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Involvement of SMAD4, but not of SMAD2, in transforming growth factor-beta1-induced trophoblast expression of matrix metalloproteinase-2

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1. ABSTRACT

Matrix metalloproteinases (MMPs) play crucial roles in extravillous trophoblast invasion. In the present study, we examined the possible role of Smad4 and Smad2 in transforming growth factor (TGF)-beta1-induced MMP-2 expression, using the well-established invasive extravillous trophoblast cell line HTR-8/SVneo. Recombinant sense *Smad4* or *Smad2* retroviral vectors were constructed by inserting full-length *Smad4* or *Smad2* cDNA into pLXSN retroviral vector. Stable PT67 packaging cell clones were isolated and viral supernatants were used to infect HTR-8/SVneo cells. Effects of retroviral expression of Smad4 and Smad2 on TGF-beta1-regulated MMP-2 expression were assessed by semi-quantitative reverse transcription-polymerase chain reaction and gelatin zymography. The results showed that over-expression of Smad4 augmented MMP-2 mRNA abundance and the secretion of pro-MMP-2, and mimicked the inductive effect of TGF-beta1 on the production of MMP-2. However, retrovirus-mediated sense *Smad2* gene transfer had no effect. These findings suggest that Smad4, but not Smad2, mediates TGF-beta1-induced MMP-2 expression in invasive extravillous trophoblasts.

2. INTRODUCTION

Extravillous trophoblast (EVT) invasion is essential to placental formation. Matrix metalloproteinases (MMPs), a family of zinc-dependent neutral endopeptidases that are capable of cleaving all extracellular matrix (ECM) components, play crucial roles during the invasion process of trophoblast cells (1, 2). Among MMPs, gelatinases (MMP-2 and MMP-9) are highly expressed by invasive trophoblasts *in vivo* (3) and are essential to the invasive ability of trophoblast cells *in vitro* (4-8). In particular, it has been reported that first-trimester villi express more MMP-2 than MMP-9, and MMP-2 is markedly down-regulated in the second trimester, highlighting that MMP-2 is the primary mediator in trophoblast invasion (9, 10). Therefore, studies on the regulatory mechanism of gelatinases by numerous cytokines and growth factors such as transforming growth factor-beta (TGF-beta) are important to an understanding of trophoblast invasion.

Ligands of the TGF-beta superfamily transmit their signals via transmembrane serine/threonine kinase

receptors (type II and type I), followed by the activation of the Smad pathway, which serves as the major intracellular signaling pathway of the TGF-beta superfamily (11). R-Smads (receptor-regulated Smads) are phosphorylated by TGF-beta receptor heteromers, of which Smad1, Smad5 and Smad8 respond to bone morphogenic proteins (BMPs) whereas Smad2 and Smad3 mediate TGF-betas/activins signals. R-Smads then form heteromeric complexes with Co-Smads (common-partner Smads) (Smad4 and Smad4beta in *Xenopus*), and are translocated into the nucleus. Inhibitory Smads (I-Smads) (Smad6 and Smad7) are antagonists of TGF-beta signaling through stably interacting with phosphorylated type I receptors and preventing R-Smads from being activated by these receptors (11, 12).

It has been well established that gelatinases are regulated by TGF-beta in first-trimester trophoblast cells (1, 6, 8, 13). Normal EVT cell invasion is controlled by deciduas-derived TGF-beta. This anti-invasive effect of TGF-beta is mediated by a down-regulation of urokinasetype plasminogen activator (uPA), and an up-regulation of tissue inhibitor of MMP (TIMP)-1 (14, 15) and plasminogen activator inhibitor 1 (PAI-1) (16). Furthermore, the regulation of gelatinases by TGF-beta may be another mechanism by which trophoblast invasion is tightly controlled. Our previous studies showed that TGF-beta1 up-regulated MMP-2 mRNA and pro-enzyme expression, whereas had no effect on MMP-9 expression in a well-established invasive EVT cell line HTR-8/SVneo (unpublished data). These results closely parallel observations made by Graham *et al*. (13) and Yudate *et al*. (6) in first-trimester trophoblasts. However, the precise regulatory mechanism of MMP-2 by TGF-beta in trophoblasts, especially the possible involvement of the Smad signaling pathway, has not yet been reported.

In this study, we investigated the roles of Smad4 and Smad2 in the regulation of MMP-2 by TGF-beta1 in human invasive EVT cell line HTR-8/SVneo by using sense *Smad4* and *Smad2* recombinant retrovirus.

3. MATERIALS AND METHODS

3.1. Reagents

Dulbecco modified Eagle medium (DMEM) was purchased from Gibco BRL (Gaithersburg, MD). RPMI 1640 medium was from Hyclone (Logan, UT). Recombinant human TGF-beta1 was from PEPRO-TECH EC Ltd. (London, England). Trizol reagent, Trizol LS reagent, LipofectAMINE^{TM} and Superscript II reverse transcriptase were from Invitrogen (Gaithersburg, MD). RNase-free DNase I was from Roche (Indianapolis, IN). The oligodeoxythymidine primers were synthesized by SBS Genetech Corp. (Beijing, China). Antibodies against phosphorylated Smad2/3, total Smad2/3, and Smad4 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Flag M2 monoclonal antibody, Geneticin (G418), and Sequabrene (Sigma version of Polybrene™) were from Sigma-Aldrich (Oakville, ON, Canada). Alkaline phosphatase (AP)- and FITC-conjugated secondary antibodies were from Zymed (San Francisco, CA). BioTrace™ nitrocellulose membranes were from Pall Corp. (East Hills, NY). Broad Range Protein Molecular Weight marker, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Promega (Madison, WI). The EndoFree® Plasmid Maxi Kit was from QIAGEN Inc. (Chatsworth, CA). DL2000 DNA molecular marker and restriction endonucleases were from TaKaRa Biotech. Co. Ltd (Dalian, China). The retroviral expression vector pLXSN and the RetroPack™ PT67 cell line (BD Biosciences Clontech, Palo Alto, CA) were generous gifts from Dr. Hou-Wen Tian, National Institute for Viral Disease Control and Prevention, Chinese Centers for Disease Control and Prevention, Beijing, China.

