# The role of E2F8 in the human placenta

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**Abstract.** Recent studies have reported that E2F transcription factor (E2F) 8, an atypical E2F transcription factor, serves a critical role in promoting the growth and development of the murine placenta. However, the function of E2F8 in the human placenta remains unknown. Invasion of extravillous trophoblasts (EVTs) into the maternal decidua is known to be important for the development of the human placenta. To investigate the role of E2F8 in human placental development, E2F8 localisation was examined in the human placenta and *E2F8* mRNA expression was detected in primary cultured EVTs. The human EVT cell line, HTR-8/SVneo, was divided into two groups and treated separately, one with retrovirus expressing short hairpin (sh)-RNA against E2F8 (*shE2F8* cells) and the other with non-target control shRNA (*shControl* cells). The cell functions, including cell cycle, proliferation, invasion and adhesion, were compared between the *shE2F8* and *shControl* cells. A histological examination revealed that E2F8 was localised in the decidua cells, EVTs, and cytotrophoblasts in the placenta. *E2F8* mRNA was confirmed to be expressed in cultured primary EVTs. No significant difference was observed in the cell cycle, proliferation or adhesion between the *shE2F8* and *shControl* cells. The invasive ability was ~2-fold higher in the *shE2F8* cells when compared with the *shControl* cells ($P<0.01$). Production of matrix metalloproteinase-1 was significantly increased in the *shE2F8* cells when compared with the *shControl* cells ($P<0.05$). Taken together, E2F8 is present in the EVTs of the human placenta, but, unlike murine placenta, it may suppress the invasiveness of EVTs. E2F8 was also present in cytotrophoblasts in cell columns, which have no invasive ability and differentiate into EVTs. In conclusion, E2F8 also exists in the human placenta, and its function may be different from that in the murine placenta, although further investigation is required.

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*Abbreviations:* CK, cytokeratin; E2F, E2F transcription factor; E, embryonic day; EVT, extravillous trophoblast; FGR, foetal growth restriction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; PCR, polymerase chain reaction; RT, reverse transcription; TGC, trophoblast giant cell; TIMP, tissue inhibitor of metalloproteinases

*Key words:* extravillous trophoblast, invasion, MMP, cell cycle

## Introduction

Foetal growth restriction (FGR) is one of main causes of perinatal morbidity and mortality (1), but currently there is no clinically effective treatment. Placental development is well known to influence foetal growth, but the pathophysiology of failure of placental development remains completely unelucidated. Recently E2F transcription factor (E2F) 7 and E2F8 have been reported to be essential for murine placental development and to regulate gene expressions as repressors of the classic activator E2F3a (2). Members of the E2F family are transcriptional factors controlling cell cycle, although E2F7 and E2F8 have recently been identified as atypical E2F family members (3). The functions of E2F7 and E2F8 have also been investigated, and they are reported to control angiogenesis (3-5).

*E2f8* expression peaks on embryonic day (E) 10.5 and E15.5 in the murine placenta; it is expressed in three major trophoblast lineages-labyrinth trophoblasts, spongiotrophoblasts, and trophoblast giant cells (TGCs)-in the murine placenta. *E2f7*$^{-/-}$;*E2f8*$^{-/-}$ murine placentas are reported to be

smaller, showing failed invasion into the maternal decidua and a poor vascular network in comparison with wild-type placentas (2). In addition, ablation of *E2f7* and *E2f8* in all trophoblasts results in FGR along with the collapse of placental architecture. The human placenta, as well as the murine placenta, is classified as chorioallantoic placenta. However, there are structural differences between the human and murine placentas, including the cell types (6). Therefore, experiments using human placental samples and human cell lines will require translation of the findings from a mouse mutant model into human placental pathology. Behaviour of TGCs is similar to that of human extravillous trophoblasts (EVTs), both of which invade maternal decidua and become polyploid (7). The function of spongiotrophoblasts remains unknown, but some spongiotrophoblast cells differentiate into TGCs and are thought to be analogous to the cytotrophoblasts of cell columns that anchor villi in the human placenta (7). Labyrinth trophoblasts are analogous in function to syncytiotrophoblasts (7). In *E2f7*$^{-/-}$;*E2f8*$^{-/-}$ murine placentas, significant defects were observed in spongiotrophoblasts and TGCs, which suggests that E2F7 and E2F8 play important roles in these cell types.

From these findings, we hypothesised that atypical E2Fs also play a critical role in the development of the human placenta, which requires successful invasion of EVTs into the maternal uterus. Insufficient EVT invasion leads to human placental pathologies, including FGR. TGCs in the mouse placenta are analogous to EVTs, and E2F8 is involved in polyploidy in TGCs (2). Therefore, in this study we focused on the role of E2F8 in human EVTs. To demonstrate the involvement of E2F8 on human placental development could lead to establish the new therapy for underdevelopment of placenta related-pathologies including hypertensive disorders of pregnancy or FGR. For this end, we investigated the localisation of E2F8 in the human placenta and its function in human EVTs.

