast cells occurs only during the early stages of gestation and is restricted to the entire maternal endometrium and the upper-third of the myometrium [6]. Trophoblast invasiveness is regulated by a complex network of cell types, mediators, and signalling pathways. For example, CTBs secrete matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), which help to balance and tightly control the invasion of EVTs into the maternal endomyometrium [7]. Several studies have shown that MMP2 and MMP9 synthesis and activation are required for trophoblast invasion [8,9].

YY1, also known as Yin Yang 1, NF-E1, UCRBP, and CF1, is a ubiquitously distributed transcription factor belonging to the *GLI*-Krüppel class of zinc finger proteins. YY1 is involved in repressing and activating a diverse number of promoters and plays an essential role in various biological functions via DNA– and protein–protein interactions with numerous partners [10]. YY1 is also involved in the regulation of a broad spectrum of cellular processes, such as embryogenesis, proliferation, and tumourigenesis [11,12]. Several studies have found that YY1 activity is essential for mammalian development, as YY1-null embryos die at the peri-implantation stages of embryogenesis [13,14]. Moreover, YY1 activity is also necessary for adult tissue development: oligodendrocyte-specific depletion of YY1 caused serious neural defects [15]. As a highly expressed gene in the placenta villi, however, YY1's role in human placentation or implantation has not yet been reported.

In this study, we found that RM patients have a significantly lower level of YY1 in their villi compared with their age-matched normal counterparts, which was consistent with the finding that YY1 knockdown inhibits trophoblast invasion and migration. Interestingly, silencing of YY1 inhibited the outgrowth capacity of human villi significantly in an explant culture model by inhibiting MMP2. These results suggested that YY1 might play a key role in trophoblast invasion and migration.

## Materials and methods

Detailed descriptions of the materials and methods may be found in the Supplementary methods.

### Patient characteristics

Thirty-one patients with RM between 23 and 35 years of age (mean age $29.4 \pm 5.9$ years), 13 patients with one miscarriage between 24 and 33 years of age (mean age $28.1 \pm 3.9$ years), and 15 patients with two miscarriages between 23 and 34 years of age (mean age $27.8 \pm 4.2$ years), who had been treated at the Department of Obstetrics and Gynecology of the International Peace Maternity & Child Health Hospital of the China Welfare Institute, Shanghai Jiao Tong University School of Medicine, China between July 2014 and February 2015, were included in this study. Patients with the following features were excluded: (1) absence of uterine abnormality or cervical incompetence on pelvic examination and ultrasound; (2) abnormal karyotype analysis of the parents or abortus; (3) symptoms of endocrine or metabolic diseases (diabetes, hyperthyroidism, and hypothyroidism).

Another 36 women between 22 and 34 years of age (mean age $27.3 \pm 5.4$ years) with normal early pregnancies were recruited as healthy controls. All of these women had experienced previous pregnancies without any history of spontaneous abortion, preterm labour, or pre-eclampsia. These patients had undergone artificial abortions to terminate their unwanted pregnancies at 6–12 weeks of gestation, and samples of villous tissues were collected and stored in liquid nitrogen. The study protocol was approved by the Medical Ethics Committee of the International Peace Maternity & Child Health Hospital of the China Welfare Institute, Shanghai. Written informed consent was 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, based on a previous description that only $3–4 \times 10^6$ trophoblasts could be harvested from one patient [16]. The obtained trophoblasts were seeded in cell culture plates for further purification based on differential adherent velocities to eliminate adherent fibroblast cells and unattached leukocytes. This method resulted in trophoblast cell lines with approximately 95% culture purity, as assessed by flow cytometry for cytokeratin 7 positivity, HLA-G positivity, and vimentin negativity. Purified trophoblasts were seeded in 12-well plates at a concentration of $6 \times 10^5$ cells/ml for further experiments.

The HTR-8/SVneo cell line [17], which was derived from human invasive EVTs, was a kind gift from Dr PK Lala (University of Western Ontario, London, Ontario, Canada). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 plus 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) with antibiotics.

### Overexpression of YY1 and construction of lentivirus

To generate a construct overexpressing YY1, the coding region sequence (CDS) of human *YY1* was cloned into the pLVX-IRES-ZsGreen vector (Clontech Laboratories, Mountain View, CA, USA) using the primers CDS F: 5′-ATTGAATTCGAGCC CTCAGCCATGGCCT-3′ and R: 5′-GCGCGGATCCC TCTTCTTTTCACTGGTTGT-3′. All constructs were verified by sequencing (Life Technologies, New York,

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USA). The PLVX-IRES-ZsGreen-YY1 construct and the control vector were purified using an Endofree Plasmid kit (Qiagen, Duesseldorf, Germany) and transfected into cells using Lipofectamine 3000 (Life Technologies). For lentivirus construction, the precursor sequence of the YY1 CDS was inserted into the pLVX-IRES-ZsGreen vector and then co-transfected with VSVG and PAX2 plasmids into HEK293 cells to produce lentivirus overexpressing YY1.

### Knockdown of *YY1*

*YY1* knockdown was performed using a specific small interfering RNA (siYY1). Unless otherwise indicated, all oligonucleotides were purchased from GenePharma Inc (Shanghai, China) and transfected into the cells at a final concentration of 100 nmol/l using Oligofectamine regent (Life Technologies).

### Extravillous explant culture

Explant culture was performed as described previously [18]. In brief, small 2–3 mm tissue samples were obtained from the tips of first-trimester human placental villi (8–10 weeks), dissected, and explanted in 24-well culture dishes pre-coated with phenol red-free Matrigel substrate. Inserts were placed into 24-well culture dishes (Costar, Cambridge, MA, USA). The explants were cultured in DMEM/F12 media with 5% FBS. Placental villi, anchored on Matrigel and successfully initiated to outgrow, were used for subsequent experiments and referred to as 24 h samples. EVT sprouting and migration from the distal end of the villous tips were recorded daily for up to 3 days. The extent of migration was measured at defined positions with the help of ImageJ Pro software. To test the effect of YY1 on the migration of EVTs, 100 nM siRNA specifically targeting YY1 or an equal concentration of control siRNA was introduced into two wells of culture media. Extravillous explants from patients with RM were incubated with lenti-ctrl or lenti-YY1 lentiviral contracts, and images after 24 and 72 h of *in vitro* culture were taken under a light microscope. All explant experiments with cultured villi were repeated three times. In each experiment, ten explants were analysed for both the YY1 siRNA and control groups ($n = 10$ in each group).

### HTA 2.0 transcriptome microarray assay

Total RNA was isolated and biotinylated cDNA prepared and hybridized to Human Transcriptome Array 2.0 GeneChips. Washed and stained GeneChips were assessed and $\log_2$ transformed, RMA signal intensities calculated and displayed as heatmaps to help visualize differential expression. Details are provided in the Supplementary methods.