3.2. DNA Expression Constructs

C-terminal Flag-tagged human *Smad4* and N-terminal Flag-tagged human *Smad2* ligated in the mammalian expression vector pRK5F were generous gifts of Dr. Rik Derynck from the Departments of Growth and Development, and Anatomy, University of California at San Francisco, San Francisco, California (17). The blunt-ended 1692-bp *Eco*RI-*Hin*dIII fragment of pRK5F-*Smad4*-C-Flag was inserted into pLXSN, resulting in the generation of C-terminally Flag-tagged full length sense *Smad4* expression vector pLXSN-*Smad4*-C-Flag. Sense full-length *Smad2* retroviral vector pLXSN-N-Flag-*Smad2* was made by inserting the blunt-ended 1437-bp *Cla*I-*Xba*I fragment of pRK5F-N-Flag-*Smad2* into the blunt-ended *Xho*I-digested retroviral vector pLXSN. The authenticities of the recombinant vectors were confirmed by diagnostic restriction digestion.

3.3. Cell Cultures

An invasive EVT cell line HTR-8/SVneo was generously provided by Dr. Charles H. Graham (Queen's University, Kingston, ON, Canada). HTR-8 trophoblast cells were developed from an explant culture of human first-trimester placenta. HTR-8/SVneo cell line was immortalized by the gene encoding simian virus 40 large T antigen, and shares many phenotypic similarities with the parental HTR-8 trophoblast cells without malignant phenotype, including *in vitro* invasive abilities (15). The cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin.

The RetroPack™ PT67 cells were maintained in 10% FBS-DMEM supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin.

3.4. Stable Transfection

For each transfection experiment, RetroPack™ PT67 cells were seeded at 5×10^5 cells/60-mm culture dish and grown to 60%-80% confluence before transfection. Empty retroviral vector pLXSN, vector constructs pLXSN-*Smad4*-C-Flag, and pLXSN-N-Flag-*Smad2* were isolated using EndoFree® Plasmid Maxi Kit and transfected into the packaging cell line by using LipofectAMINE^{TM} according to instructions of the manufacturer. Twenty-four hours following the start of transfection, serum-free DNA-containing medium was replaced by fresh growth medium

containing 10% FBS. After a further 24–h incubation, cells were trypsinized and seeded at 1×10^5 cells/60-mm culture dish in 10% FBS-DMEM containing 600 μg/ml G418 (The optimal plating density and optimal concentration of G418 were titrated prior to the transfection experiments according to the method provided by the manufacturer). Stable transfectants were selected for 2 to 3 weeks, and the resultant cell populations were transferred to 24-well plates and maintained in G418-containing medium after growing to a large scale.

3.5. Selection of High-titer Retroviruses-producing Cell Clones

To rapidly identify high-titer producer clones, we performed a procedure as Quinn and Trevor (18) reported with some modifications. The method for rapid screening high-titer retrovirus produced by packaging cell clones was based on the direct isolation of viral RNA from producer clone supernatants by modified acid guanidinium/phenol/choloroform (AGPC) method, followed by reverse transcription-polymerase chain reaction (RT-PCR) analysis. The utility of AGPC/RT-PCR method for identifying high-producer clones was demonstrated by comparing with standard NIH3T3 titration method. In our case, we used Trizol LS reagent to isolate viral RNA from candidate supernatants, followed by semi-quantitative RT-PCR analysis. Briefly, G418-free media containing retroviruses were harvested from early confluence of cells until the cells were no longer viable. In each case, ten clones were separated. The collected retroviral supernatants were centrifuged at 1,500 rpm for 10 min to remove cellular debris. The viruses in supernatants were isolated using Trizol LS reagent according to instructions of the manufacturer, and were directly semi-quantitated by RT-PCR using neomycin (neo) primers (neo resistance gene is located at 1892-2686 in the vector) and pLXSN Seq/PCR primers (located at 1398-1537 in the vector) according to the method reported previously (18, 19). The primer sequences (in the 5'-3' direction) were as follows: TCC ATC ATG GCT GAT GCA ATG CGG C (forward) and GAT AGA AGG CGA TGC GCT GCG AAT CG (reverse) for neo, with an expected amplified length of 433 bp; CCC TTG AAC CTC CTC GTT CGA CC (forward) and GAG CCT GGG GAC TTT CCA CAC CC (reverse) for pLXSN Seq/PCR, with an expected amplified length of 139 bp when there was no gene insert. No cellular DNA contamination was observed when running the PCR reaction with RT template but in the absence of reverse transcriptase. The stable virus-producing packaging PT67 cell clones with highest titers were maintained. And the viral supernatants were collected, centrifuged at 500×g for 10 min to remove cellular debris, and stored at -70°C for use.

3.6. Transduction of Target Cells (HTR-8/SVneo cell line)

Target cells were plated at a density of 2×10^5 per 60-mm dish 12–18 h before the transduction and were allowed to grow to 60%-70% confluence. Media were then replaced with enough retroviral supernatants containing 8 μg/ml polybrene. After 24 h of incubation, supernatants were removed and replaced with fresh media. Media and cells were harvested 24 h later. Retroviral supernatants containing empty vector pLXSN, media of non-transfected PT67 cells, and parental non-transduced target cells were used as controls.