## Materials and methods

*Immunohistochemistry and immunofluorescence*. A first trimester pregnant uterus was previously obtained from a patient who underwent a hysterectomy during pregnancy (8,9). Third trimester placental samples were previously obtained from three pregnant women without any obstetric complications. After formalin fixation, paraffin-embedded tissue sections were cut at a thickness of 4 $\mu$m. In the present study, for heat-induced epitope retrieval, deparaffinised sections in 10 mM EDTA buffer (pH 9.0) were heated at 95˚C for 20 min using a microwave oven. Immunohistochemical staining was performed using the Histofine SAB-PO(R) kit (Nichirei Bioscience Inc., Tokyo, Japan) by the avidin-biotin immunoperoxidase technique. Briefly, endogenous peroxidase activity was blocked by incubation with 0.3% $H_2O_2$ in methanol for 20 min, and nonspecific immunoglobulin binding was blocked by treatment with 10% normal goat serum for 10 min. The sections were incubated at 4˚C overnight with 4 $\mu$g/mL of anti-human E2F8 antibody (bs-4265R; Bioss Inc., Woburn, MA, USA). For the negative control, primary antibody was replaced by goat serum (Nichirei Bioscience Inc.). The sections were then rinsed and incubated for 10 min with biotinylated secondary antibody (Nichirei Bioscience Inc.). After washing, the sections were incubated for 5 min with horseradish peroxidase-conjugated streptavidin and treated with diaminobenzidine (DAB; Dako Agilent Technologies, Inc., Santa Clara, CA, USA) in 0.01% $H_2O_2$ for 5 min. The sections were counterstained with Meyer's haematoxylin (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

For immunofluorescence, the sections were incubated at 4˚C overnight with 4 $\mu$g/mL of anti-human E2F8 antibody and anti-human Cytokeratin AE1/AE3 antibody (ready to use, PMD072; Diagnostic BioSystems Inc., Pleasanton, CA, USA) or with an anti-human E2F8 antibody and 4 $\mu$g/mL of anti-Vimentin antibody (sc-6260; Santa Cruz Biotechnology Inc., Dallas, TX, USA). The sections were then rinsed and incubated at room temperature for 30 min with 2 $\mu$g/mL Alexa Fluor 488-conjugated anti-mouse IgG (A-11029; Thermo Fisher Scientific Inc., Waltham, MA, USA) and Alexa Fluor 568-conjugated anti-rabbit IgG (A-11036; Thermo Fisher Scientific Inc.). For the negative controls, primary antibodies were replaced by 4 $\mu$g/mL mouse nonspecific IgG control antibody (555746; BD Biosciences, San Jose, CA, USA) and 4 $\mu$g/mL rabbit nonspecific IgG control antibody (I-1,000; Vector Laboratories Inc., Burlingame, CA, USA), respectively. The slides were mounted with Fluorescence Mounting Medium (S3023; Dako Agilent Technologies Inc.). The prepared slides for immunohistochemistry and immunofluorescence were photographed with AXIO Imager A1 (Carl Zeiss Microscopy Co., Ltd., Tokyo, Japan) and a BZ9000 (Keyence Corporation, Osaka, Japan), respectively.

*Human chorionic villous explant culture*. Placental tissue specimens were obtained from healthy women at 6 to 9 weeks of gestation undergoing legal abortions (n=6). Villous explant cultures were established as previously reported (8), with slight modifications. Briefly, placental tissues were placed in ice-cold PBS, washed several times, and aseptically dissected to remove decidual tissues and foetal membranes. After the placental tissues were minced, the villous fragments were placed in 10-cm collagen 1-coated dishes (AGC Techno Glass Co., Ltd., Shizuoka, Japan). The explants were cultured in Placental Epithelial Cell Growth Medium (ready-to-use) (PromoCell, Heidelberg, Germany) with 100 U/mL penicillin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), 100 $\mu$g/mL streptomycin (Meiji Seika Pharma Co., Ltd.), and 25 $\mu$g/mL amphotericin B (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The tissue specimens were incubated at 37˚C in a 5% $CO_2$ atmosphere. The samples of primary cultured EVTs, the cell outgrowth from the explants, were collected with trypsin and filtered with a 100-$\mu$m mesh (Greiner Bio-One Co., Ltd., Frickenhausen, Germany) to extract total RNA. The present study was approved by the Ethics Committee of Nagoya University Hospital (Nagoya, Japan; approval no. 648). Written informed consent was obtained from each patient for use of the chorionic villous explant culture samples collected between October 2014 to January 2015.

*Cell culture*. The establishment of the human EVT cell line HTR-8/SVneo has been reported previously (10). The cells were grown in RPMI 1640 medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% heat-inactivated foetal calf serum (FCS; Biological Industries, Kibbutz Beit Haemek,

Israel), penicillin (100 U/mL), and streptomycin (100 $\mu$g/mL) at 37˚C in a humidified atmosphere containing 5% $CO_2$.

*Total RNA isolation and reverse transcription (RT).* Total RNA isolation was performed using the RNeasy mini kit followed by treatment with RNase-Free DNase I (Qiagen GmbH, Hilden, Germany), as suggested by the manufacturer. Total RNA from primary cultured EVTs was reverse-transcribed in a 20 $\mu$l reaction volume using ReverTra Ace (Toyobo Life Science, Osaka, Japan). Total RNA from HTR-8/SVneo was reverse-transcribed using High-Capacity cDNA Reverse Transcription kits and RNase Inhibitor (Thermo Fisher Scientific Inc.).

*RT-quantitative polymerase chain reaction (RT-qPCR) and semi-quantitative (sq)-PCR.* RT-qPCR was carried out with an Applied Biosystems® StepOne plus (Thermo Fisher Scientific Inc.) to measure mRNA expression of *E2F8, E2F7, E2F1, E2F3, TIMP-1, TIMP-2, PAI-1,* and *GAPDH* using Fast SYBR® Green Master Mix (Thermo Fisher Scientific Inc.). The cycling parameters were as follows: Holding stage of 95˚C for 20 sec, 40 cycles at 95˚C for 3 sec, 60˚C for 30 sec, one melting curve stage of 95˚C for 15 sec, 60˚C for 1 min, and 95˚C for 15 sec. The amplification specificity was confirmed by melting curve analysis. Using *GAPDH* as an endogenous reference gene, relative expression was estimated using the comparative Cq ($2^{-\Delta\Delta Cq}$) method (11). Data were automatically processed by StepOne plus software (Thermo Fisher Scientific Inc.). All of the primer sequences are listed in Table I.

