METABOLIC, ENDOCRINE, AND GENITOURINARY PATHOBIOLOGY

Fibrosis in Preeclamptic Placentas Is Associated with Stromal Fibroblasts Activated by the Transforming Growth Factor- β 1 Signaling Pathway

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Although fibrosis is one of the most prominent pathologic features of preeclamptic (PE) placentas, its mechanism remains largely unknown. Consistent with previous reports, we observed overexpression of collagen; actin, α 2, smooth muscle, aorta; connective tissue growth factor; and fibronectin in PE placentas compared with control ones. To investigate the mechanism of fibrosis in PE placentas, placental fibroblasts were isolated from PE placentas or normal pregnancies at delivery. The expression of fibrosis-related factors in fibroblasts was evaluated by real-time RT-PCR, Western blotting, enzyme-linked immunosorbent assay, and gene microarrays. An *in vitro* collagen gel contraction assay was also performed. Fibroblasts isolated from PE placentas showed higher expression levels of fibrosis-related factors compared with those from control ones. Global gene expression profiling of PE fibroblasts was contrasted with that of control ones and indicated an intimate association with transforming growth factor- β 1 (TGFB1) signaling. Furthermore, the PE fibroblasts expressed abundant phosphorylated SMAD family member 2 and showed higher expression levels of target genes of TGFB1 signaling compared with the control ones. The PE fibroblasts also had a greater ability to contract compared with the control ones. Contractility also depended on TGFB1 signaling. Our results suggest that TGFB1 signaling is activated in the fibroblasts in PE placentas and that these active fibroblasts contribute to fibrosis. (*Am J Pathol* 2018, 188: 683–695; <https://doi.org/10.1016/j.ajpath.2017.11.008>)

The human placenta is a critical organ for growth and development of the fetus, consisting of the decidua basalis from maternal components and chorionic villi from the fetus. Chorionic villi include trophoblasts and villous stroma, which is originally derived from the extraembryonic mesoderm and consists of mesenchymal cells, reticulum cells, and fibroblasts.¹

Preeclampsia (PE) is a pregnancy-specific disease that is characterized by maternal hypertension and proteinuria; it affects 5% to 10% of all pregnancies.² The first stage of PE pathogenesis is thought to be associated with failure of trophoblast invasion into the maternal decidua, resulting in insufficient vascular remodeling. This physiological change induces oxidative stress, which finally causes systemic inflammation in the maternal body.³ Histologic features of placentas in women with PE are characterized by chronic inflammation, vascular lesions, perivillous coagulation, and

villous fibrosis.^{4,5} These features suggest that dysfunction of trophoblasts is just a part of the pathology of preeclampsia and that villous stroma is the main site affected by the disease. Among the changes occurring in the villous stroma of PE, fibrosis is one of the most prominent features.^{4,5}

Fibrosis is a pathologic process, which takes place in response to tissue injury or inflammation, and is defined by overproduction of extracellular matrix (ECM) in the connective tissue.⁶ Fibrosis plays a significant role in multiple organ failures, such as systemic sclerosis, idiopathic pulmonary fibrosis, liver cirrhosis, kidney fibrosis, and cardiac fibrosis. In normal physiological conditions, fibroblasts play

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pivotal roles in ECM maintenance and wound healing in the connective tissues. However, in a pathologic situation, uncontrolled activation of fibroblasts promotes excessive production of ECM and leads to fibrosis.⁷ Activated fibroblasts might originate from tissue fibroblasts, endothelial cells, epithelial cells, pericytes, or bone marrow-derived cells.^{6,8}

The most well-known mechanism of fibrosis and fibroblast activation is the activity of transforming growth factor- β 1 (TGFB1).^{9,10} Hypoxia and hypoxia-inducible factor 1 α subunit are also known to contribute to fibrogenesis.¹¹ Hypoxia was reported to induce TGFB1 expression in fibroblasts.^{12,13} Lysophosphatidic acid, sphingosine-1-phosphate, plasma coagulation cascade, and matrix metalloproteinases were also reported to promote fibrosis by activating fibroblasts.^{14–17}

Although the mechanism of fibrosis in the villous stroma of PE placentas is largely unknown, it has been suggested that ischemia and hypoxia are involved.^{4,5} *In vitro* culture of placental fibroblasts in hypoxic conditions stimulated the expression and production of ECM, fibronectins, and collagen type 4, which suggests the activation of fibroblasts.^{18,19} TGFB signaling has been also implicated to be involved in fibrosis of PE placentas. TGFB families are expressed in decidua cells and in the extracellular regions of villous stroma.^{20,21} Some studies reported that plasma levels of TGFB in cases of PE were significantly higher than those in controls.^{22,23} Treatment of placental fibroblasts with TGFB1 led to production of ECM, fibronectins, and collagen types 1 and 4.¹⁹ As described earlier in this paragraph, there is only little known about fibrosis in PE placenta, and it is also unclear whether trophoblasts, fibroblasts, or both are involved in the fibrosis.

Herein, we focused on placental fibrosis as a feature of PE. We observed overproduction of ECM, including collagen (COL) and fibronectin (FN1), and overexpression of actin, α 2, smooth muscle, aorta (ACTA2) and connective tissue growth factor (CTGF) in PE placentas compared with control ones. Fibroblasts isolated from PE placentas showed higher expression levels of these fibrosis-related factors compared with those from control ones. Furthermore, the PE fibroblasts expressed abundant phosphorylated SMAD family member 2 (SMAD2) and showed intimate association with TGFB1 signaling by microarray analysis. The PE fibroblasts also had a greater ability to contract compared with controls, measured by an *in vitro* contraction assay, which also depended on TGFB1 signaling. These data suggest that TGFB1 signaling is activated in the fibroblasts in PE placentas and contributes to fibrosis.

Materials and Methods

Participants and Samples

Case and control pregnant Japanese women who had a prenatal checkup and gave birth at Kyushu University Hospital

between 2014 and 2016 were recruited to this study. Women with a multiple pregnancy, a fetus with malformations, or a fetus with chromosomal abnormalities were excluded. The participants were divided into two groups: a normal pregnancy (NP) group without any maternal or fetal complications, except for preterm birth ($n = 15$), and a PE group ($n = 14$) (Table 1). PE was diagnosed on the basis of the defined criteria (blood pressure reading, twice, at least 6 hours apart, with a systolic blood pressure ≥ 140 mm Hg and/or a diastolic blood pressure ≥ 90 mm Hg), with proteinuria (≥ 300 mg/day) during the second half of the pregnancy. All participants involved provided their written informed consent. This study was approved by the Ethical Committee of Kyushu University. Tissue blocks of a cross section of placenta located halfway between the cord insertion point and the lateral edge were dissected, and chorion and decidua were removed soon after deliveries and saved for histologic analysis and isolation of fibroblasts.