3.7. Indirect Immunofluorescence and Confocal Microscopy

For immunofluorescence analysis, stably transfected PT67 cells were grown on coverslips to early confluence and fixed with methanol: acetone (1:1) at –20°C for 30 min. After blocking in 3% BSA (in PBS) at room temperature for 30 min, cells were sequentially incubated at 37°C with anti-Flag primary antibody (diluted in blocking solution at a final concentration of 10 μg/ml) for 1 h, FITC-conjugated secondary antibody (diluted 1:100 in PBS) for 1 h, and 10 μg/ml propidium iodide (in PBS), with intervening rinsing with PBST (PBS containing 0.1% Tween 20) after each incubation. Controls were performed by obviating primary antibodies in each experiment. Cells were visualized with a Leica confocal microscopy device (TCS-NT) (Leica Lasertechnik GmBH, Heidelberg, Germany).

3.8. Western Blot Analysis

Whole protein extracts were isolated using Trizol reagent according to the instructions of the manufacturer. Protein concentration was determined by DU 530 UV spectrophotometer (Beckman, Fullerton State, CA). Equal amounts of protein from different treatments were boiled in 2×loading buffer (100 mM Tris, pH6.8, 4% SDS, 20% glycerol, 10% beta-mercaptoetthanol, 0.2% bromophenol blue), fractionated by electrophoresis in 12% SDS-polyacrylamide gels under reducing conditions, and transferred to nitrocellulose membranes. After blocking in 3% BSA solution, the membrane was sequentially incubated with primary antibody (0.2 μg/ml) and AP-conjugated secondary antibody. Color development was performed using NBT/BCIP. Actin content was also determined as loading control.

3.9. Semi-quantitative RT-PCR

Total cellular RNA was isolated using Trizol reagent. Genomic DNA contamination was avoided by RNase-free DNase I treatment. Reverse transcription was carried out with Superscript II reverse transcriptase from 1 μg of total RNA. Specific primers used for amplification of MMP-2 and GAPDH cDNAs were designed by the Primer Premier version 5.0 Program (PREMIER Biosoft International, Palo Alto, CA). Primer sequences (in the 5'-3' direction) were as follows: ATT TGG CGG ACT GTG ACG (forward) and GCT TCA GGT AAT AGG CAC (reverse) for MMP-2, with an expected amplified length of 442 bp; AGC CAC ATC GCT CAG ACA C (forward) and TGG ACT CCA CGA CGT ACT C (reverse) for GAPDH, with an expected amplified length of 315 bp. Semi-quantitative RT-PCR was performed as previously described (20, 21) with some modifications. Briefly, 1.5 μl of the resultant cDNAs were amplified by PCR in a 25 μl reaction mixture containing 0.2 mM each of dNTPs, 1.5 mM magnesium chloride, 10 pmol of sense and antisense primers, and 1 U Taq DNA polymerase. To compare intensities of RT-PCR products in a semi-quantitative way,

we determined the exponential phase of amplification by performing (i) 25-30-35-40 cycles for neo (ii) 25-30-35-40 cycles for pLXSN Seq/PCR (iii) 25-30-32-35-40 cycles for MMP-2, and (iv) 15-20-23-25-30 cycles for GAPDH. Final amplification cycles in the linear curve were 35 for neo, 35 for pLXSN Seq/PCR, 35 for MMP-2, and 23 for GAPDH. As a negative control, each sample was run through PCR in the absence of cDNA. PCR products were visualized on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide (EB) by UV transillumination. Semiquantitative analysis was performed using a computerized densitometer to obtain MMP-2/GAPDH ratios.

3.10. Zymography

Gelatinase activity was analyzed by zymography as previously described (3) with some modifications. In brief, equal amounts of supernatants were incubated with 4×loading buffer (0.25 M Tris, pH 6.8, 40% glycerol, 8% SDS, 0.4% bromophenol blue) at 37°C for 30 min before being electrophorized in 10% SDS-polyacrylamide gels impregnated with 1 mg/ml gelatin. After electrophoresis, gels were washed 2×30 min at room temperature in Triton X-100 buffer (2.5% Triton X-100, 50 mM Tris, pH7.5), incubated for 20 h at 37°C in Ca^{2+} substrate buffer (50 mM Tris, 0.2 M NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% Triton, pH 7.5), stained for 60 min with 0.2% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, and destained with 10% acetic acid. The molecular weights of the gelatinases were determined by comparison with protein standards.

3.11. Statistical Analysis

Signal intensities were determined by MetaView image analyzing system version 4.50 (Universal Imaging Corp., Downington, PA). Values are expressed as means ± SEM of three experiments. Statistical analysis were carried out by one-way ANOVA, using the SPSS 10.0 software program (SPSS Inc., Chicago, IL), and differences were considered to be significant at $P<0.05$.

4. RESULTS

4.1. Generation of Stable Retroviral Packaging Cell Clones

The PT67 packaging cells were transfected with retroviral vectors pLXSN, pLXSN-*Smad4*-C-Flag, and pLXSN-N-Flag-*Smad2*. PT67 clones were isolated by selection in active G418. Individual stably transfected clones were expanded and rapidly screened by semi-quantitative RT-PCR with neo and pLXSN PCR/Seq primers as described in *Materials and Methods*. Detection of retroviral neo genes produced by screened packaging cell clones pLXSN-PT67, Smad4-PT67, and Smad2-PT67 are shown in Figure 1A. Further identification of these clones with pLXSN PCR/Seq primers (Figure 1B) showed that PCR products from Smad4-PT67 and Smad2-PT67 contained expected full-length *Smad4* and *Smad2* genes, respectively, while PCR product from pLXSN-PT67 only showed an expected band of 139 bp with no gene insert.