To estimate the amount of cDNA for sqPCR, the Cq value of *GAPDH* was obtained by RT-qPCR, because it is inversely correlated with the amount of template cDNA present in the reaction. Equal amounts of cDNA were used as templates for PCR. sqPCR was performed on the cDNA of primary cultured EVTs by using a Veriti Thermal Cycler (Thermo Fisher Scientific Inc.) with Blend taq (Toyobo Life Science), as previously reported (12).

The sqPCR conditions were as follows: Pre-denaturation at 94˚C for 5 min, 35 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec, and extension at 72˚C for 1 min. The amplification products were electrophoresed on 15% polyacrylamide gels.

*Knockdown of E2F8 expression.* To knockdown *E2F8* expression, HTR-8/SVneo cells were infected with retrovirus expressing shRNA against E2F8 or non-target control shRNA. Oligonucleotides encoding shRNA specific to human E2F8 (5'-GCAGCCAATGATACCTCAAAG-3') (*shE2F8*) and luciferase for control (5'-CTTACGCTGAGT ACTTCGA-3') (*shControl*) were cloned into a retroviral expression vector pSIREN-RetroQ (Takara Bio, Inc., Otsu, Japan). Insertion of expression constructs was confirmed by DNA sequence analysis. 293T cells (RCB220; RIKEN BioResource Center, Tsukuba, Japan) were co-transfected with the pSIREN-RetroQ encoding either *shE2F8* or *shControl* in combination with the pVPack-GP and pVPack-Ampho vectors (Agilent Technologies, Inc., Santa Clara, CA, USA) using Lipofectamine 3000 (Thermo Fisher Scientific Inc.). After 6 h, the culture medium of 293T cells was replaced with fresh RPMI 1640 supplemented with 10% FCS, penicillin (100 U/mL), and streptomycin (100 $\mu$g/mL). The 293T cell supernatant was collected after 48 h. The supernatant and 8 $\mu$g/mL Polybrene (Nacalai Tesque Inc., Kyoto, Japan) were added to HTR-8/SVneo cells when the cell density reached about 50%. After 20 h of incubation, the infected cells were selected in RPMI 1640 with 10% FCS, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, and 1 $\mu$g/mL puromycin (Nacalai Tesque Inc.). Subsequent experiments were performed using the pooled populations of puromycin-resistant cells after drug selection.

*Proliferation assay.* The cells were plated in 96-well plates in triplicate at a density of 1,650 cells in a 100 $\mu$l volume and cultured with RPMI 1640 supplemented with 10% FCS. After 1, 2, and 3 days in culture, cell proliferation was examined using a Cell Counting Kit-8 (Dojindo Molecular Technologies Inc., Kumamoto, Japan) according to the manufacturer's instruction. Absorbance was measured at 450 nm using a microplate reader (Thermo Scientific™ Multiskan™ FC; Thermo Fisher Scientific Inc.). The experiments were repeated three times.

*Cell cycle analysis.* Cells were seeded at $5x10^5$ cells per 10-cm culture dish, cultured for 72 h, trypsinised, and fixed with 70% ethanol in PBS. RNase (0.25 mg/ml; Thermo Fisher Scientific Inc.) and propidium iodide (50 $\mu$g/mL; Sigma-Aldrich; Merck KGaA) were added to the fixed cells, and incubated for 30 min on ice. Then they were analysed with an Attune Acoustic Focusing Cytometer (Thermo Fisher Scientific Inc.). The experiment was repeated three times.

*Invasion assay.* Cell invasion was determined by the ability of the cells to cross the 8-$\mu$m pores of polycarbonate membranes (6.5 mm filter; 8 $\mu$m pore size; Corning Costar Inc., Corning, NY, USA) coated with 5 $\mu$g/well Matrigel (Becton Dickinson; BD Biosciences). In brief, the *shControl* and *shE2F8* cells ($6x10^4$ cells/well) were placed in the upper chamber, and 700 $\mu$l of RPMI 1640 medium containing 10% FCS was added to the lower chamber. Samples were then incubated at 37˚C under a 5% $CO_2$ atmosphere for 16 h, and the cells on the upper surface of the membrane were removed with a sterile cotton swab. The cells which had moved through the membrane to the lower surface, were stained with Giemsa Stain Solution (Wako Pure Chemical Industries, Ltd.). The surfaces of the membrane were photographed using a BX43 microscope with a DP21 camera and cellSens software (Olympus Corp., Tokyo, Japan) at magnification, x4 and the number of invading cells were counted in a 2.3-$mm^2$ area. The experiment was performed in triplicate and repeated three times.

*Gelatin zymography.* To determine the activity of the secreted proteases involved in cell invasion and migration, supernatant from *shE2F8* and *shControl* cells was assayed by zymography. These cells ($2x10^5$ cells) were seeded onto 3.5-cm culture dishes and cultured as described above. Twenty-four h after seeding, the cells were washed with serum-free medium and replaced with another 500 $\mu$l of serum-free RPMI 1640. After 24 h incubation, the supernatant was collected and centrifuged to remove cells. Tris-Glycine SDS Sample Buffer without reducing agent was added to the supernatant, and 20 $\mu$l of the sample was electrophoresed

Table I. List of primers.