### Confocal imaging

HTR-8 cells were cultured on poly-L-lysine-coated coverslips in 24-well plates, transfected with siCtrl or siYY1, cultured for 48 h, washed three times with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde–PBS for 10 min, and stained with primary antibodies (1:200 dilution for all antibodies, except 1:500 for anti-YY1), using standard immunofluorescence protocols.

### Wound healing assay

HTR-8 cells were transfected with siCtrl, siRNA or YY1 overexpression plasmid and plated. After 48 h, when the cells reached 80% confluence, scratch wounds were created with a pipette tip; cells were washed three times with PBS; and wound gaps were imaged and measured 48 h later.

### Reverse transcription quantitative PCR (RT-qPCR)

RNA was extracted from cells using the TRIzol reagent (Life Technologies, Grand Island, NY, USA) and cDNA generated using a PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Shiga, Japan) with random or oligo-dT primers. Quantitative PCR was performed using an SYBR Green kit (Takara Bio). For *in vitro* experiments, relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to *GAPDH*. For clinical data, relative expression was calculated using the $2^{-\Delta Ct}$ method normalized against *GAPDH*. The primers were as follows: *YY1* F: 5′-AGAATAAGAAGTGGGAGCAGAAGC-3′, R: 5′-ACGAGGTGAGTTCTCTCCAATGAT-3′; and *GAPDH* F: 5′-CACTGGGCTACACTGAGCAC-3′, R: 5′-AGTGGTCGTTGAGGGCAAT-3′.

### Western blotting

Antibodies recognizing YY1 (ab109237; Abcam, Cambridge, UK) and MMP2 (ab37150; Abcam) were used to determine protein levels using western blot. GAPDH (ab181602; Abcam) was detected as a loading control.

### Nuclear protein extraction and chromatin immunoprecipitation (ChIP)

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

### Immunohistochemistry

Immunohistochemical staining was performed as previously described [19]. Human villous tissues were labelled with rabbit anti-YY1 antibodies (ab109237, dilution 1:500; Abcam).

### Quantibody® human MMP array

Human MMP antibody array analysis was performed using the Quantibody Human MMP Array 1

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(RayBiotech Inc, Norcross, GA, USA) as detailed in the Supplementary methods.

### 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 [20]. Details are provided in the Supplementary methods.

### Gelatin zymography

Gelatinolytic activity was analysed using 10% (w/v) polyacrylamide gels containing 0.5 mg/ml gelatin (Sigma, St Louis, MO, USA). Details are provided in the Supplementary methods.

### Statistical analysis

Data were analysed using an independent sample *t*-test for comparison between the two groups. Comparison among multiple groups was carried out by one-way ANOVA followed by Tukey's *post-hoc* test. Correlations were analysed using Spearman's rank correlation test. Data are presented as means ± SD. All $p$ values are two-sided. $p < 0.05$ was considered statistically significant. All statistical calculations were performed using SPSS for Windows, Version 16.0 (SPSS Inc, Chicago, IL, USA).

## Results

### YY1 is down-regulated in trophoblasts from RM patients

A previous study demonstrated that insufficient proliferation and invasion of CTBs is associated with early or late RM [21]. To explore the genes involved in the pathogenesis of RM, we analysed the expression profiles of trophoblasts derived from RM and healthy controls using whole-genome microarrays. A heatmap analysis clearly showed that transcripts of *CDC20*, *CTSF*, *CCR7*, *NUF2*, and some others were at higher levels in trophoblasts from the RM group. By contrast, transcripts of *IGFBP1*, *YY1*, *FGF7*, *CCNA2*, and some others were less abundant in primary trophoblasts (Figure 1A). We validated our microarray results using reverse transcription (RT) qPCR and analysed the list according to gene function. We used RT-qPCR to evaluate the modulation of 16 genes in the same trophoblasts (Figures 1B and 1C). Both microarray and RT-qPCR results showed similar modulations. Interestingly, the level of transcripts for *YY1*, encoding a transcription factor involved in complex biological functions, including apoptosis, tumourigenesis, development, and differentiation [10–12], was lower in trophoblasts of the RM group compared with the HC (healthy controls) group. Furthermore, analysis using the online STRING database revealed that YY1 might interact with proteins involved in epigenetic regulation (Supplementary Figure 1). These results indicated that YY1 expression was significantly inhibited in chorionic villi from RM patients and implied that YY1 might be involved in the pathogenesis of RM.

### YY1 is decreased in EVTs and CTBs from RM patients

RT-qPCR and western blotting analyses of first-trimester chorionic villous tissues were performed to explore whether YY1 is involved in the pathogenesis of RM. Our results showed that YY1 expression was significantly decreased in the chorionic villous tissues of RM patients compared with healthy controls (Figures 2A and 2B). Immunohistochemical analysis of paraffin-embedded first-trimester chorionic villous tissues and third-trimester placental tissues was performed to further investigate the localization of YY1 in chorionic villous tissue. Strong expression of YY1 was observed in normal chorionic villous tissue in the controls, with the staining mainly distributed in CTBs, but not in STBs. A weak positive signal for YY1 was detected in the chorionic villous tissue in the RM group and in trophoblasts from third-trimester placental tissue (Figures 2C and 2D). In addition, immunofluorescence staining was used to confirm the expression of YY1 in the paraffin-embedded sections from first-trimester human villi and maternal decidua. Fluorescence staining using an anti-YY1 antibody revealed that YY1 was mainly expressed in CTBs (Figures 2E and 2F). Further, double immunofluorescence staining for cytokeratin 7 (CK7) with YY1 was performed; strong staining for YY1 was observed in invasive EVTs (CK7-positive) in the maternal decidua of the HC group compared with that of the RM group. Subsequently, the percentage of $CK7^+/YY1^+$ invading trophoblasts in decidua tissue of RM and HC samples was calculated (Figures 2G and 2H), which indicated that impaired YY1 expression in RM patients might decrease the EVT cells' ability to invade the decidua layer. Taken together, these results implied that the YY1 level in CTBs and EVTs was decreased in patients with RM and the decrease might be correlated with trophoblast proliferation and invasion.