Immunohistochemistry and Image Analysis

Placenta tissue blocks were fixed with formalin and embedded in paraffin wax. Sections (4 μ m thick) were subjected to standard Masson's trichrome staining to visualize COL deposition. For immunohistochemistry, sections were deparaffinized, rehydrated, and heated in Histofine (pH 9; Nichirei, Tokyo, Japan) to activate antigen. After endogenous peroxidase activity had been blocked by 3% hydrogen peroxide, the sections were blocked with Protein Block Serum-Free Ready-To-Use (Agilent Technologies, Santa Clara, CA) and were incubated with a primary antibody at 4°C overnight. The EnVision+ dual-link system—horseradish peroxidase (Agilent Technologies) was used for the addition of a secondary antibody and color development. Hematoxylin was used for counterstaining nuclei. Rabbit primary antibodies used were against ACTA2 (1:300; ab5694), CTGF (1:400; ab6992), and FN1 (1:300; ab23750) (all from Abcam, Cambridge, UK). The images were captured using a microscope (BZ-8100; Keyence, Osaka, Japan). One hundred fields with 10 $\times$ amplification were captured, and areas with and without positive signals were calculated using a BZ analyzer and Hybrid Cell Count (Keyence). The ratios of signal-positive areas/total tissue areas were calculated.

Cell Culture

Placental fibroblasts were isolated, as reported.^{12,24} In brief, 5 to 10 g of villous tissue was minced and digested with 0.1% collagenase solution for 30 minutes at 37°C using a rotating mixer. Dispersed cells were filtered through a 160- μ m metal mesh and collected by centrifugation at 500 $\times$ g for 5 minutes. Then, the precipitate was gently suspended by pipetting with 2 mL of Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (FBS; Merck KGaA, Darmstadt, Germany) and plated. The cells were maintained in DMEM

Table 1 Clinical Characteristics of Placental Fibroblasts

Sample ID	Type	Maternal age, years	Delivery week	Delivery mode	Infant body weight, g	Infant sex	SGA
NP-1	NP	32	38	CS	2830	F	—
NP-2	NP	38	38	CS	2490	F	—
NP-3	NP	34	37	CS	2820	M	—
NP-4	NP	38	32	CS	2052	F	—
NP-5	NP	31	37	CS	2930	F	—
NP-6	NP	37	31	CS	1792	M	—
NP-7	NP	25	25	CS	622	F	—
NP-8	NP	36	38	CS	2800	F	—
NP-9	NP	35	37	CS	3280	M	—
NP-10	NP	42	37	CS	2885	M	—
NP-11	NP	36	39	TVD	3410	M	—
NP-12	NP	24	37	CS	2285	M	—
NP-13	NP	36	28	CS	960	M	—
NP-14	NP	28	41	TVD	3265	F	—
NP-15	NP	37	26	TVD	970	M	—
PE-1	PH-LOS	33	40	Forceps	2395	F	+
PE-2	PH-LOS	41	41	TVD	3440	F	—
PE-3	PH-E0	35	32	CS	1900	F	—
PE-4	PH-E0	43	23	CS	300	M	+
PE-5	PH-E0S	33	25	CS	595	M	+
PE-6	PH-E0	41	29	CS	855	F	+
PE-7	PH-LOS	38	37	Forceps	2500	F	—
PE-8	PH-E0	29	31	CS	1048	F	+
PE-9	pH-LO	34	39	CS	3025	M	—
PE-10	PH-LO	34	40	CS	2880	M	—
PE-11	PH-LO	39	34	CS	1348	M	+
PE-12	PH-E0S	33	25	CS	595	M	—
PE-13	pH-LO	24	37	CS	3508	F	—
PE-14	PH-LO	36	35	CS	2330	M	—

F, female; M, male; +, Present; —, absent; CS, cesarean section; E0, early onset; E0S, early onset, superimposed; ID, identification; LO, late onset; LOS, late onset, superimposed; NP, normal pregnancy; PE, preeclampsia; PH, proteinuria and hypertension in a severe criteria; pH, proteinuria and hypertension in a mild criteria; SGA, small for gestational age; TVD, transvaginal delivery.

containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a 5% CO₂ atmosphere. Placental fibroblasts at passages 2 to 8 were used for experiments. As used in our previous study, HTR-8/SVneo and TCL-1 trophoblast cells were obtained and maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin, and streptomycin.²⁵

Real-Time RT-PCR

Total RNA was purified from fibroblasts with RNeasy Mini Kits (Qiagen, Hilden, Germany). Isolated RNA was reverse transcribed into cDNA using ReverTra Ace-α (Toyobo, Osaka, Japan). Real-time RT-PCR was performed using SYBR Premix ExTaqII (Takara, Shiga, Japan). The sequences for primers are shown in Table 2. Relative expression levels were normalized to hypoxanthine phosphoribosyl-transferase 1 and calculated using the $2^{-\Delta\Delta C_t}$ method. Real-time RT-PCR for FN1 and secreted protein acidic and cysteine rich was performed using TaqMan primers and probe (FN1, Hs01549973_m1; secreted protein acidic and cysteine

rich, Hs00234160_m1; Thermo Fisher Scientific, Waltham, MA) and TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). Gene expression levels were obtained using individual values normalized to 18S rRNA (Hs99999901_s1; Thermo Fisher Scientific).

Western Blotting

Placental fibroblasts were harvested and lysed with Cell Lysis Buffer (Merck KGaA) with Protease Inhibitor Mixture (Nacalai Tesque) and phosphatase inhibitors (Nacalai Tesque). A total of 40 µg protein was loaded per lane, separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking with Blocking One or Blocking One-P solutions (Nacalai Tesque) in tris-buffered saline containing 0.1% Tween 20 for 30 minutes at 37°C, the membranes were incubated with rabbit primary antibodies against COL1 (1:5000; 600-401-103-0.5; Rockland, Inc., Gilbertsville, PA), fibroblast activation protein (FAP; 1:1000; ab28244; Abcam), phospho-SMAD2 (1:1000; number 3108; Cell

Table 2 Primers Used for the Real-Time RT-PCR Analysis

Target gene	Accession no.	Sequence		Amplicon, bp
		Forward primer	Reverse primer	
<i>COL1A1</i>	NM_000088.3	5'-AGGTCCCCCTGGAAAGAA-3'	5'-AATCCTCGAGCACCCCT-3'	96
<i>ACTA2</i>	NM_001141945.2	5'-CTGAAGTACCCGATAGAACATGG-3'	5'-TGAGAAAGAGTGGTGCCAGATT-3'	76
<i>CTGF</i>	NM_001901.2	5'-CTTGCGAAGCTGACCTGGAA-3'	5'-AAAGCTCAAACCTTGATAGGCTTG-3'	90
<i>IL15</i>	NM_000585.4	5'-GTCTTCATTTTGGGCTGTTTCAGT-3'	5'-CCTCACATTCTTTGCATCCAGATTCT-3'	316
<i>COL8A1</i>	NM_001850.4	5'-ACACGTTCAACCACTCACCC-3'	5'-CAGCGGCTTGATCCCATAGT-3'	291
<i>FGF1</i>	NM_001257212.1	5'-CACATTCTAGCTGCAGCTCAG-3'	5'-TGCTTTCTGGCCATAGTGAGTC-3'	264
<i>IGFBP3</i>	NM_001013398.1	5'-GGGTAGGAGGGACAGAGAGA-3'	5'-CAGCAAGCCATTCTCTCTTC-3'	247
<i>FBN2</i>	NM_001999.3	5'-CGAGGAGATGGGAGTCTGTC-3'	5'-GGGGTTAGGTCTGAAGCCTT-3'	174
<i>MMP9</i>	NM_004994.2	5'-CCCGGACCAAGGATACAG-3'	5'-GGCTTTCTCTCGGTACTG-3'	850
<i>ADAM15</i>	NM_001261465.1	5'-CCGGAAGTGGGCTTCAAGA-3'	5'-GAACGCTCCCAAGATGTAC-3'	246
<i>ITGA2</i>	NM_002203.3	5'-GCAAACCTCTGCAACCCAG-3'	5'-CGGTTCTCAGGAAAGCCACT-3'	277
<i>PGF</i>	NM_001293643.1	5'-GGGAACAGCATCGCCGCA-3'	5'-TGCAAGGTCCTAAAGATCCGT-3'	194
<i>HPRT1</i>	NM_000194.2	5'-GGCAGTATAATCCAAAGATGGTCAA-3'	5'-GTCAAGGCATATCTTACAACAAAC-3'	131

Sequence information can be obtained at <https://www.ncbi.nlm.nih.gov/nucore>.