| Genes | Direction | Primer sequences (5'-3') | Length (bp) |
|---|---|---|---|
| *E2F1* | Forward | CTCCTGAGACCCAGCTCCAA | 114 |
| | Reverse | ATCCCACCTACGGTCTCCTCA | |
| *E2F3* | Forward | GTATGATACGTCTCTTGGTCTGC | 78 |
| | Reverse | CAAATCCAATACCCCATCGGG | |
| *E2F7* | Forward | AAAGGGACTATTCCGACCCAT | 168 |
| | Reverse | ACTTGGATAGCGAGCTAGAAACT | |
| *E2F8* | Forward | AAGTACGCCGAGCAGATTATG | 127 |
| | Reverse | ATGTCTGGGTGTCCATTTGGG | |
| *TIMP-1* | Forward | CGGCCTTCTGCAATTCCGACC | 146 |
| | Reverse | GGATGTCAGCGGCATCCCCTA | |
| *TIMP-2* | Forward | CTCGGCAGTGTGTGGGGTC | 137 |
| | Reverse | TGGGTGGTGCTCAGGGTGTC | |
| *PAI-1* | Forward | CAGACCAAGAGCCTCTCCAC | 181 |
| | Reverse | GACTGTTCCTGTGGGGTTGT | |
| *GAPDH* | Forward | CATCCATGACAACTTTGGTATCGT | 107 |
| | Reverse | CCATCACGCCACAGTTTCC | |

E2F, E2F transcription factor; TIMP, tissue inhibitor of metallopeptidase; PAI-1, plasminogen activator inhibitor-1.

on 10% SDS-polyacrylamide gels containing 0.03% gelatin. After electrophoresis, the gels were washed three times with wash buffer [50 mM Tris-HCl (pH 7.4), 2.5% Triton X-100] and incubated at 37˚C in reaction buffer [50 mM Tris-HCl (pH 7.4), containing 10 mM $CaCl_2$] for 24 h. The gels were fixed with 50% methanol and 10% acetic acid, stained with a solution of 0.2% Coomassie Brilliant Blue in 50% methanol and 10% acetic acid for 30 min and then washed with 20% methanol and 10% acetic acid. The gelatinases were then detected as unstained bands. Matrix metalloproteinase (MMP) marker (Primary Cell, Ishikari, Japan) containing pro-MMP-9, pro-MMP-2, and MMP-2 was loaded into the gels. The gels were digitised with ImageQuant LAS 4010 (GE Healthcare UK Ltd., Little Chalfont, UK) using a white transilluminator, and the band intensity was measured using ImageQuant TL (GE Healthcare UK Ltd.). The experiment was repeated three times.

*Assay of adhesion to extracellular matrix-coated dish*. Experiments were performed as previously reported (13). Briefly, $4x10^4$ cells were plated in 100 $\mu$l of serum-free RPMI 1640 in 96-well microtiter plates coated with fibronectin, laminin, collagen 1, or collagen 4 (Corning Inc.). Then the culture plates were centrifuged at 500 rpm for 30 sec and incubated at 37˚C in a humidified atmosphere with 5% $CO_2$ for 2 h. After incubation, the plates were washed three times with assay buffer to remove non-adherent cells. Then, the adherent cells were evaluated for cell viability using the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). Absorbance was measured at 490 nm using a microplate reader, Viento 808 IU (BioTek Instruments, Inc., Winooski, VT, USA). Three individual experiments were performed in triplicate.

*Mass spectrometry analysis*. *shE2F8* and *shControl* cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, cOmplete™ EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich; Merck KGaA)] and sonicated for 5 sec on ice. The lysates were then centrifuged for 30 min at 13,000 rpm and the supernatant fraction collected. Their total protein concentrations were assessed by the BCA assay (Pierce™ BCA Protein Assay kit-Reducing Agent Compatible; Thermo Fisher Scientific, Inc.), using bovine serum albumin for generating the standard curve. The procedure was performed in accordance with a previous report (14).

The protein samples were digested with trypsin and subjected to mass spectrometry analysis using the Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific Inc.) in combination with an Advance LC System (Bruker Corporation, Billerica, MA, USA) Then, the samples were injected into the Advance LC System equipped with a MonoCap C18 0.1 mm in diameter and 150 mm in length (GL Sciences Inc., Tokyo, Japan). Finally, multiple MS/MS spectra were submitted to the Mascot program, version 2.5.1 (Matrix Science Inc., Boston, MA, USA) for the MS/MS ion search. The MS data were analysed using the Mascot software (Matrix Science, Wyndham Place, UK) with a threshold of a 1.2-fold change to identify differentially expressed proteins in *shE2F8* cells compared to those in *shControl* cells, in accordance with a previous report (15,16). The proteins thus identified were categorised using the Database for Annotation, Visualization and Integrated Discovery (DAVID, david.abcc.ncifcrf.gov, and version 6.8). Proteomap was generated to visualise the differential contribution of biological pathways (bionic-vis.biologie.uni-greifswald.de/, v2.0).

*Western blot analysis*. Cells were lysed in sample buffer [50 mM Tris-HCl (pH 6.8), 5% glycerol, 2% SDS] and boiled at 95˚C for 3 min. The whole-cell lysates were resolved by

10% SDS-PAGE and transferred onto a Polyvinylidene Difluoride membrane (Immobilon-P; EMD Millipore, Billerica, MA, USA). MMP-1 was detected using 0.092 $\mu$g/mL rabbit anti-human MMP-1 polyclonal antibody (ab137332; Abcam, Cambridge, UK). Horseradish peroxidase-conjugated antibody was used as a secondary antibody (0.019 $\mu$g/mL, NA934; GE Healthcare UK Ltd.). The 0.2 $\mu$g/mL mouse anti-beta actin monoclonal antibody (017-24573; WAKO Pure Chemical Industries, Ltd.) was used for standardising the amount of sample loaded. The chemiluminescent signals were detected using ECL Plus (GE Healthcare UK Ltd.) and scanned using ImageQuant LAS 4010 (GE Healthcare UK Ltd.). The band intensity was measured using ImageQuant TL (GE Healthcare UK Ltd.) and the experiment was performed seven times.