Vector function was then assessed by detecting production of Flag and expression of Smad4/Smad2 proteins. Obvious Flag signals were detected in Smad4-PT67 and Smad2-PT67 cell clones, as shown both by immunofluorescence (Figure 1C) and Western blot (Figure 1D). The expression level of Smad4 by Smad4-PT67 cells was significantly higher than that by un-transfected PT67 cells and pLXSN-PT67 cells ($P<0.05$, Figure 1E). The expression level of Smad2 by Smad2-PT67 cells was also significantly higher compared to that in un-transfected PT67 cells and pLXSN-PT67 cells ($P<0.05$, Figure 1E).

4.2. Transduction of HTR-8/SVneo cells

HTR-8/SVneo cells were infected with the retroviral supernatants from the PT67 packaging cell clones, and named Smad4-HTR, Smad2-HTR, and pLXSN-HTR. Control experiment with supernatant from un-transfected PT67 cells (named con-HTR) was also included. To confirm the success of transduction, Smad4 and Smad2 protein contents were detected by Western blot, using anti-Flag and anti-Smad antibodies. Retroviral-mediated gene transfer was successful, as indicated by the expression of Flag tag in Smad4-HTR and Smad2-HTR (Figure 2A). Furthermore, compared with un-transduced HTR-8/SVneo cells, con-HTR, and pLXSN-HTR, the expression level of Smad4 (or Smad2) by Smad4-HTR (or Smad2-HTR) was significantly higher ($P<0.05$, Figure 2B).

4.3. TGF-beta1 Up-regulates MMP-2 mRNA Abundance and Secretion

We first detected abundant mRNA expression of TGF-beta receptors types I and II in HTR-8/SVneo cells (data not shown) using the primers reported previously (22). To confirm whether exogenous TGF-beta1 can activate the Smad signaling pathway in HTR-8/SVneo cells, we assessed the changes in phosphorylated and total Smad2/3 contents at various incubation duration after TGF-beta1 challenge. Continuous increase in the content of phosphorylated Smad2/3 (corrected for total Smad2/3 level) was observed when cells were exposed to TGF-beta1 ($P<0.05$ versus basal at 30, 45, 60, 90, 120, and 180 min), with a maximal increase detectable at 90 min (Figure 3).

As shown in Figure 4, TGF-beta1 significantly up-regulated MMP-2 mRNA abundance ($P<0.05$, Figure 4A, left two lanes) and secretion ($P<0.05$, Figure 4B, left two lanes) in HTR-8/SVneo cells. Pro-MMP-9 (92 kDa) and pro-MMP-2 (72 kDa) were detected in the serum-free culture medium of HTR-8/SVneo, as shown both by our previous unpublished data and report by Qiu *et al*. (23). Treatment with TGF-beta1 had no significant effect on the secretion of pro-MMP-9 (data not shown).

4.4. Smad4 but Not Smad2 is Involved in the Up-regulation of MMP-2 by TGF-beta1

A possible involvement of Smad4 or Smad2 in the up-regulation of MMP-2 by TGF-beta1 was further investigated. Treatment with PT67 conditioned medium (con-0 or con-T) and empty retrovirus (p-0 or p-T) did not result in any significant effect on MMP-2 mRNA abundance and secretion compared with corresponding controls (0 or T) (Figure 4A and B). MMP-2 mRNA abundance and secretion were significantly higher ($P<0.05$) in Smad4-HTR cells (S4-0) than in untreated HTR-