Signaling Technology, Danvers, MA), SMAD2 (1:1000; number 5339; Cell Signaling Technology), and ACTA2 (1:3000; ab5694; Abcam) at 4°C overnight. For internal control, we also determined the expression of glyceraldehyde-3-phosphate dehydrogenase (1:2000; FL-335; Santa Cruz Biotechnology, Dallas, TX). After washing four times with tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with a goat anti-rabbit horse-radish peroxidase-conjugated secondary antibody for 1 hour at 37°C, followed by treatment with Clarity western electrochemiluminescence substrate detection kits (Bio-Rad, Hercules, CA), and subjected to a ChemiDoc XRS Touch Imaging System (Bio-Rad).

Immunofluorescence of Cells in Culture

Fibroblasts were seeded into multichamber culture slides (Lab-Tek, Naperville, IL) at a density of 5×10^4 cells per chamber. The cells were fixed in 4% paraformaldehyde and blocked in Protein Block Serum-Free Ready-To-Use for 10 minutes. The cells were incubated overnight at 4°C with primary antibodies: mouse anti-pan-keratin (1:400; C11; Cell Signaling Technology) and rabbit anti-vimentin (1:500; ab45939; Abcam). The cells were incubated for 30 minutes at room temperature with suitable combinations of the following secondary antibodies: Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:500; A21206; Invitrogen Life Sciences, Carlsbad, CA) or Alexa Fluor 594-conjugated goat anti-mouse IgG (1:500; A11005; Invitrogen Life Sciences). They were then mounted in ProLong Gold antifade reagent with DAPI (P36935; Invitrogen Life Sciences). Images were acquired using a BZ-8100 microscope (Keyence).

TGFB1 Treatment of Placental Fibroblasts

For the study of TGFB1 stimulation, placental fibroblasts maintained in DMEM with 10% FBS were transferred to

serum-free DMEM for 24 hours. Then, recombinant human TGFB1 (number 100-21; PeproTech, Rocky Hill, NJ) was added to the serum-free DMEM at a final concentration of 10 ng/mL, and the fibroblasts were cultured for another 24 hours before sample collection.

Knockdown of TGFB1 Gene Expression in Placental Fibroblasts

A validated siRNA for transforming growth factor beta receptor 1 (TGFB1) (103324; Thermo Fisher Scientific) was used to compare with negative control siRNA (AM4611; Thermo Fisher Scientific). Lipofectamine RNAiMAX was used to transfect the siRNAs to placental fibroblasts (Thermo Fisher Scientific). Forty-eight hours after transfection in DMEM with 10% FBS, the fibroblasts were used for collagen gel contraction assay or collected directly for mRNA and protein assays.

Enzyme-Linked Immunosorbent Assay

The supernatant of serum-free DMEM incubated with fibroblasts for 24 hours was used to determine the concentrations of secreted TGFB1 and FN1. In the case of TGFB1 stimulation, after 24 hours' incubation with serum-free DMEM, fibroblasts were treated with or without TGFB1 (10 ng/mL) for another 24 hours. The concentrations of TGFB1 and FN1 were analyzed by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Assays were performed following instructions provided by the manufacturer. The concentrations were determined in triplicate wells.

Collagen Gel Contraction Assay

Collagen lattices were prepared using type I collagen (Cellmatrix, type 1-A; Kurabo, Osaka, Japan) to a final concentration of 2.1 mg/mL with 20 mmol/L HEPES,

5 mmol/L NaOH, and $1 \times$ DMEM. Aliquots of 300 μ L of this mix were polymerized for 30 minutes at 37°C in 24-well plates. Placental fibroblasts suspended in 300 μ L serum-free DMEM (1×10^5 cells/mL) were loaded onto the collagen lattices. After 12 hours' incubation, TGFB1 (final concentration, 10 ng/mL) was added. One hour after the addition, the collagen gels were released mechanically from the underlying dishes, and the diameters of the gels were measured at 6, 12, and 24 hours later. The relative diameter of the gels was calculated using the following formula: $100 \times (\text{gel diameter})/(\text{well diameter})$.

Whole-Genome Expression Analysis by Microarrays

DNA microarray analysis was performed using SurePrint G3 Human Gene Expression Microarrays 8×60 K version 2 (Agilent Technologies). In brief, total RNA from fibroblasts maintained in DMEM with 10% FBS was prepared with the RNeasy Mini Kit. Relative target intensity was calculated using Agilent Feature Extraction Software version 9.5.1.1 (Agilent Technologies). The data were deposited at the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/geo/>; accession number GSE99007). We generated a heat map using MeV software version 4.8.1 (Multiple Experiment Viewer; <http://www.tm4.org>) and a hierarchical clustering method to sort the genes. Gene Set Enrichment Analysis (<http://www.broadinstitute.org/gsea/>

[index.jsp](#)) of the expression data was used to assess enrichment of the up-regulated genes from GSE40839²⁶ and GSE63659.²⁷

Statistical Analysis

All experiments were repeated at least three times, with similar results in each instance. Results were analyzed using the nonparametric *U*-test, *t*-test, paired *t*-test, or Wilcoxon signed-rank test. $P < 0.05$ was considered significant.

Results

Fibrosis and the Expression of FRFs Are Prominent in PE Placentas

To examine the involvement of fibrosis in PE pathogenesis, collagen fibers in placenta tissue sections from PE and NP cases were visualized by Masson's trichrome staining procedure. Positive staining was observed in villous stroma of placentas (Figure 1, A and B). The ratio of the positive staining area was significantly higher in the PE group than in the NP group ($P = 0.002$) (Figure 1C). On the basis of this result, the expression of levels fibrosis-related factors (FRFs), such as ACTA2, CTGF, and FN1, were next examined in the placental tissues. ACTA2 is a marker for myofibroblasts and active fibroblasts.²⁸ CTGF plays a central role in fibrosis and

Figure 1 Collagen deposition and fibrosis-related proteins in normal pregnancy (NP) and preeclamptic (PE) placentas. **A** and **B**: Placenta tissues from 14 NP and 11 PE cases were evaluated by Masson's trichrome staining to visualize collagen (COL) deposition. **C**, **F**, **I**, and **L**: Ratio of signal-positive area/total tissue area is shown as % expression area. **D**, **E**, **G**, **H**, **J**, and **K**: Collagen depositions are blue, and cytoplasm is red; immunohistochemistry staining of NP and PE placentas of 6 to 10 randomly selected cases are shown for actin, $\alpha 2$, smooth muscle, aorta (ACTA2; **D** and **E**), connective tissue growth factor (CTGF; **G** and **H**), and fibronectin (FN1; **J** and **K**). * $P < 0.05$, ** $P < 0.01$. Scale bars = 200 μ m (**A**, **B**, **D**, **E**, **G**, **H**, **J**, and **K**).

up-regulates the expression of FN1 and collagen type 4.²⁹ FN1 is an ECM protein playing an important role in the development of fibrotic disease.³⁰ Immunohistochemistry showed that the expression of ACTA2 was localized around intravillous vessels and in villous stroma (Figure 1, D and E), the expression of CTGF was localized in villous stroma (Figure 1, G and H), and the expression of FN1 was scattered in fibrous villous stroma (Figure 1, J and K). Ratios of the immunopositive area for ACTA2, CTGF, and FN1 were significantly higher in the PE group than in the NP group ($P < 0.05$) (Figure 1, F, I, and L).