### YY1 promotes the proliferation and invasion of trophoblasts *in vitro*

As YY1 was expressed in the CTBs and EVTs in first-trimester placentas [22], we next investigated whether YY1 is involved in the proliferation and migratory capacity of trophoblasts. To this end, we used HTR-8/SVneo (HTR-8) cells, a first-trimester human EVT-derived cell line. The cells were transfected with siYY1 or the YY1-overexpressing vector. YY1 expression was decreased after transfection of siYY1 and up-regulated after transfection of the YY1-overexpressing vector (Figure 3A). The MTT assay demonstrated that overexpression of YY1 increased the proliferation of HTR-8 cells, whereas

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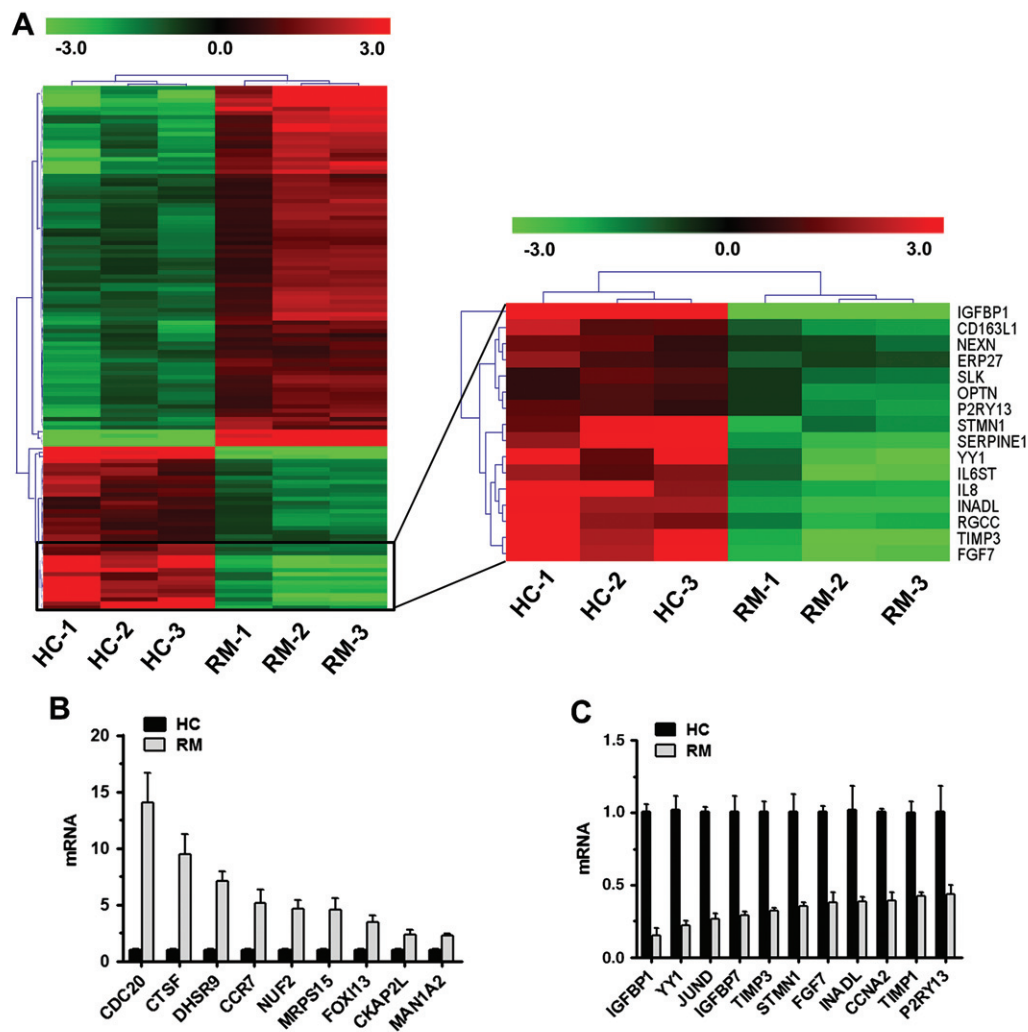


**Figure 1.** MRNA expression profiling in trophoblasts isolated from patients with recurrent miscarriage and healthy control subjects. (A) Heatmap of normalized expression levels of trophoblast cell genes isolated from recurrent miscarriage (RM) patients and healthy controls (HC). Green indicates low expression levels; red indicates high expression levels. (B, C) Real-time PCR was performed to determine the mRNA levels of the typical genes in cytotrophoblasts isolated from the RM and HC groups.

knockdown of YY1 decreased HTR-8 proliferation (Figure 3B). To further confirm the role of YY1 in trophoblast invasion and migration, wound healing and Matrigel cell invasion assays were conducted. The results revealed that YY1 overexpression significantly increased the migratory and invasive ability of HTR-8 cells, whereas knockdown of YY1 obviously reduced migration and invasion (Figures 3C–3G and Supplementary Figure 2). These results suggested to us that YY1 might play a key role in trophoblast proliferation and invasion.

## YY1 regulates the migration of EVTs in an extravillous explant culture model

To further confirm the role of YY1 in trophoblast invasion and migration *in vivo*, extravillous explants from first-trimester villi from the RM and control groups (8–10 weeks of gestation) were cultured on Matrigel-coated dishes. The distance of outgrowth on the Matrigel surface was measured at 24 and 72 h and used as a marker of EVT migration ability. Following 24 h of culture, the explants were anchored onto the Matrigel and started to exhibit outgrowth. No significant difference was observed between the control and RM groups at this point. At 72 h of *in vitro* culture, the RM explants had migrated shorter distances compared with the control explants (Figures 4A and 4B). Interestingly, a whole-mount immunofluorescence assay showed that the YY1 level was obviously decreased in the control EVT explants compared with the RM explants (Figure 4C); the percentage of $CK7^+/YY1^+$ invading trophoblasts in the RM and HC groups was also calculated and this indicated that impaired YY1 expression in RM patients decreases the invasion ability of EVT cells (Figure 4D). In a further experiment, explants were freshly obtained from healthy villi and separated into two groups. One group was treated with siCtrl and the other group was treated with siYY1. After 24 h of culture, these Matrigel explants started to display outgrowth, but there were no significant differences between the siCtrl and siYY1-treated groups. Following 72 h of *in vitro* culture, however, the siYY1-treated