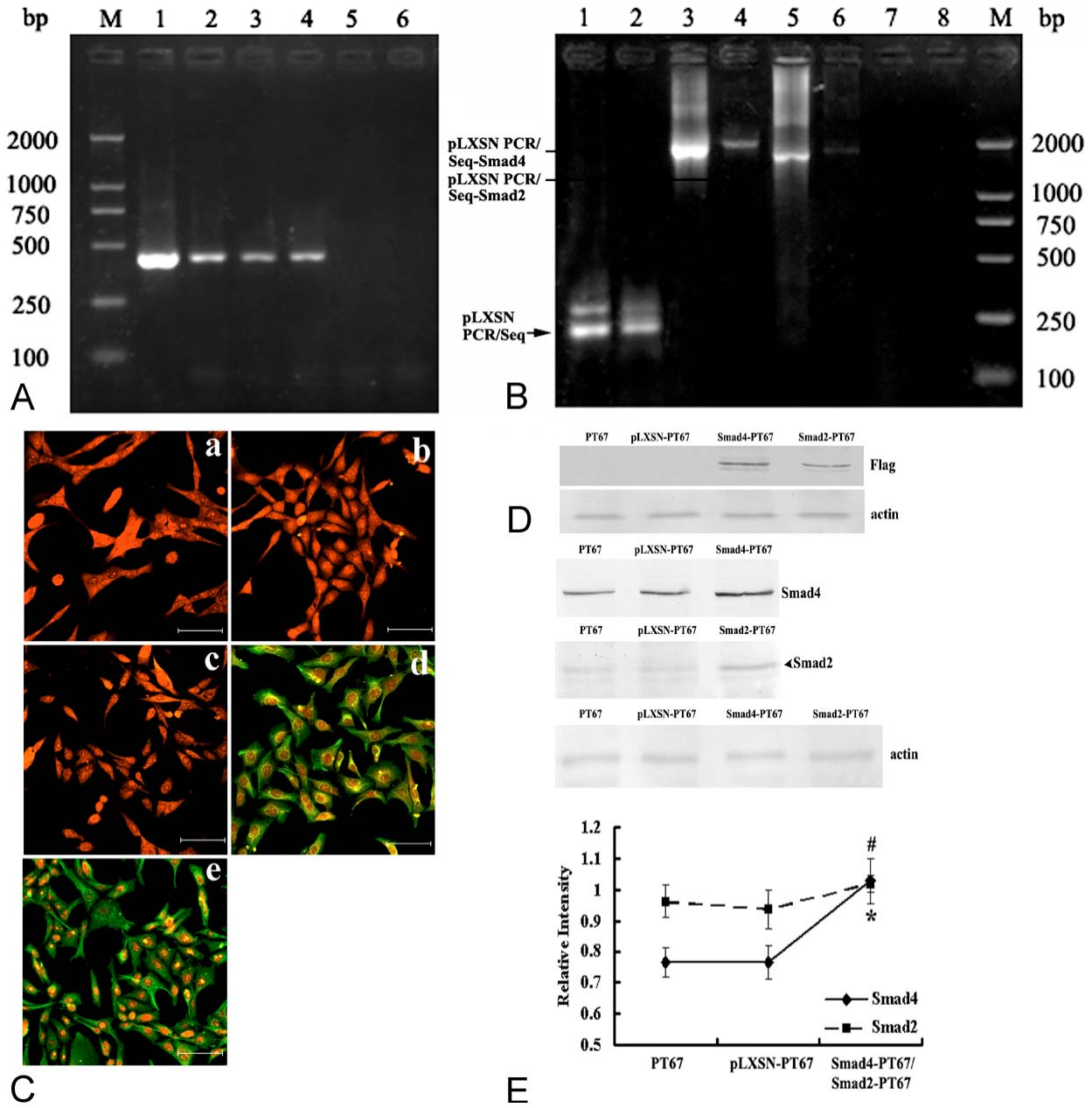

Figure 1. Identification of retrovirus production by stable packaging PT67 cell clones. (A) Neomycin detection by RT-PCR in the medium of screened PT67 packaging cells. M, DL2000 marker. 1, positive control of PCR amplification using pLXSN vector as the template. 2, Smad4-PT67 packaging cell clone. 3, Smad2-PT67 packaging cell clone. 4, pLXSN-PT67 packaging cell clone. 5, negative control of PCR amplification using RT product from the medium of un-transfected PT67 cells. 6, negative control of PCR amplification using RT product without reverse transcriptase. (B) Detection of insert mRNAs expression by RT-PCR using pLXSN PCR/Seq primers. M, DL2000 marker. 1, positive control of PCR amplification using pLXSN vector as the template. 2, pLXSN-PT67. 3, positive control, using pLXSN-*Smad4*-C-Flag vector as the template. 4, Smad4-PT67. 5, positive control, using pLXSN-N-Flag-*Smad2* vector as the template. 6, Smad2-PT67. 7, negative control of PCR amplification using RT product from the medium of un-transfected PT67 cells. 8. negative control of PCR amplification using RT product without reverse transcriptase. (C) Detection of Flag expression of Smad4-PT67 and Smad2-PT67 cells by immunofluorescence. a, negative control with PBS replacing anti-Flag antibody. b, un-transfected PT67 cell. c, pLXSN-PT67. d, Smad4-PT67. e, Smad2-PT67. Bar=100 μm. (D) Western blot detection of Smad4 and Smad2 expression in Smad4-PT67 and Smad2-PT67 cells, respectively, using anti-Flag antibody. (E) Western blot analysis of Smad4 and Smad2 contents, using anti-Smad4 and -Smad2 antibody, respectively. Actin was used as a loading control. The results are representative of three independent experiments. Values were presented as ratio of densitometric readings for target protein to corresponding actin. Each value represents mean ± SEM of three independent experiments. Points with different letters are significantly different ($P<0.05$), with A and B representing significant differences between groups for Smad4, and a and b representing significant differences between groups for Smad2.

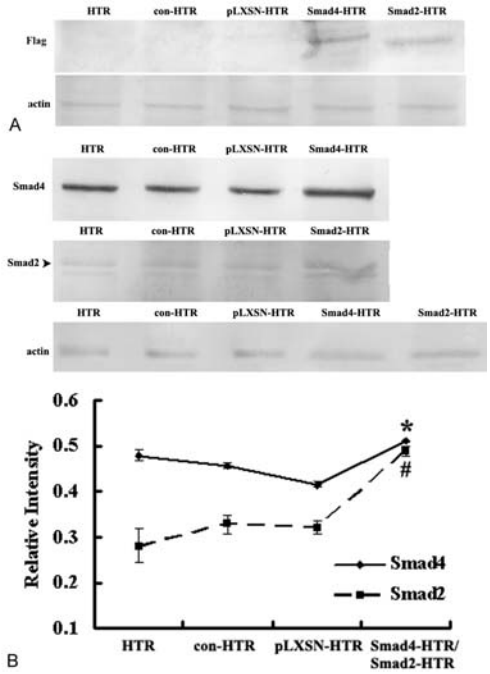

Figure 2. Transduction of HTR-8/SVneo cells. (A) Western blot showing Smad4 and Smad2 expression in sense *Smad4* and *Smad2* recombinant retroviruses transduced HTR-8/SVneo cells, Smad4-HTR and Smad2-HTR, as determined by anti-Flag antibody. (B) Western blot analysis of Smad4 and Smad2 expression in Smad4-HTR and Smad2-HTR, using anti-Smad4 and -Smad2 antibody, respectively. Actin was used as a loading control. The results are representative of three independent experiments. Values were presented as ratio of densitometric readings for target protein to corresponding actin. Each value represents mean ± SEM of three independent experiments. Values with different letters are significantly different (P<0.05), with A and B representing significant differences between groups for Smad4, and a and b representing significant differences between groups for Smad2.