Characterization of Placental Fibroblasts Isolated from Term Placentas

The data in Figure 1 suggested that abnormal fibrosis is a significant feature of PE placentas. Excessive production of ECM, and consequent fibrosis, is known to be promoted by aberrant activation of fibroblasts.⁷ Therefore, fibroblasts were isolated from PE and NP placentas, and their characteristics were evaluated. To minimize the effects from culture conditions, the fibroblasts were analyzed within the first 8 cell culture passages. Fibroblasts originate from mesenchymal cells and should be positive for mesenchymal markers and negative for epithelial markers. At first, the fibroblasts isolated were confirmed for their homogeneity by immunocytochemistry. All of the fibroblasts were positive for a mesenchymal marker, vimentin, and negative for an epithelial marker, pan-keratins (Figure 2A). The mRNA expression of epithelial markers, cadherin 1, and vimentin in the fibroblasts was confirmed by real-time RT-PCR analysis in comparison with trophoblast cell lines, HTR-8/SVneo

cells, and TCL-1 cells (Figure 2, B and C). There were no remarkable differences in morphology or in the expression levels of mesenchymal/epithelial markers in between the fibroblasts from PE and those from NP cases (Figure 2).

Expression Levels of FRFs Are Higher in the Fibroblasts from PE Placentas

Next, the expression levels of FRFs were compared between the fibroblasts from NP and PE placentas. The comparative mRNA levels of COL1A1, ACTA2, CTGF, and FN1 were higher in the fibroblasts from PE than in those from NP placentas ($P < 0.05$) (Figure 3, A–D). The fibroblasts from PE placentas all expressed high protein levels of COL1A1, ACTA2, and FAP (Figure 3E). FAP is a transmembrane serine protease, possessing type 1 collagen–specific collagenase activity, and it is recognized as another marker of activated fibroblasts.³¹ The protein secretion level of FN1 in the culture medium was assessed by enzyme-linked immunosorbent assay. The fibroblasts from PE placentas secreted larger amounts of FN1 than those from NP cases ($P = 0.002$) (Figure 3F). These data indicated that the fibroblasts isolated from PE placentas were in a more activated state than those from NP placentas, even after several passages.

Expression of FRFs in the Placental Fibroblasts Does Not Depend on Placental Region of Sampling or Passage Numbers

It was previously reported that variations in the morphology and gene expression of different placental regions were

Figure 2 Characterization of fibroblasts isolated from placentas. **A:** Protein expression of vimentin (VIM) and pan-keratin in fibroblasts from normal pregnancy (NP) and preeclamptic (PE) cases were evaluated by immunocytochemistry. Representative results of cases NP-9 and PE-2 are shown. **Leftmost panels:** Phase-contrast images of cultured fibroblasts. **Rightmost panels:** Merged images of immunocytochemical staining of VIM and pan-keratin counterstained with DAPI. **B and C:** mRNA analysis of cadherin 1 (**B**) and VIM (**C**) in isolated fibroblasts from NP and PE cases and from the trophoblast HTR8SVneo and TCL1 cell lines. Scale bars = 50 μ m (**A**). Original magnification, $\times 20$ (**leftmost column**).

Figure 3 Expression of fibrosis-related factors in fibroblasts from normal pregnancy (NP) and preeclamptic (PE) placentas. mRNA analysis of fibroblasts from NP and PE cases by real-time RT-PCR. **A–D**: Fibroblast samples from six NP (NP-1, NP-3, NP-4, NP-5, NP-6, and NP-11) and six PE (PE-1, PE-2, PE-4, PE-5, PE-7, and PE-8) cases treated with or without 10 ng/mL TGFB1 for 24 hours were used to analyze their mRNA levels of collagen 1A1 (COL1A1; **A**), actin, α 2, smooth muscle, aorta (ACTA2; **B**), connective tissue growth factor (**C**), and fibronectin (FN1; **D**). **E**: Protein expression levels of COL1, ACTA2, fibroblast activation protein (FAP), phosphorylated SMAD2 (p-SMAD2), and total SMAD2 in fibroblasts from NP and PE cases maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. **F** and **G**: Concentrations of FN1 (**F**) and TGFB1 (**G**) secreted in serum-free culture media for 24 hours were evaluated by enzyme-linked immunosorbent assay. * $P < 0.05$, ** $P < 0.01$. ID, identification.

dependent on the oxygen or nutrient gradient from the center to the lateral edge.³² The regional differences of gene expression levels among PE fibroblasts from sites adjacent to the cord insertion site, halfway between the cord insertion point and the lateral edge and beside the lateral edge, were evaluated. However, no region-specific expression of FRFs was observed in fibroblasts (Supplemental Figure S1, A–D).

The passage number–dependency of gene expression was also examined in NP and PE fibroblasts. Three lines from NP and PE fibroblasts were cultured from passage 3 (passage 4 for some lines) to 8, and samples were collected at each passage. However, no remarkable difference in FRF expression was observed among the fibroblasts at each cell passage (Supplemental Figure S1, E–I).

Genome-Wide Profiling of Gene Expression—Associated PE Fibroblasts with Fibrosis and TGFB1 Signaling

To investigate the differential expression patterns of mRNAs in the fibroblasts between NP and PE placentas, a

genome-wide RNA microarray analysis was performed. Hierarchical clustering of all genes formed depending on the gene expression patterns of PE and NP samples distinguished these two groups. The Tree View showed that each group appeared to have a specific gene profile (Figure 4A). Gene expression profiling in placental fibroblasts showed that 626 genes were differentially expressed between PE and NP samples with LIMMA (twofold or greater; $P \leq 0.05$). Up-regulated ($n = 358$) and down-regulated ($n = 268$) genes showed significant differences between the PE and NP fibroblasts.