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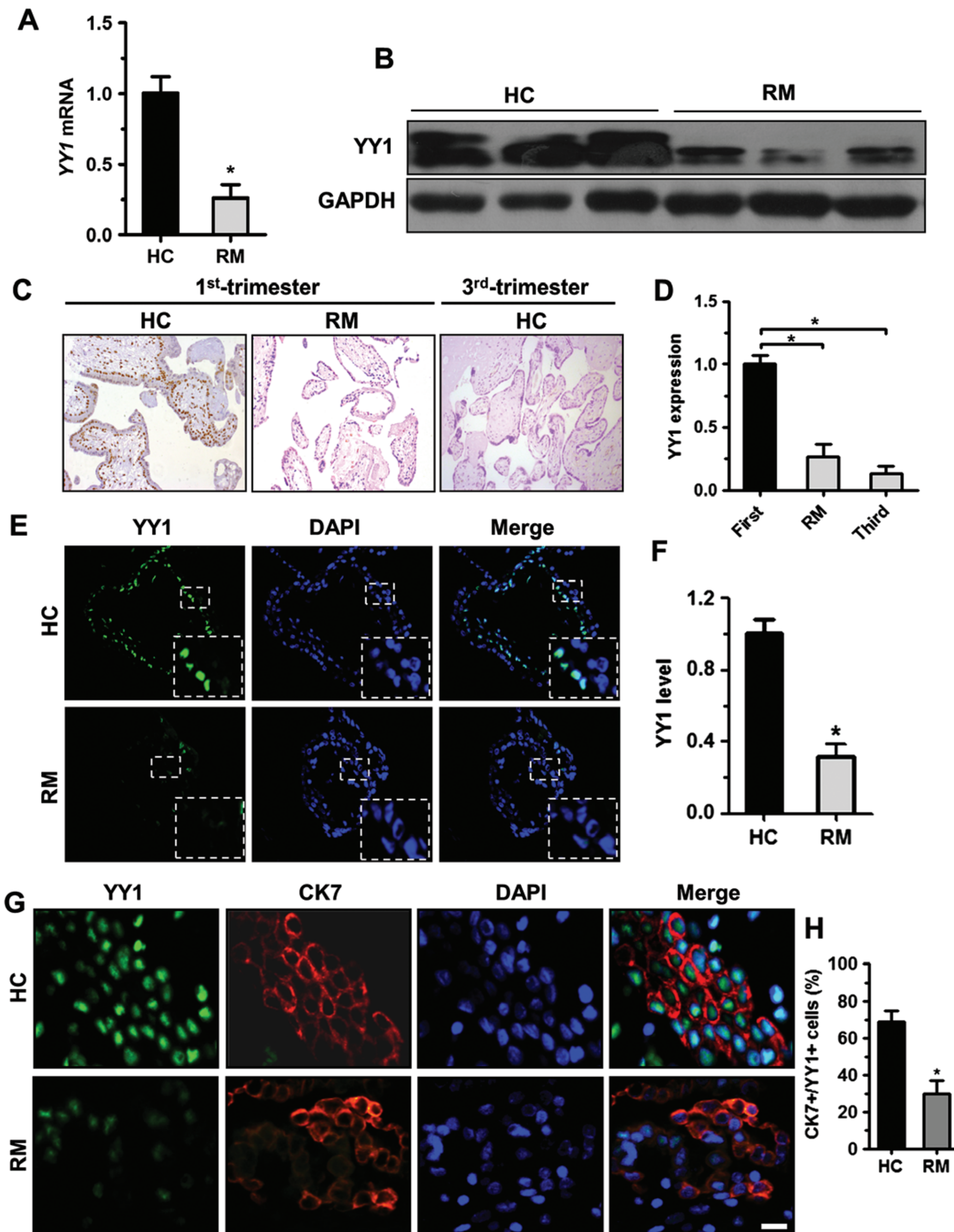


Figure 2. *YY1* is down-regulated in cytotrophoblasts and extravillous trophoblasts in RM patients. (A, B) The *YY1* levels in first-trimester villous tissues and third-trimester placental tissues from the RM and HC groups were determined by RT-qPCR and western blotting ($n = 6$). (C, D) Single staining of maternal villi [cytotrophoblasts (CTBs) and syncytiotrophoblasts (STBs)] using rabbit IgG anti-human YY1 antibodies and developed with a labelled streptavidin biotin + horseradish peroxidase (HRP) kit. The sections were counterstained with haematoxylin and positive cells were quantified using ImagePro-plus 6.0. Panel: original magnification × 40; $n = 12$. (E, F) Representative immunofluorescence images of YY1 in frozen first-trimester villous sections (6–8 weeks of gestation). Green indicates fluorescence signals specific to anti-YY1 antibodies and blue indicates nuclei. Fluorescence intensity of the signalling was assessed by Leica confocal SP8 software. Large panel: original magnification × 100; small panel: original magnification × 200; $n = 15$. (G) Representative immunofluorescence images of YY1 in frozen first-trimester decidual tissue sections (6–8 weeks of gestation). Fluorescence signals specific to anti-YY1 antibodies appear green; to the CK7 staining, red; and to the DAPI-stained nuclei, blue. Panel: original magnification × 100; $n = 15$. (H) The number of $YY1^+$ cells and $CK7^+$ cells was calculated, respectively, using Leica confocal SP8 software and then the percentage of $CK7^+$ cells normalized to the number of $YY1^+$ cells in the decidual tissue of RM and HC samples was assessed.

explants migrated less far than the siCtrl-treated explants (Figure 4E and Supplementary Figure 3A). To further clarify the regulation of trophoblast invasion by YY1, explants freshly obtained from the villi of RM samples were cultured in 24-well dishes for 24 h and then treated with lenti-ctrl and lenti-YY1 lentiviral constructs. The results showed that lenti-YY1-treated explants migrated significantly further than the lenti-ctrl-treated explants (Figure 4F and Supplementary Figure 3B).