*Statistical analysis*. R2.15.2 software was used for statistical analysis (cran.r-project.org/bin/windows/base/old/2.15.2/). The data were expressed as the mean ± standard deviation and statistical analyses were performed with a Student's t-test or Welch's t-test, according to whether equal variance was assumed or not, respectively. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*E2F8 expression in human placenta*. In the first trimester, the localisation of E2F8 was investigated, and double staining of cytokeratin (CK), as an epithelial marker, and vimentin, as a stromal marker, was performed. E2F8 was expressed in EVTs and decidual cells, co-stained with CK and vimentin, respectively (Fig. 1A). E2F8 expression was strong in cytotrophoblasts in the cell columns and villi, but weak in syncytiotrophoblasts (Fig. 1B). Then, we also confirmed the expression of E2F8 in EVTs in the third trimester (Fig. 1B). It was found that *E2F8* mRNA was expressed in primary cultured EVTs from 6 to 9 weeks of gestation (n=6) and HTR-8/SVneo cells, an EVT cell line (Fig. 1C).

*E2F8 silencing in HTR-8/SVneo*. To elucidate the role of E2F8 in EVTs, HTR-8/SVneo cells were stably infected with a retrovirus expressing shRNA targeting *E2F8*. *E2F8* mRNA was clearly reduced in *shE2F8* cells compared with *shConrtol* cells (Fig. 2A, $P<0.01$). In *shE2F8* cells, the expression of other E2F family proteins, including E2F7, E2F1, and E2F3, was also examined to exclude the effects of the changed expression levels of these factors. *E2F8* silencing did not significantly affect expression of *E2F7*, *E2F1*, or *E2F3* (Fig. 2A, P=0.461, P=0.550, and P=0.386, respectively). Then, the role of E2F8 in the cell cycle, proliferation, and invasion in HTR-8/SVneo cells was examined. *E2F8* silencing did not significantly affect the cell cycle (Fig. 2B) or proliferation (Fig. 2C). However, the number of invaded cells in the *shE2F8* cells was significantly larger than that in *shControl* cells (Fig. 2D, $P<0.01$).

*The effect of E2F8 silencing on molecules involved in HTR-8/SVneo invasion and adhesion*. We examined whether MMP-2, MMP-9, *TIMP-1*, *TIMP-2* and *PAI-1* expression was altered by *E2F8* silencing. These molecules are well known as factors related to the invasive potential of EVTs. The expression of pro-MMP-2 and pro-MMP-9 in *shE2F8* cells were found to be slightly higher than that in *shControl* cells, but not significantly changed (Fig. 3A, P=0.546 and P=0.556, respectively). *E2F8* silencing did not significantly affect the expression of *TIMP-1*, *TIMP-2,* or *PAI-1* (Fig. 3B, P=0.097, P=0.962, and P=0.765, respectively).

We also investigated the effect of *E2F8* silencing on the adhesive ability to collagen 1, collagen 4, fibronectin, and laminin, but found no significant changes (Fig. 3C, P=0.475, P=0.544, P=0.425, and P=0.509, respectively).

The proteomic profile of *shE2F8* cells was analysed and compared with that of *shControl* cells. The 1,293 proteins were identified as differentially expressed (data not shown). MMP-1 was listed as one of upregulated proteins in *shE2F8* cells (Fig. 3D), which was validated by western blot analysis. The MMP-1 expression in *shE2F8* cells was significantly higher than that in *shControl* cells (Fig. 3E, $P<0.05$).

## Discussion

The present study demonstrates E2F8 expression and function in human EVT cells. The localisation of E2F8 was also shown in cytotrophoblasts from the cell columns and EVTs in the human placenta in the first and third trimesters. Thus, E2F8 also exists in an invasive type of trophoblast in both the human placenta and murine placenta. Additionally, E2F8 was detected in decidual cells, as previously reported (17).

However, the function of E2F8 in human EVTs does not seem to be consistent with that in murine TGCs. In the mouse model, E2F8 plays a critical role in placental development. Based on these findings, we speculated that decrease of E2F8 in human EVTs might have a negative effect on invasive ability, as successful EVT invasion into maternal decidua is essential for the development of human placenta. Therefore, we investigated whether *E2F8* knockdown might decrease the invasive ability of EVTs, but unexpectedly *E2F8* suppression significantly increased their invasion. These results suggest that *E2F8* suppression would promote placental growth with increased EVT invasion. From these findings, E2F8 does not seem to be critical for successful invasion of human EVTs. A previous study reported that E2F3 expression in FGR placentas was lower than that in the placentas of normal control (18). Another research demonstrated that E2F1 was significantly downregulated in placenta with severe early-onset preeclampsia (19), which is known to be caused by failed EVT invasion. Thus, E2F1 and E2F3 could promote placental growth in human, although their function in human EVTs is not fully known. On the other hand, in murine placenta E2F3 is thought to have a negative role in placental growth (20). E2F1 and E2F3 are known to be essential for cellular proliferation as classical E2F proteins (21), but E2F8 functionally antagonises the classical E2F proteins. These findings, together with the results of the present study, suggest that E2F8 could play a negative role in human placental development, which is consistent with the finding that E2F1 and E2F3 could be important in the human placenta. We speculated that upregulated invasion in *shE2F*8 cells might be related to *E2F1* and *E2F3* expression, but *E2F1* and *E2F3* expression was unchanged in *shE2F8* cells. A previous research also suggested that the function of E2F8 in human