Gene Set Enrichment Analysis showed remarkably positive correlations in the changes in gene expression profiles of fibroblasts from PE versus NP placentas and of those from idiopathic pulmonary fibrosis versus normal lung tissues (Figure 4B).²⁶ Gene Set Enrichment Analysis also showed remarkably positive correlation in changes in gene expression profiles of fibroblasts from PE versus NP placentas and of those from lung fibroblasts stimulated with TGFB1 versus nontreated controls (Figure 4C).²⁷

Figure 4 Whole-genome expression analysis of fibroblasts from normal pregnancy (NP) and preeclamptic (PE) cases. **A**: Heat map originated from data of whole-genome expression analysis of fibroblasts from cases NP-1, NP-2, NP-3, PE-1, PE-2, PE-3, and PE-4 using gene microarrays. **B**: Gene Set Enrichment Analysis (GSEA) of up-regulated genes in idiopathic pulmonary fibrosis (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE40839²⁶) compared with the changes in their expression in PE fibroblasts relative to that in NP fibroblasts. **C**: GSEA of up-regulated genes in lung fibroblasts stimulated with TGFβ1 (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE63659²⁷) compared with the changes in their expression in PE fibroblasts relative to that in NP fibroblasts. **D–M**: mRNA analysis of 10 genes in fibroblasts from NP and PE cases: *IL15* (D), *COL8A1* (E), *FGF1* (F), *IGFBP3* (G), *ADAM15* (K), *ITGA2* (L), and *PGF* (M). * $P < 0.05$, ** $P < 0.01$. FDR, false-discovery rate; NES, normalized enrichment score.

Ten genes with remarkable expression changes were selected to validate their altered expression in PE samples by real-time RT-PCR (Figure 4, D–M). Among these genes, the expressions of *IL15* and *PGF* are known to be associated with PE.^{33,34} *FGF1*, *IGFBP3*, and *ADAM15* are associated with pulmonary or cardiac fibrosis.^{35–37} *COL8A1*, *IGFBP3*, *SPARC*, *FBN2*, *MMP9*, and *ITGA2* were reported to be affected by or to affect TGFβ–SMAD signaling.^{36,38–42} These data suggested that fibrosis and activated TGFβ signaling are the characteristic features of PE fibroblasts.

Involvement of TGFβ1 Signaling in Activated Fibroblasts from PE Placentas

Supported by the results in Figure 4, it was further examined whether TGFβ1 signaling was activated in PE fibroblasts. Phosphorylated SMAD2 (p-SMAD2) was first examined in serum-free conditions, as used for mRNA analysis; however, no detectable levels were observed in the fibroblasts. Therefore, 10% FBS conditions were used for the protein analysis shown in Figure 3E, which shows more than a detectable level of p-SMAD2 in most of the fibroblast

samples from PE placentas. This result suggested that TGF- β —SMAD signaling was activated in the PE fibroblasts. To explore the mechanism of aberrant activation of TGF β signaling in the PE fibroblasts, serum-free cell culture supernatants from the NP and PE fibroblasts were examined for their TGF β 1 levels. Larger amounts of TGF β 1 were secreted from the PE fibroblasts than from the NP fibroblasts (Figure 3G).

To explore the responsiveness of the fibroblasts to TGF- β 1, both the PE and NP fibroblasts were treated with 10 ng/mL of TGF β 1 for 24 hours. Increased levels of COL1A1, ACTA2, CTGF, FN1, and FAP were observed in both types of fibroblasts in response to TGF β 1 treatment ($P < 0.05$) (Figure 3, A–D, and Figure 5A). FN1 secretion was induced by TGF β 1 treatment in NP fibroblasts ($P = 0.009$) (Figure 5B). As expected, these responses were accompanied by up-regulation of p-SMAD2 (Figure 5A). These data suggest that both the NP and PE fibroblasts respond to TGF β 1 and that the aberrant activation of TGF β 1—SMAD pathways was a characteristic of the PE fibroblasts.

Knockdown of TGFBR1 was also used to demonstrate the involvement of TGF β 1—SMAD signaling in activated fibroblasts from PE cases. Successful knockdown of

TGFBR1 in PE fibroblasts was verified by real-time RT-PCR (Figure 5C). As expected, knockdown of TGFBR1 resulted in a decrease in the p-SMAD2 level (Figure 5D). Furthermore, knockdown of TGFBR1 reduced the protein levels of COL1 and FAP and the mRNA levels of COL1A1, CTGF, and FN1 (Figure 5, D–J). These data also suggested that the aberrant activation of TGF β 1—SMAD pathway in PE fibroblasts was involved in the fibrogenesis of PE.

Fibroblasts from PE Cases Have a Greater Ability to Contract *in Vitro*

To assess the force-mediated matrix remodeling of placental fibroblasts *in vitro*, fibroblasts from NP and PE cases were loaded onto collagen lattices. The relative diameters of the collagen lattices were monitored at the defined time points. The fibroblasts from PE cases showed a greater ability to contract than those from NP cases (Figure 6A). The impact of TGF β 1 on collagen contraction was also examined. As expected, addition of TGF β 1 to the fibroblasts from NP cases enhanced collagen contraction (Figure 6B). By contrast, knockdown of TGFBR1 in the PE fibroblasts suppressed this contraction (Figure 6C). These data also

Figure 5 Effects of treatment with TGF β 1 or knockdown of *TGFBR1* gene expression in placental fibroblasts. **A:** Protein levels of collagen 1 (COL1), fibroblast activation protein (FAP), phosphorylated SMAD2 (p-SMAD2), and total SMAD2 in fibroblasts from cases normal pregnancy (NP)-3 and preeclampsia (PE)-6 treated with or without 10 ng/mL TGF β 1 for 24 hours were evaluated by Western blotting. **B:** Fibronectin (FN1) secretion into serum-free Dulbecco's modified Eagle's medium by fibroblasts treated with or without TGF β 1 was evaluated by enzyme-linked immunosorbent assay. **C:** Fibroblasts from cases PE-4, PE-5, and PE-6 were transfected with siRNA to knockdown TGFBR1 (siTGFBR1). The mRNA levels of TGFBR1 in each fibroblast type were compared with control siRNA (siCtrl) by real-time RT-PCR. **D:** Protein levels of COL1, FAP, p-SMAD2, and total SMAD2 in fibroblasts from cases PE-3, PE-5, and PE-6 transfected with siCtrl and siTGFBR1 were evaluated. **E–J:** mRNA levels of COL1A1 (**E** and **H**), connective tissue growth factor (**F** and **I**), and FN1 (**G** and **J**) in fibroblasts from cases PE-4 (**E–G**) and PE-5 (**H–J**) transfected with siCtrl and siTGFBR1 were evaluated. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

suggested that the fibroblasts from PE placentas had been activated functionally and that TGFBR1 signaling was involved in this process.

Discussion

At the placental site of PE, deficiency of trophoblast invasion into the maternal decidua is considered to be the first stage of the two-stage theory. In the second stage, histologic features characterized by villous fibrosis become apparent.^{4,5} Although there is little known about the mechanism of placental fibrosis, TGFBR1 is considered a trigger.^{12,19} TGFBR1 is also implicated to regulate trophoblast differentiation and invasion, which determine the first stage of preeclampsia.^{43,44} Little is known about fibrosis in PE placentas, and it is also unclear whether trophoblasts, fibroblasts, or both are responsible for this pathologic change.

In the present study, placental fibrosis was studied as a feature of PE and the fibroblasts from PE placentas were characterized. Histology showed that the PE placentas overproduced collagen fibers and overexpressed ACTA2, CTGF, and FN1 (Figure 1). These FRFs, such as COL1A1, ACTA2, CTGF, and FN1, have been suggested to be involved in PE-related fibrosis in the placenta.^{45–47} Among these factors, CTGF was reported to be expressed in villous trophoblasts, consistent with our result (Figure 1G).¹² However, our data also showed that the stromal expression of CTGF was dominant in preeclampsia cases (Figure 1H). These results led us to focus on villous stromal mesenchyme, which is thought to have a central role in placental fibrosis. In addition, trophoblasts might directly or indirectly affect fibrosis in the villous stroma, and this

remains to be studied in the future. Interestingly, primary culture of the fibroblasts isolated from PE placentas also showed higher expression levels of FRFs than in those from NP placentas (Figure 3). These data suggest that the fibroblasts from PE placentas had been aberrantly activated and were kept activated, even after several passages in culture. The active state of PE fibroblasts was also demonstrated from a functional aspect (Figure 6A).