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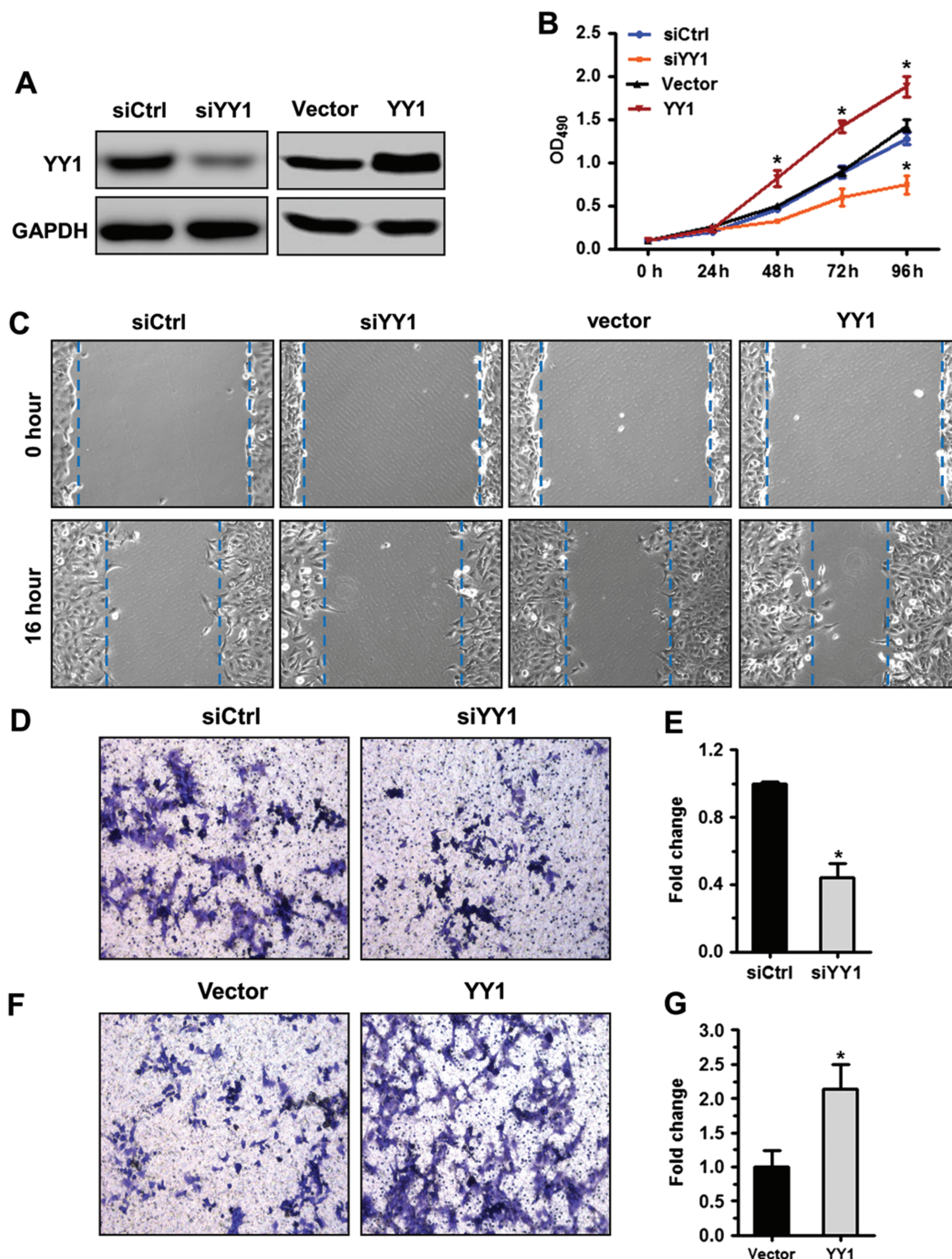


Figure 3. Knockdown of *YY1* decreases trophoblast proliferation and migration. (A) Western blot analysis of *YY1* expression in HTR-8 cells transfected with siCtrl, siYY1, control vector or YY1-overexpressing vector after 48 h. (B) HTR-8 cells were transfected with one of the above-mentioned four vectors and incubated for 48 h. Cell proliferation was measured after 48 h using the MTT assay. $^*p < 0.05$ compared with siCtrl or control vector. (C) Overexpression of *YY1* in HTR-8 cells resulted in increased wound closure ability compared with the vector control cells. *YY1* knockdown drastically reduced the rate of wound closure in comparison with the scrambled control cell line. Original magnification × 100. (D–G) Overexpression of YY1 in HTR-8 cells significantly increased cell invasion compared with the vector control cell line (left). Knockdown of *YY1* reduced cell invasion compared with the scrambled control cell line. Original magnification × 200. $^*p < 0.05$ versus siCtrl or control vector.

## YY1 promotes MMP2 expression in trophoblasts

Proteolysis of the extracellular matrix (ECM) by MMPs plays a crucial role in the regulation of cell motility. Gelatinases (MMP2 and MMP9) have been implicated in remodelling the ECM during the process of trophoblast invasion [23]. To determine whether YY1 expression affects MMP production by trophoblasts, we applied antibody array technology to determine the expression profiles of seven MMPs and three TIMPs in the supernatant of HTR-8 cells knocked down for YY1. As shown in Figure 5A, MMP2 expression was decreased in the supernatants of HTR-8 cells and TIMP2 expression was increased, while the levels of MMP1, MMP3, MMP9, MMP13, MMP10, TIMP1, and TIMP4 did not change. Other MMPs were not detected in the supernatant of HTR-8 cells. Furthermore, using an ELISA test, we confirmed

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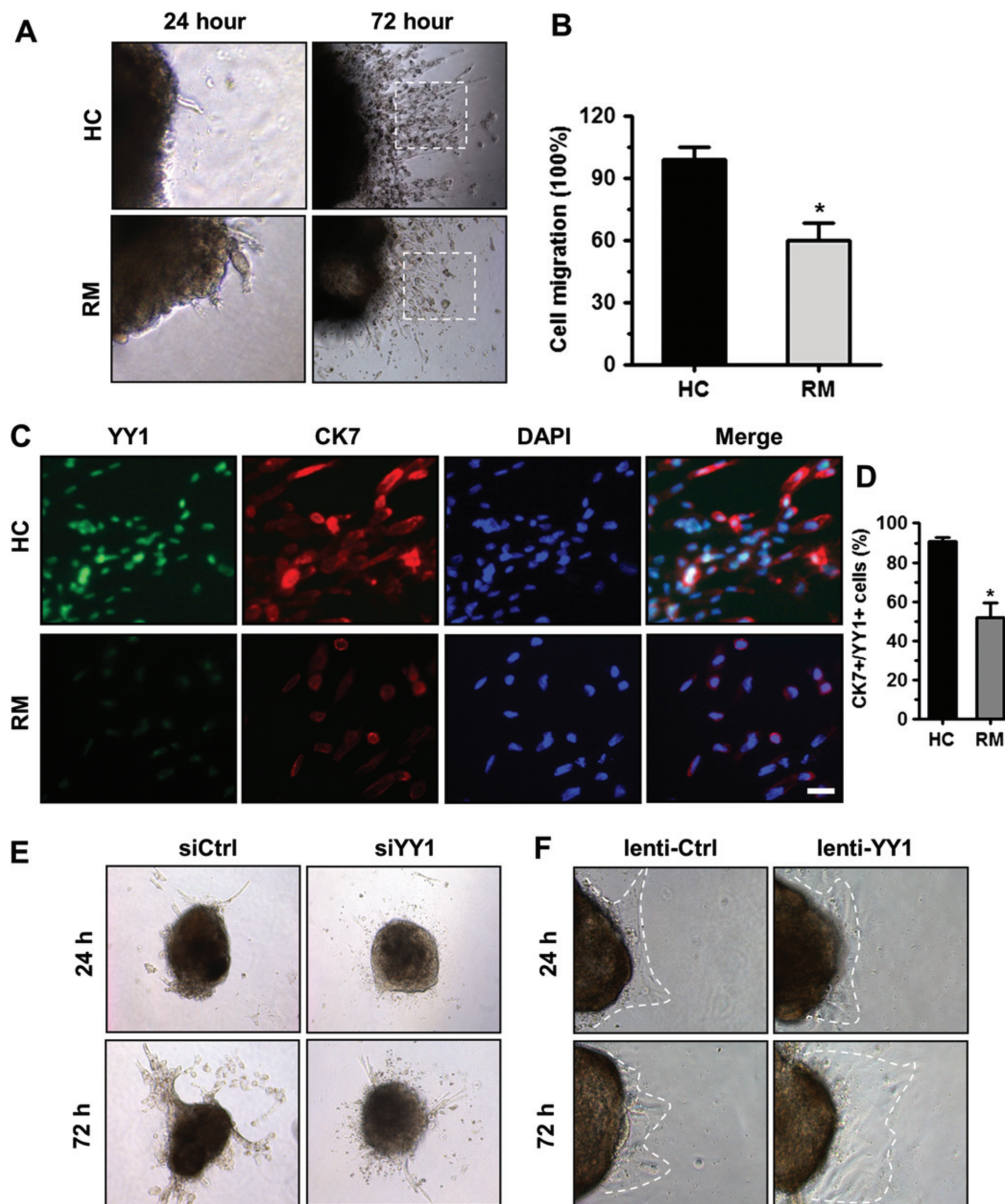