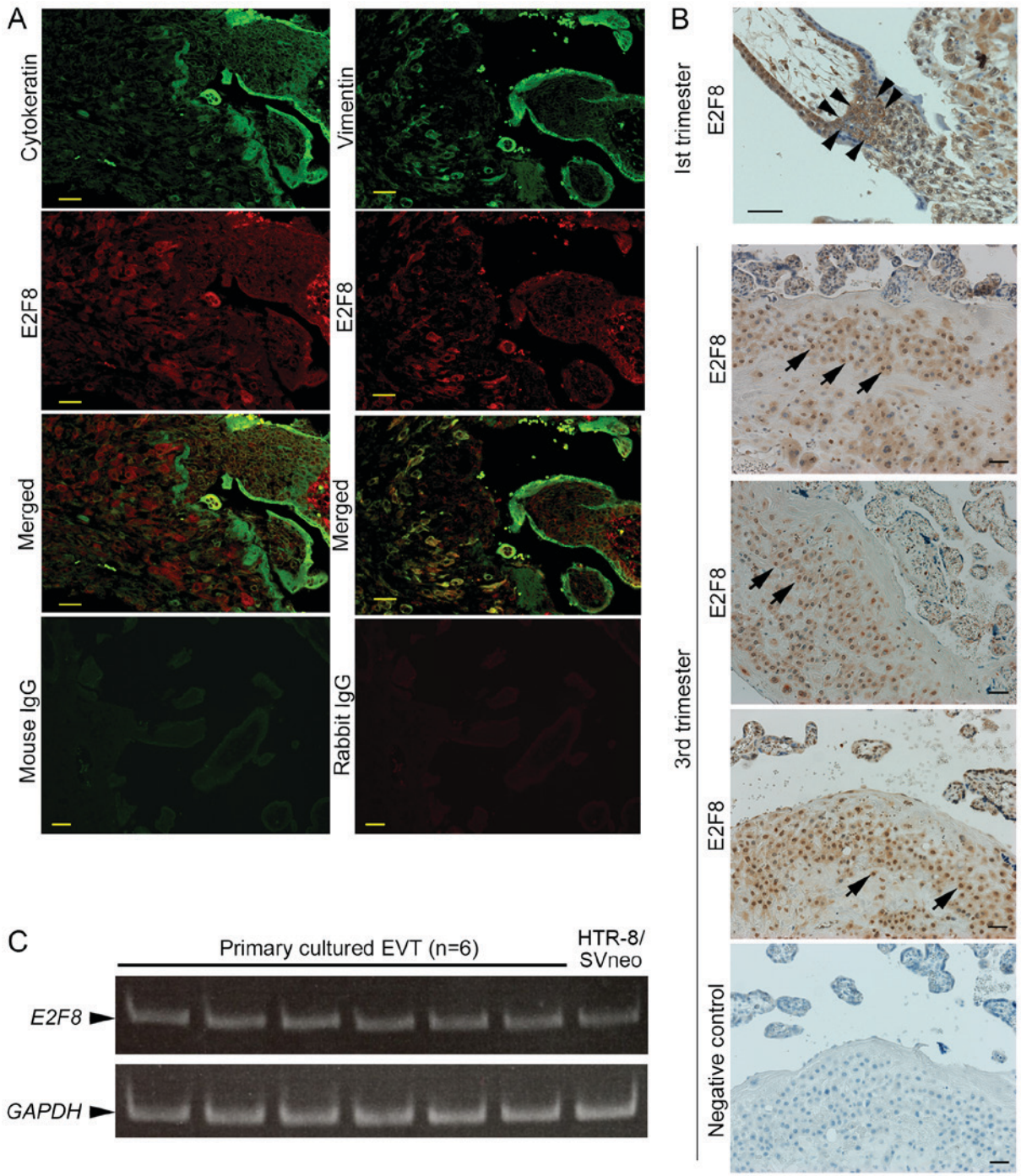



Figure 1. Localisation of E2F8 in the human placenta. (A) Double immunofluorescence staining was performed on samples of a first trimester human placenta. Staining was conducted for Cytokeratin (green), an epithelial cell marker, and E2F8 (red), or Vimentin (green), a marker of stromal cells, and E2F8 (red). The specimen exhibiting no staining with nonspecific rabbit or mouse IgG served as the negative controls. Magnification, x200; Scale bars, 40 $\mu$m. (B) Immunohistochemical staining of E2F8 in first and third trimester human placentas. E2F8 was detected in cytotrophoblasts (arrowheads) from the first trimester cell column and third trimester EVTs (arrows). The negative control is presented in the bottom panel (Magnification, x200; scale bars, 40 $\mu$m). (C) *E2F8* mRNA expression in cultured primary EVTs (6 to 9 weeks of gestation; n=6) was determined by semi-quantitative polymerase chain reaction. HTR-8/SVneo is a human extravillous trophoblast cell line derived from first trimester human trophoblasts. E2F, E2F transcription factor; EVTs, extravillous trophoblasts; IgG, immunoglobulin G.

decidualisation was opposite of that in mice. Decidualisation is an important change during conception, the first step of pregnancy. Though E2F8 plays a role in the development of the human placenta, this may not align with the role of E2F8 in the murine placenta.

The increased invasiveness caused by *E2F8* suppression would be related with increased MMP-1 expression in *shE2F8* cells. A previous study provided evidence to support that impaired invasion of EVTs in preeclampsia and FGR could result from reduced production of MMP-1 (22). Other reports demonstrated that MMP-1 is involved in invasion of EVTs or HTR-8/SVneo (23,24). The E2F transcription family proteins have the consensus winged helix DNA-binding motif sequence (25). It was reported that E2F transcription