Samples collected from the placental disk, halfway between the cord insertion point and the lateral edge, were used for histologic analysis and the isolation of fibroblasts. At present, there are no standards for placental sampling to satisfy all researchers. Regarding the placental disk, variations in morphology and gene expression in different placental regions were reported to be dependent on the oxygen or nutrient gradient from the center to the lateral edge.³² Although there have been several attempts to optimize placental sampling by multiple site sampling or randomization of sampling sites, site-directed sampling to exclude the interregional differences has been broadly accepted.^{32,48–50} Experiments were conducted to evaluate the regional differences of gene expression among PE fibroblasts from sites adjacent to the cord insertion site, halfway between the cord insertion point and the lateral edge, and beside the lateral edge. However, no region-specific expression of FRFs was observed in fibroblasts (Supplemental Figure S1, A–D).

For exploring useful fibrosis-specific markers involved in PE-related fibrosis, a genome-wide gene expression analysis was applied by microarray. Gene expression profiling of the PE fibroblasts indicated strong associations with those seen in cases of idiopathic pulmonary fibrosis and TGFBR1 stimulation (Figure 4, B and C). Among the differentially

Figure 6 Collagen gel contraction by placental fibroblasts. **A:** Fibroblasts from three normal pregnancy (NP; NP-2, NP-7, and NP-8) and three preeclampsia (PE; PE-4, PE-5, and PE-6) cases were evaluated for their *in vitro* contraction ability. **B:** Fibroblasts from four NP cases (NP-2, NP-3, NP-7, and NP-13) were treated with or without 10 ng/mL TGFBR1 to evaluate their contraction *in vitro*. **C:** Fibroblasts from three PE cases (PE-4, PE-5, and PE-6) were transfected with control siRNA (siCtrl) or siRNA to knockdown TGFBR1 (siTGFBR1), and their contraction was evaluated *in vitro*. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

expressed genes, 10 were selected to validate their expression levels (Figure 4, D–M). Among these, the expressions of *IL15* and *PGF* are known to be associated with PE. Previous studies reported the expression of *IL15* and *PGF* in the placental mesenchyme,^{51,52} but the association of *IL15* and *PGF* in fibroblasts with preeclampsia remains unclear.^{33,34} In addition to *FGF1*, *IGFBP3*, and *ADAM15*, known to be associated with tissue fibrosis,^{35–37} *COL8A1* and *SPARC*, which are reported to be targets of TGFβ1 signaling, were involved.^{36,38,42} These data suggest that fibrosis and activated TGFβ1 signaling are critical characteristics of PE fibroblasts.

Factors other than PE might affect gene expression in the placenta. Whole-genome analysis reported that gene expression of the placenta correlated with sex, delivery mode, and birth weight.⁴⁸ *IL15* production from placental tissue explants was reported to be higher in term placental tissues than first-trimester tissues. Furthermore, an increase in *IL15* production was observed in placentas with the presence of labor at term.⁵³ All of the data were analyzed to evaluate any correlation of gene expression with maternal age, delivery week, delivery mode, infant body weight, or infant sex. However, no significant differences correlated with these factors. One limitation of our study was the sample size, which was too small to analyze potential association with each of these factors.

To exclude the effects of serum in culture, p-SMAD2 levels were first examined in serum-free conditions, as used for mRNA analysis; however, no detectable levels were observed in the fibroblasts (Figure 5A). Therefore, 10% FBS was used for protein analysis in Figure 3E. With this limitation—although it was not remarkable—most of the PE fibroblasts expressed abundant p-SMAD2 (Figure 3E). These data also strengthen our contention that aberrant activation of TGFβ–SMAD pathways was involved in the activation of fibroblasts from PE placentas.

Although the basal expression levels of FRFs were higher in the PE fibroblasts, the expression levels were enhanced in both NP and PE fibroblasts after exogenous TGFβ1 treatment (Figure 3, A–D, and Figure 5A). These data also indicated that the NP and PE fibroblasts have a similar responsiveness to TGFβ1 treatment. Induced levels of *COL1A1*, *ACTA2*, *CTGF*, *FN1*, and *FAP* are also known to occur in other fibroblast types stimulated by TGF-β1.^{54–56} Furthermore, the fibroblasts from PE placentas secreted a larger amount of TGFβ1 into the culture medium than those from NP placentas (Figure 3G). These results suggest that the autoinduction of TGFβ1–SMAD signaling might be involved in the activation of the PE fibroblasts, as has been reported in fibroblasts from other organs.^{57,58} Because a higher plasma level of TGFβ1 has been reported in patients with PE,^{22,23} the increased plasma level of TGFβ1 might also participate in the activation of their fibroblasts. Furthermore, whether villous trophoblasts express and secrete TGFβs is currently controversial; however, TGFβs secreted from trophoblasts might also be involved in the activation of fibroblasts.^{20,21,59,60}

In conclusion, we isolated fibroblasts from NP and PE placentas and clarified their characteristics. A detailed gene expression analysis revealed that higher levels of fibrosis-related genes were expressed in the PE fibroblasts and that TGFβ1 signaling was activated aberrantly. These novel insights may help us to elucidate the mechanism of placental dysfunction in PE and to develop a molecular strategy to treat affected patients.

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Supplemental Data

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References

- Kaufmann P, Stark J, Stegner HE: The villous stroma of the human placenta, I: the ultrastructure of fixed connective tissue cells. *Cell Tissue Res* 1977, 177:105–121
- Hernandez-Diaz S, Toh S, Cnattingius S: Risk of pre-eclampsia in first and subsequent pregnancies: prospective cohort study. *BMJ* 2009, 338:b2255
- Roberts JM, Hubel CA: The two stage model of preeclampsia: variations on the theme. *Placenta* 2009, 30 Suppl A:S32–S37
- Ducray JF, Naicker T, Moodley J: Pilot study of comparative placental morphometry in pre-eclamptic and normotensive pregnancies suggests possible maladaptations of the fetal component of the placenta. *Eur J Obstet Gynecol Reprod Biol* 2011, 156:29–34
- Devisme L, Merlot B, Ego A, Houfflin-Debarge V, Deruelle P, Subtil D: A case-control study of placental lesions associated with pre-eclampsia. *Int J Gynaecol Obstet* 2013, 120:165–168
- Zeisberg M, Kalluri R: Cellular mechanisms of tissue fibrosis, 1: common and organ-specific mechanisms associated with tissue fibrosis. *Am J Physiol Cell Physiol* 2013, 304:C216–C225
- Kendall RT, Feghali-Bostwick CA: Fibroblasts in fibrosis: novel roles and mediators. *Front Pharmacol* 2014, 5:123
- Ueha S, Shand FH, Matsushima K: Cellular and molecular mechanisms of chronic inflammation-associated organ fibrosis. *Front Immunol* 2012, 3:71
- Border WA, Okuda S, Languino LR, Sporn MB, Ruoslahti E: Suppression of experimental glomerulonephritis by antiserum against transforming growth factor beta 1. *Nature* 1990, 346:371–374
- Leask A: Potential therapeutic targets for cardiac fibrosis: TGFβ, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. *Circ Res* 2010, 106:1675–1680
- Higgins DF, Kimura K, Bernhardt WM, Shrimanker N, Akai Y, Hohenstein B, Saito Y, Johnson RS, Kretzler M, Cohen CD, Eckardt KU, Iwano M, Haase VH: Hypoxia promotes fibrogenesis in vivo via HIF-1 stimulation of epithelial-to-mesenchymal transition. *J Clin Invest* 2007, 117:3810–3820