Figure 4. YY1 promotes trophoblast outgrowth in extravillous explant cultures. (A) Extravillous explants were obtained from HCs and RM patients at 6–8 weeks of gestation and cultured on Matrigel. (B) Statistical assay of the migration distance of villous tips (%). Data are presented as means ± SD of three independent experiments. (C) The extravillous explants were cultured on Matrigel for 72 h. Immunofluorescence staining using anti-YY1 antibodies showed an obvious decrease in the YY1 protein level in the RM group compared with the HC group. Green fluorescence signals indicate bound anti-YY1 antibodies; CK7 staining is visualized as red; and the DAPI-stained nuclei are blue. (D) The number of $YY1^+$ cells and $CK7^+$ cells was calculated, respectively, using Leica confocal SP8 software and then the percentage of $CK7^+$ cells normalized to the number of $YY1^+$ invading trophoblasts in RM and HC samples was assessed. (E) Extravillous explants were maintained in culture on Matrigel. Serial pictures of the explants incubated with siYY1 or siCtrl were taken under a light microscope after 24 and 72 h of culture *in vitro*. (F) Extravillous explants from RM patients were maintained in culture on Matrigel. Serial pictures of the explants incubated with lenti-ctrl or lenti-YY1 lentivirus were taken under a light microscope after 24 and 72 h of culture *in vitro*.

that the level of MMP2 expression was significantly decreased in the supernatant of HTR-8 cells treated with the siYY1 siRNA, while the level of TIMP2 was increased (Figures 5B–5G). To determine whether YY1 expression affects the production of active MMP2 by trophoblasts, we used gelatin zymography to measure the MMP2 activities in the conditioned medium of the siYY1 or YY1-overexpressing trophoblasts. The results showed that YY1 overexpression promoted MMP2 activity compared with the vector-transfected cells. Conversely, knockdown of YY1 inhibited the MMP2 activity (Figures 5H and 5I). HTR-8 cells or chorionic villi explants were obtained freshly from RM samples that were also stably infected with lenti-ctrl vector or lenti-YY1 lentiviral vector. As expected, overexpression of YY1 significantly promoted the level of the MMP2 protein (Figures 5J and 5K) *in vitro*. These results suggest that YY1 promotes the migration and invasion of trophoblasts by regulating the expression of MMP2.

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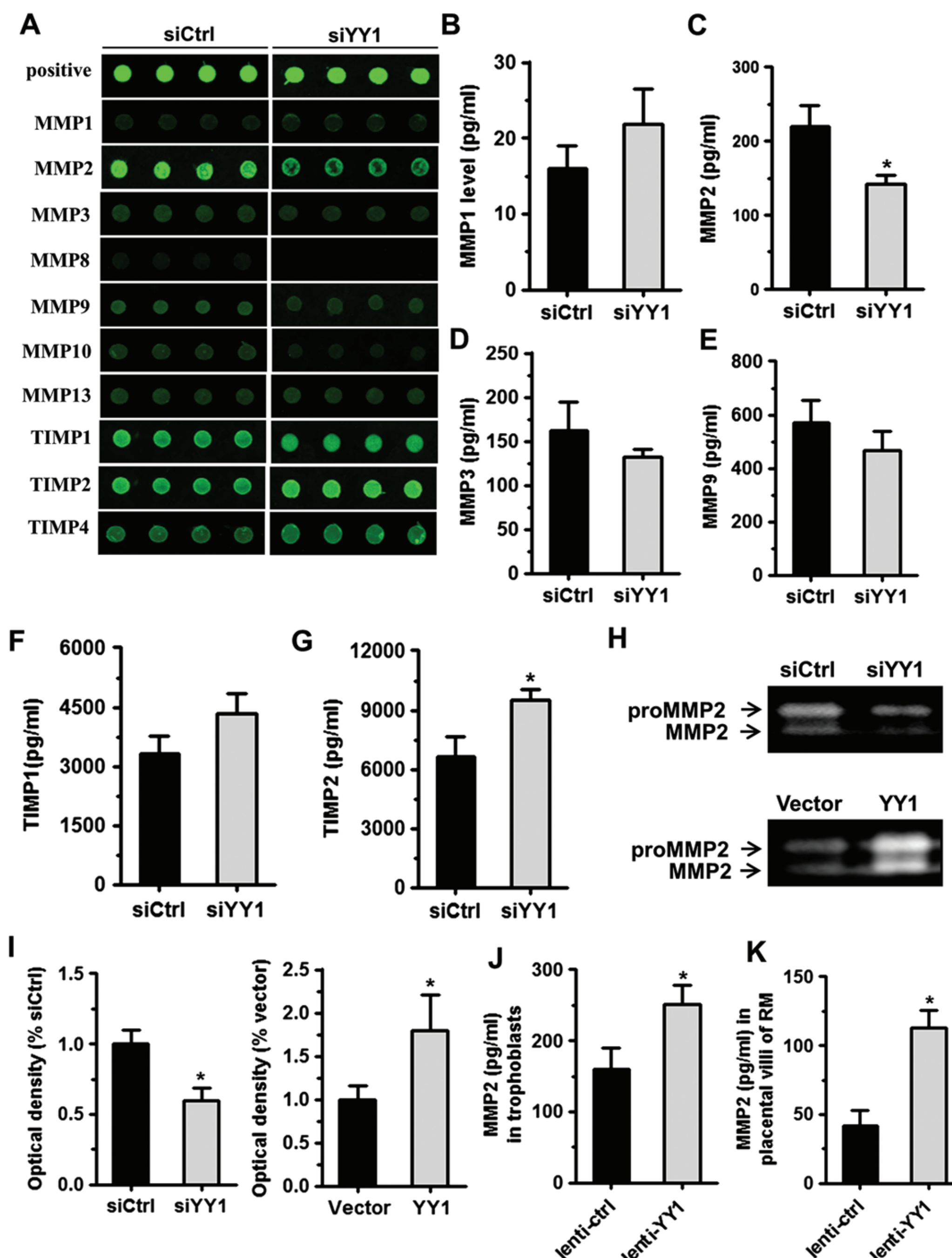