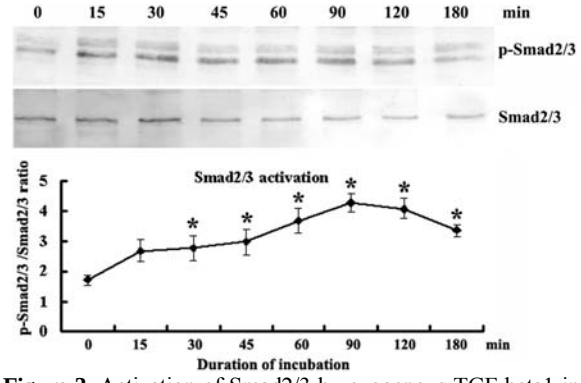

Figure 3. Activation of Smad2/3 by exogenous TGF-beta1 in HTR-8/SVneo cells. Cells were treated with 10 ng/ml TGF-beta1 for 0, 15, 30, 45, 60, 90, 120, or 180 min. The levels of phosphorylated Smad2/3 were quantitated by densitometric scanning and corrected for the levels of Smad2/3 in the same samples. Each value represents mean ± SEM of three independent experiments. Difference is considered to be significant at P<0.05 (*) versus basal (0 min).

/SVneo cells (0), con-HTR (con-0), or pLXSN-HTR cells (p-0), as shown by RT-PCR (Figure 4A) and zymography (Figure 4B). These effects were further enhanced significantly upon the stimulation with TGF-beta1 (P<0.05 versus S4-0, Figure 4A and B). Treatment with TGF-beta1 elicited no significant difference between sense *Smad4* (S4-T), HTR-8/SVneo (T), con-HTR (con-T), and pLXSN-HTR cells (p-T) (Figure 4A and B).

Smad2 gene transfer failed to show any regulatory effect on MMP-2 mRNA abundance or the secretion of its proenzyme (compared with parental HTR-8/SVneo cells, con-HTR, or pLXSN-HTR cells; Figure 4A and B).

5. DISCUSSION

MMP-2 is an essential mediator in trophoblast invasion (9, 10, 24, 25). In the present study, we showed that TGF-beta1 up-regulated MMP-2 content, an observation in line with reports on first-trimester trophoblasts by other laboratories (6, 13). These data support the concept that together with other cytokines and growth factors (8, 25-28), TGF-beta can regulate MMP-2 expression and potentially function in the invasive process of trophoblast cells.

Although regulatory effects of TGF-beta on the expression of MMP-2 in invasive trophoblasts have been documented, the mechanism(s) by which the growth factor exerts its action, especially the possible involvement of the Smad signaling pathway, are not well understood. However, the roles of the Smad signaling in the regulation of MMPs expression in other cell types have been evaluated. For instance, cultured rat mesangial cells (Msc) transfected with *Smad2* show increased MMP-2 and TIMP-2 expression and enzymatic activity, while over-expression of *Smad3* gene resulted in slight increase in TIMP-2 expression and enzymatic activity. These effects are enhanced upon TGF-beta1 stimulation (29). In dermal fibroblasts, exogenous Smad3 and Smad4 mimic the inhibitory effect of TGF-beta on MMP-1 gene expression, while dominant negative mutants of *Smad3* or *Smad4* abrogate negative regulation of MMP-1 transcription by TGF-beta (30). Smad3 mediates TGF-beta-induced MMP-13 expression in human gingival fibroblasts (31). Smad7 can abrogate the regulatory activity of TGF-beta on MMP-1 (32), MMP-2 (33), MMP-9 (34), and TIMP-2 (29) in various cell types.

Our results showed for the first time that like TGF-beta1, retroviral expression of sense *Smad4* increased MMP-2 mRNA abundance and the secretion of pro-MMP-2 in HTR-8/SVneo cells. These responses were further enhanced by the presence of the growth factor. These data suggest that Smad4, as the common-partner Smad, is essential for the induction of MMP-2 expression by TGF-beta1 in invasive trophoblast cells. Our results also show that upon treatment with TGF-beta1, sense *Smad4* had no effect on MMP-2 expression compared with parental, PT67-medium-treated, or empty-vector-treated HTR-8/SVneo cells. While the reason(s) for this lack of response