12. Rimón E, Chen B, Shanks AL, Nelson DM, Sadovsky Y: Hypoxia in human trophoblasts stimulates the expression and secretion of connective tissue growth factor. *Endocrinology* 2008, 149:2952–2958
13. Falanga V, Zhou L, Yufit T: Low oxygen tension stimulates collagen synthesis and COL1A1 transcription through the action of TGF-beta1. *J Cell Physiol* 2002, 191:42–50
14. Tager AM, LaCamera P, Shea BS, Campanella GS, Selman M, Zhao Z, Polosukhin V, Wain J, Karimi-Shah BA, Kim ND, Hart WK, Pardo A, Blackwell TS, Xu Y, Chun J, Luster AD: The lysophosphatidic acid receptor LPA1 links pulmonary fibrosis to lung injury by mediating fibroblast recruitment and vascular leak. *Nat Med* 2008, 14:45–54
15. Takuwa N, Ohkura S, Takashima S, Ohtani K, Okamoto Y, Tanaka T, Hirano K, Usui S, Wang F, Du W, Yoshioka K, Banno Y, Sasaki M, Ichi I, Okamura M, Sugimoto N, Mizugishi K, Nakanuma Y, Ishii I, Takamura M, Kaneko S, Kojo S, Satouchi K, Mitumori K, Chun J, Takuwa Y: S1P3-mediated cardiac fibrosis in sphingosine kinase 1 transgenic mice involves reactive oxygen species. *Cardiovasc Res* 2010, 85:484–493
16. Chambers RC, Laurent GJ: Coagulation cascade proteases and tissue fibrosis. *Biochem Soc Trans* 2002, 30:194–200
17. Hemmann S, Graf J, Roderfeld M, Roeb E: Expression of MMPs and TIMPs in liver fibrosis: a systematic review with special emphasis on anti-fibrotic strategies. *J Hepatol* 2007, 46:955–975
18. Chen CP, Aplin JD: Placental extracellular matrix: gene expression, deposition by placental fibroblasts and the effect of oxygen. *Placenta* 2003, 24:316–325
19. Chen CP, Yang YC, Su TH, Chen CY, Aplin JD: Hypoxia and transforming growth factor-beta 1 act independently to increase extracellular matrix production by placental fibroblasts. *J Clin Endocrinol Metab* 2005, 90:1083–1090
20. Lyall F, Simpson H, Bulmer JN, Barber A, Robson SC: Transforming growth factor-beta expression in human placenta and placental bed in third trimester normal pregnancy, preeclampsia, and fetal growth restriction. *Am J Pathol* 2001, 159:1827–1838
21. Simpson H, Robson SC, Bulmer JN, Barber A, Lyall F: Transforming growth factor beta expression in human placenta and placental bed during early pregnancy. *Placenta* 2002, 23:44–58
22. Madazli R, Aydin S, Uludag S, Vildan O, Tolun N: Maternal plasma levels of cytokines in normal and preeclamptic pregnancies and their relationship with diastolic blood pressure and fibronectin levels. *Acta Obstet Gynecol Scand* 2003, 82:797–802
23. Ozkan ZS, Simsek M, Ilhan F, Deveci D, Godekmerdan A, Sapmaz E: Plasma IL-17, IL-35, interferon-gamma, SOCS3 and TGF-beta levels in pregnant women with preeclampsia, and their relation with severity of disease. *J Matern Fetal Neonatal Med* 2014, 27:1513–1517
24. Liu G, Asanoma K, Takao T, Tsukimori K, Uchi H, Furue M, Kato K, Wake N: Aryl hydrocarbon receptor SNP-130 C/T associates with dioxins susceptibility through regulating its receptor activity and downstream effectors including interleukin 24. *Toxicol Lett* 2015, 232:384–392
25. Takao T, Asanoma K, Kato K, Fukushima K, Tsunematsu R, Hirakawa T, Matsumura S, Seki H, Takeda S, Wake N: Isolation and characterization of human trophoblast side-population (SP) cells in primary villous cytotrophoblasts and HTR-8/SVneo cell line. *PLoS One* 2011, 6:e21990
26. Lindahl GE, Stock CJ, Shi-Wen X, Leoni P, Sestini P, Howat SL, Bou-Gharios G, Nicholson AG, Denton CP, Grutters JC, Maher TM, Wells AU, Abraham DJ, Renzoni EA: Microarray profiling reveals suppressed interferon stimulated gene program in fibroblasts from scleroderma-associated interstitial lung disease. *Respir Res* 2013, 14:80
27. Wettlaufer SH, Scott JP, McEachin RC, Peters-Golden M, Huang SK: Reversal of the transcriptome by prostaglandin E2 during myofibroblast dedifferentiation. *Am J Respir Cell Mol Biol* 2016, 54:114–127
28. Kalluri R, Zeisberg M: Fibroblasts in cancer. *Nat Rev Cancer* 2006, 6:392–401
29. Shi-Wen X, Leask A, Abraham D: Regulation and function of connective tissue growth factor/CCN2 in tissue repair, scarring and fibrosis. *Cytokine Growth Factor Rev* 2008, 19:133–144
30. Bhattacharyya S, Tamaki Z, Wang W, Hinchcliff M, Hoover P, Getsios S, White ES, Varga J: Fibronectin EDA promotes chronic cutaneous fibrosis through Toll-like receptor signaling. *Sci Transl Med* 2014, 6:232ra250
31. Kelly T, Huang Y, Simms AE, Mazur A: Fibroblast activation protein-alpha: a key modulator of the microenvironment in multiple pathologies. *Int Rev Cell Mol Biol* 2012, 297:83–116
32. Burton GJ, Sebire NJ, Myatt L, Tannetta D, Wang YL, Sadovsky Y, Staff AC, Redman CW: Optimising sample collection for placental research. *Placenta* 2014, 35:9–22
33. Hu W, Wang H, Wang Z, Huang H, Dong M: Elevated serum levels of interleukin-15 and interleukin-16 in preeclampsia. *J Reprod Immunol* 2007, 73:166–171
34. Weel IC, Baergen RN, Romao-Veiga M, Borges VT, Ribeiro VR, Witkin SS, Bannwart-Castro C, Peracoli JC, De Oliveira L, Peracoli MT: Association between placental lesions, cytokines and angiogenic factors in pregnant women with preeclampsia. *PLoS One* 2016, 11:e0157584
35. MacKenzie B, Korfei M, Henneke I, Sibinska Z, Tian X, Hezel S, Dilai S, Wasnick R, Schneider B, Wilhelm J, El Agha E, Klepetko W, Seeger W, Schermuly R, Gunther A, Bellusci S: Increased FGF1-FGFRc expression in idiopathic pulmonary fibrosis. *Respir Res* 2015, 16:83
36. Pilewski JM, Liu L, Henry AC, Knauer AV, Feghali-Bostwick CA: Insulin-like growth factor binding proteins 3 and 5 are overexpressed in idiopathic pulmonary fibrosis and contribute to extracellular matrix deposition. *Am J Pathol* 2005, 166:399–407
37. Nadadur RD, Umar S, Wong G, Eghbali M, Iorga A, Matori H, Partow-Navid R, Eghbali M: Reverse right ventricular structural and extracellular matrix remodeling by estrogen in severe pulmonary hypertension. *J Appl Physiol* (1985) 2012, 113:149–158
38. Loeffler I, Hopfer U, Koczan D, Wolf G: Type VIII collagen modulates TGF-beta1-induced proliferation of mesangial cells. *J Am Soc Nephrol* 2011, 22:649–663
39. Olivieri J, Smaldone S, Ramirez F: Fibrillin assemblies: extracellular determinants of tissue formation and fibrosis. *Fibrogenesis Tissue Repair* 2010, 3:24
40. Warburton D, Shi W, Xu B: TGF-beta-Smad3 signaling in emphysema and pulmonary fibrosis: an epigenetic aberration of normal development? *Am J Physiol Lung Cell Mol Physiol* 2013, 304:L83–L85
41. Girgert R, Martin M, Kruegel J, Miosge N, Temme J, Eckes B, Muller GA, Gross O: Integrin alpha2-deficient mice provide insights into specific functions of collagen receptors in the kidney. *Fibrogenesis Tissue Repair* 2010, 3:19
42. Shibata S, Ishiyama J: Secreted protein acidic and rich in cysteine (SPARC) is upregulated by transforming growth factor (TGF)-beta and is required for TGF-beta-induced hydrogen peroxide production in fibroblasts. *Fibrogenesis Tissue Repair* 2013, 6:6
43. Zhao MR, Qiu W, Li YX, Zhang ZB, Li D, Wang YL: Dual effect of transforming growth factor beta1 on cell adhesion and invasion in human placenta trophoblast cells. *Reproduction* 2006, 132:333–341
44. Cheng JC, Chang HM, Leung PC: Transforming growth factor-beta1 inhibits trophoblast cell invasion by inducing Snail-mediated down-regulation of vascular endothelial-cadherin protein. *J Biol Chem* 2013, 288:33181–33192
45. Rukosuev VS, Nanaev AK, Milovanov AP: Participation of collagen types I, III, IV, V, and fibronectin in the formation of villi fibrosis in human term placenta. *Acta Histochem* 1990, 89:11–16
46. Todros T, Marziani D, Lorenzi T, Piccoli E, Capparuccia L, Perugini V, Cardaropoli S, Romagnoli R, Gesuita R, Rolfo A, Paulesu L, Castellucci M: Evidence for a role of TGF-beta1 in the expression and regulation of alpha-SMA in fetal growth restricted placentae. *Placenta* 2007, 28:1123–1132