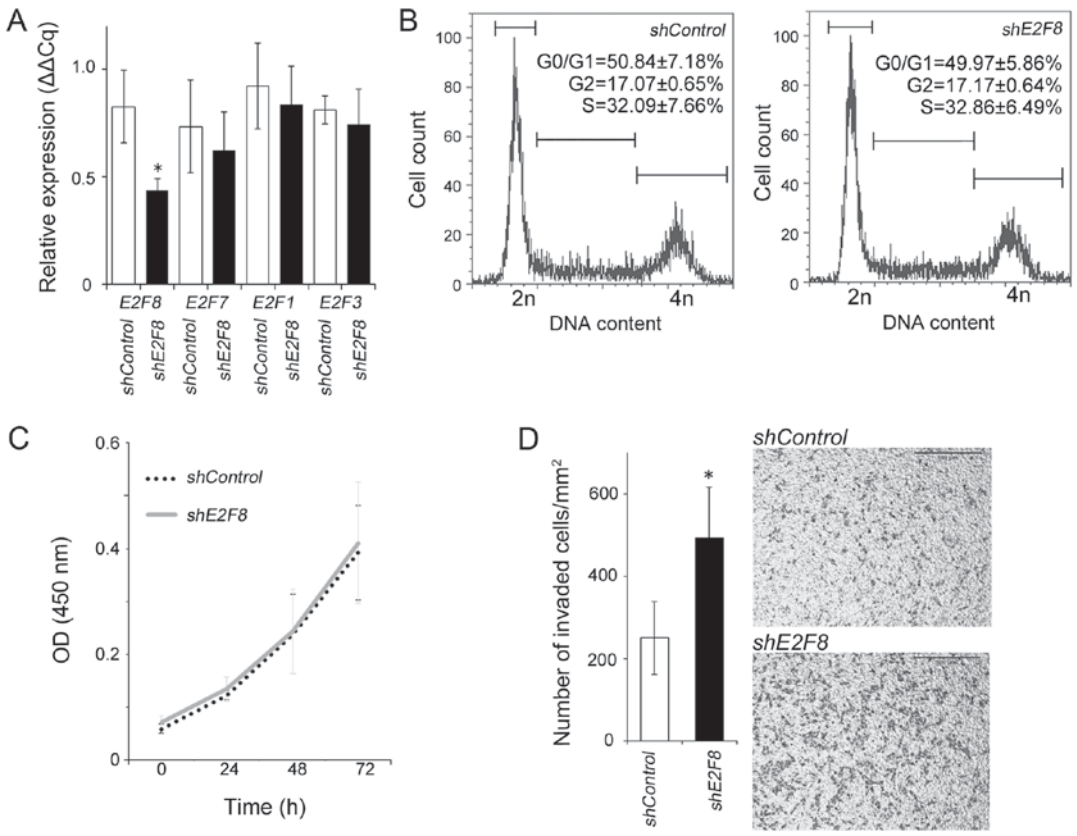


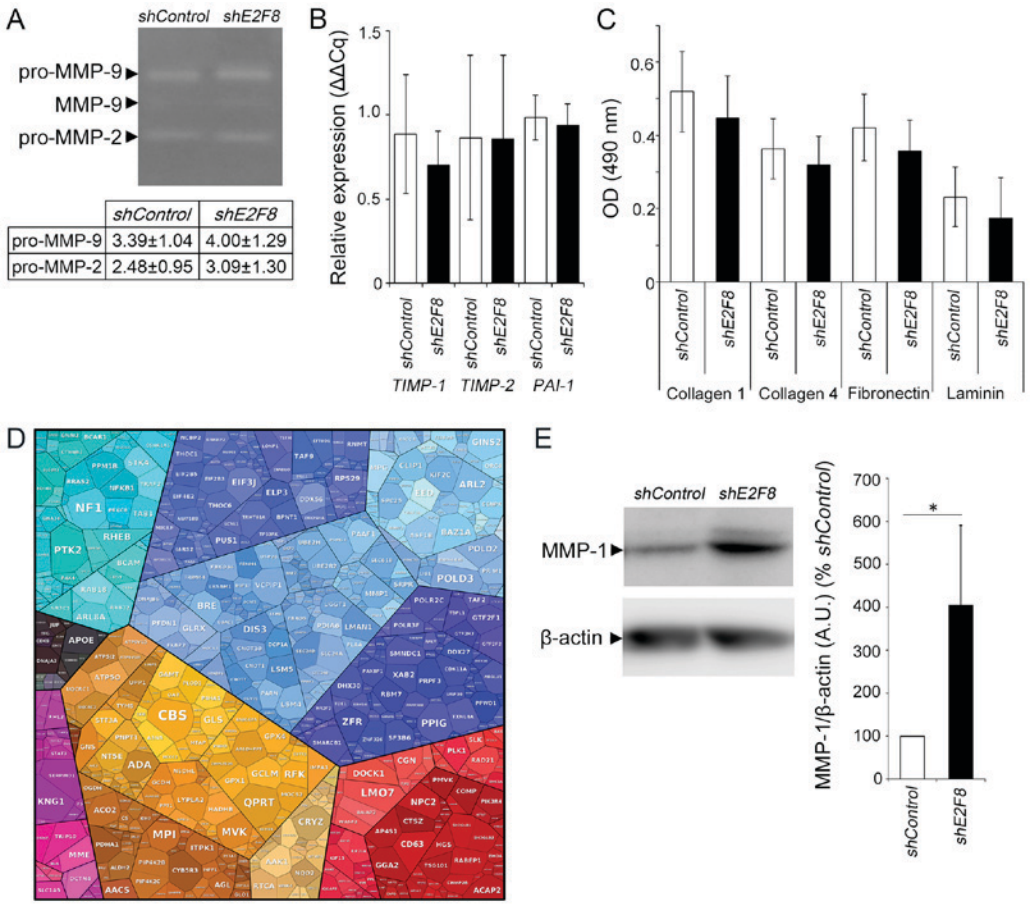

Figure 2. Effects of *E2F8* knockdown on the mRNA expression of E2F family members, and cell proliferation and invasion in HTR-8/SVneo. (A) mRNA levels of *E2F8*, *E2F7*, *E2F1*, and *E2F3* as determined by reverse transcription-quantitative polymerase chain reaction analysis of *shE2F8* and *shControl* cells. The *E2F8* mRNA levels significantly decreased in *shE2F8* cells when compared with those of *shControl* cells; however, no significant difference in *E2F7*, *E2F1* or *E2F3* mRNA levels was observed between the two groups. The results are expressed as the mean ± standard deviation (n=4/group). (B) Cell cycle progression was analysed by flow cytometry 72 h following plating. Data are shown as the percentage of the corresponding phases (n=3). (C) Time course of cell proliferation was measured by Cell Counting Kit-8. Cell growth curves were not significantly different between the *shControl* and *shE2F8* cells. *shControl* and *shE2F8* cells are represented as dotted and solid lines, respectively. (D) The number of invaded cells (per $mm^2$) was significantly increased in *shE2F8* cells when compared with *shControl* cells. Representative images of transwell invasion assays with *shControl* (upper image) and *shE2F8* (lower image) cells are presented. Magnification, x100; Scale bar, 500 $\mu$m. The results are expressed as the mean ± standard deviation. *P<0.01 vs. *shControl*. E2F, E2F transcription factor; sh-, short hairpin RNA; OD, optical density.

factors commonly bind to MMP promoter regions and regulate MMP gene expression (26,27). These findings suggest that E2F8 might regulate the MMP-1 promoter negatively and suppress its expression, which might lead to inhibition of EVT invasion.

E2F8 expression in EVTs and suppression of the invasive ability of EVTs seem paradoxical, but E2F8 might act in coordination with E2F1 and E2F3 to control EVT invasion. Moreover, a part of the findings in murine placenta is consistent with our results. In murine placenta, *E2f7* and *E2f8* ablation in TGCs, spongiotrophoblasts, or both caused no significant change in placental architecture (2), which might mean that E2F8 could not either be correlated with trophoblast invasion in murine placenta. The ablation of *E2f7* and *E2f8* in all trophoblasts including trophoblast progenitor cells in mice showed placental abnormalities including FGR. In the present study, *E2F8* knockdown could not be performed in other trophoblasts, including cytotrophoblasts and syncytiotrophoblasts, because their cell lines remain unestablished. E2F8 might play some role in those trophoblasts and concomitant human placental function. The present study demonstrates that E2F8 expression was high in cytotrophoblasts in the cell columns during the first trimester. Cytotrophoblasts are thought to have a stem cell-like feature corresponding to that of murine spongiotrophoblasts. Based on these findings, we speculate that reduction in E2F8 expression in cytotrophoblasts might promote cell differentiation into invasive EVTs, although further investigation is required.

Furthermore, there is another potential limitation of this study. The role of E2F8 was investigated in HTR-8/SVneo cells. It is an EVT cell line that is used worldwide, but it is also known to have a signature different from that of primary EVT cells (28). Additionally, in this study, the role of E2F7 was not investigated, but E2F7 has been reported to be have a synergistic function with E2F8 (29,30). Thus, the effect of *E2F8* suppression on EVT functions in the present study might be minimal because of E2F7 functions. It is important to understand the involvement of both E2F7 and E2F8 on human placental development *in vivo*, and further investigation is required for this end. We intend to investigate the role of E2F7 and correlation between E2F7 and E2F8 by suppressing *E2F7* or both *E2F7* and *E2F8* in our future studies.