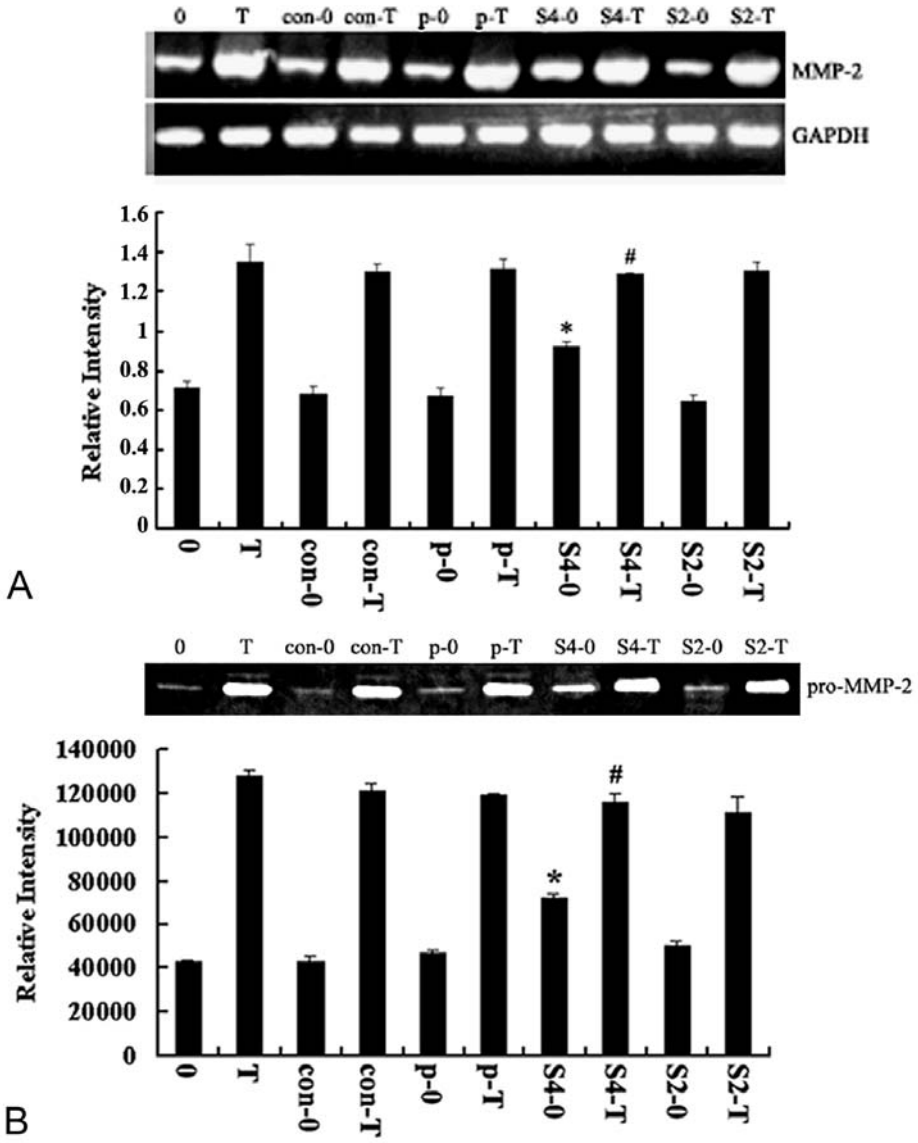

Figure 4. Semi-quantitative RT-PCR (A) and zymographic analysis (B) of the effects of transduction on TGF-beta1-regulated MMP-2 expression in HTR-8/SVneo cells. 0, HTR-8/SVneo cells treated with vehicle for 24 h. T, HTR-8/SVneo cells treated with 10 ng/ml TGF-beta1 for 24 h. con-0, HTR-8/SVneo cells treated with PT67 conditioned medium for 24 h. con-T, HTR-8/SVneo cells treated with 10 ng/ml TGF-beta1 in PT67 conditioned medium for 24 h. p-0, pLXSN-HTR cells treated with vehicle for 24 h. p-T, pLXSN-HTR cells treated with 10 ng/ml TGF-beta1 for 24 h. S4-0, Smad4-HTR cells treated with vehicle for 24 h. S4-T, Smad4-HTR cells treated with 10 ng/ml TGF-beta1 for 24 h. S2-0, Smad2-HTR cells treated with vehicle for 24 h. S2-T, Smad2-HTR cells treated with 10 ng/ml TGF-beta1 for 24 h. The results are representative of three independent experiments. Each bar graph represents mean ± SEM of three independent experiments. Difference is considered to be significant at $P<0.05$ (*) versus basal (0) and $P<0.05$ (#) versus sense *Smad4* transduction (S4-0). The significant differences ($P<0.05$) between groups treated with vehicle or TGF-beta1 are not shown in the graphs. In RT-PCR experiments, GAPDH was used as the internal control and values were presented as ratio of densitometric readings for target gene to corresponding GAPDH.

is not immediately apparent, the possibility that this could be a consequence of a maximal stimulation of the MMP-2 system by high concentration of TGF-beta1, cannot be excluded.

The Smad pathway is of prime importance to the TGF-beta family signaling, and the direct binding of Smad complex to Smad binding element (SBE) results in gene transcriptional responses. More commonly, however, the interaction of Smads with transcriptional co-activators, such as forkhead activin signal transducer (FAST) (35), p300 (36), and activator protein-1 (AP-1) (37), is also required for the gene activation. For many TGF-beta-regulated genes, SBEs are located adjacent to AP-1 sites

(38). Smads can bind directly to Jun family members (39). This complex, consisting of c-Jun, c-Fos, Smad3, and Smad4, binds to the AP-1 site or SBE in target gene promoter and activates gene transcription (37, 40). We found that in HTR-8/SVneo cells, sense *Smad2* had no effect on MMP-2 mRNA abundance and the secretion of pro-MMP-2, indicating that Smad2 is not involved in the induction of the responses by TGF-beta1. It has been demonstrated that the AP-1 site mediates MMP-2 transcription through the binding of Fra1-JunB and FosB-JunB heterodimers, members of the Fos and Jun families (41), and over-expression of c-Jun directly increases MMP-2 expression in breast cancer cell lines (42). Therefore, the possible involvement of other transcriptional co-activators (e.g. AP-1) together with Smad2 in TGF-beta1-induced MMP-2 expression deserves to be further elucidated.

TGF-beta1 signals are mediated through cytoplasmic Smad2 or Smad3, followed by formation of heteromeric complex with Smad4 and translocation of the complex into the nucleus (11). Although Smad2 and Smad3 are structurally similar, they have distinct roles in TGF-beta signaling (43). The functional difference between Smad2 and Smad3 can be attributed to the loop 2 in the amino-terminal of Smad2 MH1 domain, which interferes with Smad2-DNA binding (44, 45). However, Smad3 can directly bind to DNA through the beta-hairpin loop in its MH1 domain (46). In addition, most amino-terminal region of the MH1 domain also contributes to the functional difference between Smad2 and Smad3 (47). It has been reported that adenoviral expression of Smad3, but not Smad2, augments TGF-beta-induced MMP-13 expression in human gingival fibroblasts (31). Therefore, the possible involvement of Smad3 in the induction of MMP-2 by TGF-beta1 in HTR-8/SVneo cells will be a project of further studies.