47. Oh SY, Song SE, Seo ES, Kim KH, Choi SJ, Suh YL, Sadovsky Y, Roh CR: The expression of connective tissue growth factor in pregnancies complicated by severe preeclampsia or fetal growth restriction. *Placenta* 2009, 30:981–987
48. Sood R, Zehnder JL, Druzin ML, Brown PO: Gene expression patterns in human placenta. *Proc Natl Acad Sci U S A* 2006, 103:5478–5483
49. Lee HJ, Jeong SK, Na K, Lee MJ, Lee SH, Lim JS, Cha HJ, Cho JY, Kwon JY, Kim H, Song SY, Yoo JS, Park YM, Kim H, Hancock WS, Paik YK: Comprehensive genome-wide proteomic analysis of human placental tissue for the Chromosome-Centric Human Proteome Project. *J Proteome Res* 2013, 12:2458–2466
50. Wolfe LM, Thiagarajan RD, Boscolo F, Tache V, Coleman RL, Kim J, Kwan WK, Loring JF, Parast M, Laurent LC: Banking placental tissue: an optimized collection procedure for genome-wide analysis of nucleic acids. *Placenta* 2014, 35:645–654
51. Abumaree MH, Al Jumah MA, Kalionis B, Jawdat D, Al Khaldi A, AlTalabani AA, Knawy BA: Phenotypic and functional characterization of mesenchymal stem cells from chorionic villi of human term placenta. *Stem Cell Rev* 2013, 9:16–31
52. Khaliq A, Li XF, Shams M, Sisi P, Acevedo CA, Whittle MJ, Weich H, Ahmed A: Localisation of placenta growth factor (PlGF) in human term placenta. *Growth Factors* 1996, 13:243–250. color plates I-II, pre.bk cov
53. Agarwal R, Loganath A, Roy AC, Wong YC, Ng SC: Expression profiles of interleukin-15 in early and late gestational human placenta and in pre-eclamptic placenta. *Mol Hum Reprod* 2001, 7:97–101
54. Denys H, Derycke L, Hendrix A, Westbroek W, Gheldof A, Narine K, Pauwels P, Gespach C, Bracke M, De Wever O: Differential impact of TGF-beta and EGF on fibroblast differentiation and invasion reciprocally promotes colon cancer cell invasion. *Cancer Lett* 2008, 266:263–274
55. Kucich U, Rosenbloom JC, Shen G, Abrams WR, Hamilton AD, Sebt SM, Rosenbloom J: TGF-beta1 stimulation of fibronectin transcription in cultured human lung fibroblasts requires active geranylgeranyl transferase I, phosphatidylcholine-specific phospholipase C, protein kinase C-delta, and p38, but not erk1/erk2. *Arch Biochem Biophys* 2000, 374:313–324
56. Mun JH, Kim YM, Kim BS, Kim JH, Kim MB, Ko HC: Simvastatin inhibits transforming growth factor-beta1-induced expression of type I collagen, CTGF, and alpha-SMA in keloid fibroblasts. *Wound Repair Regen* 2014, 22:125–133
57. Bascom CC, Wolfshohl JR, Coffey RJ Jr, Madisen L, Webb NR, Purchio AR, Derynck R, Moses HL: Complex regulation of transforming growth factor beta 1, beta 2, and beta 3 mRNA expression in mouse fibroblasts and keratinocytes by transforming growth factors beta 1 and beta 2. *Mol Cell Biol* 1989, 9:5508–5515
58. Flanders KC, Holder MG, Winokur TS: Autoinduction of mRNA and protein expression for transforming growth factor-beta S in cultured cardiac cells. *J Mol Cell Cardiol* 1995, 27:805–812
59. Caniggia I, Taylor CV, Ritchie JW, Lye SJ, Letarte M: Endoglin regulates trophoblast differentiation along the invasive pathway in human placental villous explants. *Endocrinology* 1997, 138:4977–4988
60. Ando N, Hirahara F, Fukushima J, Kawamoto S, Okuda K, Funabashi T, Gorai I, Minaguchi H: Differential gene expression of TGF-beta isoforms and TGF-beta receptors during the first trimester of pregnancy at the human maternal-fetal interface. *Am J Reprod Immunol* 1998, 40:48–56