Figure 5. Knockdown of YY1 decreases the expression of MMPs in primary trophoblasts. (A) Primary trophoblasts were transfected with siCtrl or siYY1 oligonucleotides for 48 h, and the MMP and TIMP levels in the cell supernatant were quantified using Quantibody Human MMP Array 1. *$p < 0.05$ versus siCtrl. (B–G) MMP1, MMP2, MMP3, MMP 9, TIMP1 or TIMP2 expression levels in the supernatant of primary trophoblasts transfected with siCtrl or siYY1 were determined using an ELISA Kit (R&D Systems). (H, I) Serum-free culture media of primary trophoblasts transfected as indicated with siCtrl, siYY1, vector, and YY1-overexpression plasmid were collected for gelatin zymography. (J) The level of MMP2 expression in the supernatant of primary trophoblasts transfected with lenti-ctrl or lenti-YY1 lentivirus was determined using an ELISA kit (R&D Systems). (K) The level of MMP2 expression in the supernatant of the explants from RM patients incubated with lenti-ctrl or lenti-YY1 lentivirus was determined by ELISA.

## YY1 is a transcriptional activator for MMP2 in trophoblasts

To further clarify the molecular mechanism by which YY1 regulates trophoblast migration and invasion, four YY1 transcriptional binding sites were identified in the *MMP2* promoter region using the TRANSFAC tool [24]. These YY1 binding sites (namely, UP1, UP2, UP3, and UP4) were located in the conserved region (−77 bp to −1.3 kb) upstream of *MMP2* (Figure 6A). Next, we performed chromatin immunoprecipitation (ChIP) experiments in trophoblast cells to determine whether YY1 binds directly to the *MMP2* genomic locus. The results showed that YY1 binds directly to the promoter region of *MMP2* via UP1 and UP2 (Figure 6B). These results

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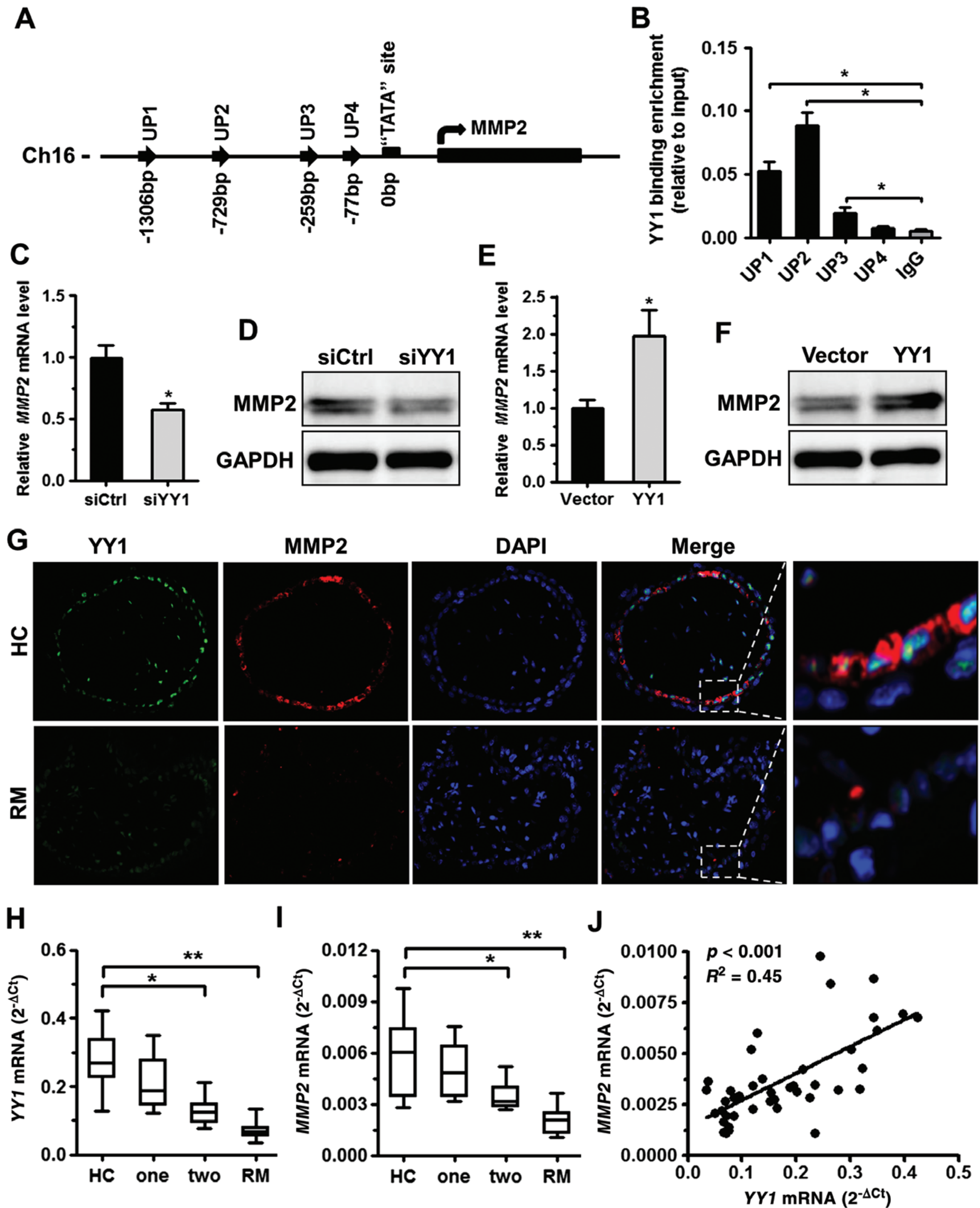


Figure 6. YY1 represses MMP2 expression in trophoblasts. (A) The diagram illustrates the YY1-binding site in the promoter region of MMP2. (B) Primary trophoblasts ($5 \times 10^6$) were isolated from HCs at 6–8 weeks of gestation and a ChIP assay was performed using 2 µg of YY1-specific antibody to immunoprecipitate the transcriptionally active regions of DNA. The purified DNA was amplified by RT-qPCR using primer sets specific to UP1–UP4 of MMP2. (C–F) Western blotting and RT-qPCR analyses of MMP2 expression in HTR-8 cells transfected as indicated with siCtrl, siYY1, and control vector or YY1 expression vector. (G) Representative immunofluorescence images of YY1 in frozen first-trimester villous sections obtained at 6–8 weeks of gestation. Fluorescence signals specific to anti-YY1 antibodies appear green; anti-MMP2 antibodies appear red; and the DAPI-stained nuclei appear blue. (H, I) The *YY1* and *MMP2* mRNA expression levels in the villous tissue of patients with one ($n = 13$) or two ($n = 15$) pregnancy losses, RM ($n = 31$), and HCs ($n = 36$) were determined by RT-qPCR. (J) The *MMP2* mRNA expression level measured in the villous tissue of patients ($n = 49$) was measured using RT-qPCR and correlated with the *YY1* mRNA expression level in the villous tissue in corresponding patients ($n = 49$).