In conclusion, E2F8 is expressed in EVTs and cytotrophoblasts in the cell columns of the human placenta, and may be involved in invasion of EVTs, which is important for human placental development. Further research is required to reveal




| | shControl | shE2F8 |
|---|---|---|
| pro-MMP-9 | 3.39±1.04 | 4.00±1.29 |
| pro-MMP-2 | 2.48±0.95 | 3.09±1.30 |

Figure 3. Effects of *E2F8* knockdown on the mRNA and protein expressions involved in matrix degradation. (A) MMP-2 and MMP-9 activity by gelatin zymography comparing *shE2F8* and *shControl* cells. Pro-MMP-2 and pro-MMP-9 activity were quantified by band intensity. Values are expressed as the mean ± standard deviation of three independent experiments. (B) mRNA expression levels of *TIMP-1*, *TIMP-2* and *PAI-1* were not changed in *shE2F8* cells, as determined by reverse transcription-quantitative polymerase chain reaction analysis. Statistical bars in the graph are expressed as the mean ± standard deviation of four independent experiments. (C) *shControl* or *shE2F8* cells were seeded on collagen 1, collagen 4, fibronectin and laminin-coated plates. The extent of cell adhesion is presented as absorbance at 490 nm. Values are expressed as the mean ± standard deviation of independent experiments performed in triplicate. (D) Proteomic analysis of *shE2F8* and *shControl* cells. Proteomap of gene ontology annotation, where the areas for each protein reflect the magnitude of the fold change in *shE2F8* cells in comparison with *shControl* cells. (E) Western blot analysis of MMP-1 in *shE2F8* and *shControl* cells. The bar graph presents MMP-1 band intensity normalised to that of b-actin. Values are expressed as the mean ± standard deviation of independent experiments performed in septuplicate. *P<0.05, as indicated. E2F, E2F transcription factor; sh-, short hairpin RNA; OD, optical density; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metallopeptidase; PAI, plasminogen activator inhibitor-1.

the role of E2F8, as well as other E2Fs, in human placental development.

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**Availability of data and materials**

The analysed datasets generated during the present study are available from the corresponding author upon reasonable request.

**Authors' contributions**

TK designed the experiments, analysed the data and wrote the paper. MM and RM performed the experiments, analysed the data and wrote the paper. YM performed the experiments, and analysed and interpreted the data. TU, KI, KN, TN, HT and SS analysed and interpreted the data. EY, AI, TS and FK reviewed and interpreted the data. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of Nagoya University Hospital (Aichi, Japan; approval nos. 648 and 2017-0302). Written informed consent was obtained from each patient for use of the chorionic villous explant culture samples collected between October 2014 to January 2015. The requirement for written informed consent for the use of first trimester uterus and third trimester placental samples was waived due to the retrospective nature of these experiments.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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