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suggested that UP1 and UP2 mediate *MMP2* expression by YY1. Furthermore, overexpression of YY1 increased the expression of *MMP2* in HTR-8 cells, while YY1 knockdown significantly decreased its expression (Figures 6C–6F and Supplementary Figures 4A–4D). These results suggest that YY1 is a transcriptional activator of *MMP2*.

Immunofluorescence staining was used to further confirm MMP2 expression in paraffin-embedded villi. The result revealed that MMP2 expression in the trophoblasts of the RM group was significantly lower than that in the trophoblasts of the control group (Supplementary Figure 5). Furthermore, double immunofluorescent staining for MMP2 and YY1 revealed decreased staining for YY1 and MMP2 in cytotrophoblasts from villi tissue of the RM group compared with that of the HC group (Figure 6G). We further examined the expression levels of *MMP2* and *YY1* in villi from patients with one pregnancy loss, two pregnancy losses, RM, and in healthy controls. Consistent with the results of YY1 down-regulation, *MMP2* mRNA was significantly decreased in the villi of the RM group (Figures 6H and 6I). Linear correlation analysis showed that the *MMP2* mRNA level was positively correlated with the *YY1* level in villous tissue (Figure 6J). These results indicated that *YY1* and *MMP2* expression levels were down-regulated in the villous tissue of the RM group, which correlated with the pathogenesis of RM.

To further assess the clinical significance of YY1, explants were freshly obtained from villi and treated with either siCtrl or YY1 siRNA. Whole-mount immunofluorescence staining of both villous samples showed the silencing efficiency of YY1. CK7 is a marker for cytotrophoblast cells, and it was used to identify trophoblast cells in the outgrowth area of the villous tip. Compared with the siCtrl-treated group, the *YY1* and *MMP2* levels were significantly decreased in EVTs treated by its specific siRNA (Supplementary Figures 6A–6D). These results validated the hypothesis that YY1 targets MMP2 *in vivo*.

## Discussion

Recurrent miscarriage (RM) affects approximately 1–3% of women during their reproductive years and is usually defined as three or more consecutive spontaneous abortions before 20 weeks of gestation [21,25,26]. RM is associated with a high level of psychological distress and is emotionally taxing for couples. However, the mechanism of RM remains poorly understood. Many studies have found that insufficient proliferation and invasion of cytotrophoblasts are associated with early and late RM [3]. In this study, we explored the function of YY1 in human villi and trophoblasts during the early stage of pregnancy.

Invasion of trophoblast cells into the endometrial stroma and inner-third of the myometrium is essential for the development of definitive maternal–fetal circulation and for pregnancy success in humans [27]. The regulation of MMP activity at the maternal–fetal interface appears to be critical for successful implantation and placentation. Trophoblast cells constitutively produce MMPs and are thus invasive by nature [28,29]. A previous study found that MMP2 and MMP9 are strongly localized to the placental bed in early pregnancy, primarily to EVT cells at 6–8 weeks of gestation, and these proteins appear to regulate trophoblast invasion [30]. In particular, MMP2 has been implicated in the remodelling of the ECM during the trophoblast invasion process [19]. Previously, Rylski *et al* reported that in rat non-depolarized brain neurons, the transcriptional regulator YY1 is bound to the proximal MMP9 promoter and represses MMP9 transcription by recruiting HDAC3 [31]. In contrast, in our study, we firstly found that in human trophoblasts, YY1 was recruited and bound to the promoter region of *MMP2* (but not *MMP9*), and further promotes the expression of *MMP2*. Moreover, gene rescue assays and gelatin zymography experiments further confirmed that YY1 plays a key role in regulating trophoblast invasion by up-regulating MMP2 expression. Thus, YY1 is a novel transcriptional activator of MMP2 expression in trophoblasts. TIMPs, which are the major endogenous inhibitors of MMP activity in tissues, inhibit trophoblast invasion. In this study, knockdown of *YY1* increased the expression of TIMP2, which in turn inhibited MMP2 activity and decreased trophoblast invasion.

In the present study, we demonstrated that *YY1* is highly expressed in CTBs and EVTs at the maternal–fetal interface. Interestingly, notably higher levels of YY1 were produced by trophoblasts in the normal group compared with the RM group, which was associated with lower MMP2 production by trophoblasts. In addition, knockdown of YY1 using an siRNA effectively inhibited trophoblast outgrowth and EVT migration in an *in vitro* extravillous explant culture model. The correlation between the decreased expression of YY1 and RM provides a pathological criterion that may be applied for the diagnosis and treatment of potential miscarriages.

## Acknowledgments

This work was supported by the National Basic Research Program of China (2013CB967404 to YL), the National Natural Science Foundation of China (81401218 to F-JT, 81370767 to JY, 81501250 to X-CL, and 81125004 and 31171439 to YL), the Fund for Outstanding Academic Leaders in Shanghai, China (12XD1406600 and 2013–049 to YL), the Joint Key Project of New Frontier Technology in Shanghai Municipal Hospitals, Shanghai Municipal (SHDC12014129 to YL), the Shanghai Natural Science Fund Project (14ZR1443800 to F-JT), and the Shanghai Jiao Tong University Medicine–Engineering Fund (YG2013ZD04 to YL).

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## Author contribution statement

F-JT and Y-XC performed most of the experiments. X-CL and FW performed parts of the third experiment. C-MQ and X-LM performed parts of the fifth experiment. YL and JY supervised this study.

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## SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

**Supplementary methods.**

**Figure S1.** Network of YY1 interacting proteins, as determined by the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database.

**Figure S2.** YY1 promoted trophoblast migration.

**Figure S3.** YY1 promoted trophoblast migration from extravillous explants.

**Figure S4.** YY1 promoted MMP2 expression in trophoblasts.

**Figure S5.** Level of MMP2 in villous sections from RM patients was significantly decreased.

**Figure S6.** YY1 siRNA inhibits EVT migration by down-regulating MMP2.

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