In conclusion, we have demonstrated that retroviral expression of Smad4, but not of Smad2, mediates TGF-beta1-induced MMP-2 mRNA expression and secretion in the invasive EVT cell line HTR-8/SVneo.

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7. REFERENCES

1. Shimonovitz S, A. Hurwitz, V. Barak, M. Dushnik, E. Y. Adashi, E. Anteby & S. Yagel: Cytokine-mediated regulation of type IV collagenase expression and production in human trophoblast cells. *J Clin Endocrinol Metab* 81, 3091-3096 (1996)

2. Kahari V. M & U. Saarialho-Kere: Matrix metalloproteinases and their inhibitors in tumour growth and invasion. *Ann Med* 31, 34-45 (1999)

3. Wang H, Q. Li, L. Shao & C. Zhu: Expression of Matrix Metalloproteinase (MMP) -2, -9, -14 and Tissue Inhibitor of Metalloproteinases (TIMP)-1, -2, -3 in the Endometrium and Placenta of Rhesus Monkey (Macaca mulatta) During Early Pregnancy. *Biol Reprod* 65, 31-40 (2001)

4. Aplin J. D: Implantation, trophoblast differentiation and hemochorial placentation: mechanistic evidence in vim and in vitro. *J Cell Sci* 99, 681-692 (1991)

5. Strickland S & W. G. Richards: Invasion of the trophoblasts. *Cell* 71, 355-357 (1992)

6. Yudate T, K. Isaka, Y. Kosugi, M. Koshiishi, K. Shiraishi, M. Hosaka & M. Takayama: Analysis of the mechanism of trophoblast infiltration. *Nippon Sanka Fujinka Gakkai Zasshi* 48, 191-198 (1996)

7. Lala P. K & G. S. Hamilton: Growth factors, proteases and protease inhibitors in the maternal-fetal dialogue. *Placenta* 17, 545-555 (1996)

8. Meisser A, D. Chardonnens, A. Campana & P. Bischof: Effects of tumour necrosis factor-alpha, interleukin-1alpha, macrophage colony stimulating factor and transforming growth factor beta on trophoblastic matrix metalloproteinases. *Mol Hum Reprod* 5, 252-260 (1999)

9. Niu R, T. Okamoto, K. Iwase, S. Nomura & S. Mizutani: Quantitative analysis of matrix metalloproteinases-2 and -9, and their tissue inhibitors-1 and -2 in human placenta throughout gestation. *Life Sci* 66, 1127-1137 (2000)

10. Seval Y, G. Akkoyunlu, R. Demir & M. Asar: Distribution patterns of matrix metalloproteinase (MMP)-2 and -9 and their inhibitors (TIMP-1 and TIMP-2) in the human decidua during early pregnancy. *Acta Histochem* 106, 353-362 (2004)

11. Massague J: TGFbeta signal transduction. *Annu Rev Biochem* 67, 753-791 (1998)

12. Miyazono K, P. ten Dijke & C. H. Heldin: TGFbeta signaling by Smad proteins. *Adv Immunol* 75, 115-157 (2000)

13. Graham C. H, I. Connelly, J. R. MacDougall, R. S. Kerbel, W. G. Stetler-Stevenson & P. K. Lala: Resistance of malignant trophoblast cells to both the anti-proliferative and antiinvasive effects of transforming growth factor-beta. *Exp Cell Res* 214, 93-99 (1994)

14. Graham C. H & P. K. Lala: Mechanism of control of trophoblast invasion in situ. *J Cell Physiol* 148, 228-234 (1991)

15. Graham C. H, T. S. Hawley, R. G. Hawley, J. R. MacDougall, R. S. Kerbel, N. Khoo & P. K. Lala: Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp Cell Res* 206, 204-211 (1993)

16. Graham C. H: Effect of transforming growth factor beta in the plasminogen activator system in cultures first trimester human cytotrophoblast. *Placenta* 18, 137-143 (1997)

17. Zhang Y, X. Feng, R. We & R. Derynck: Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature* 383, 168-172 (1996)

18. Quinn T. P & K. T. Trevor: Rapid quantitation of recombinant retrovirus produced by packaging cell clones. *Biotechniques* 23, 1038-1044 (1997)
19. Morgan R. A, K. Cornetta & W. F. Anderson: Applications of the polymerase chain reaction in retroviral-mediated gene tranfer and the analysis of gene-marked human TIL cells. *Hum Gene Ther* 1, 135-149 (1990)
20. Gros J, C. C. Gerhardt & A. D. Strosberg: Expression of human beta3-adrenergic receptor induces adipocyte-like features in CHO/K1 fibroblasts. *J Cell Sci* 112, 3791-3797 (1999)
21. Qian D, H. Y. Lin, H. M. Wang, X. Zhang, D. L. Liu, Q. L. Li & C. Zhu: Normoxic induction of the hypoxic-inducible factor-1alpha by interleukin-1beta involves the extracellular signal-regulated kinase 1/2 pathway in normal human cytotrophoblast cells. *Biol Reprod* 70, 1822-1827 (2004)
22. Xu G, C. Chakraborty & P. K. Lala: Expression of TGF-beta signaling genes in the normal, premalignant and malignant human trophoblast: loss of Smad 3 in choriocarcinoma cells. *Biochem Biophys Res Commun* 287, 47-55 (2001)
23. Qiu Q, M. Yang, B. K. Tsang & A. Gruslin: EGF-induced trophoblast secretion of MMP-9 and TIMP-1 involves activation of both PI3K and MAPK signalling pathways. *Reproduction* 128, 355-363 (2004)
24. Isaka K, S. Usuda, H. Ito, Y. Sagawa, H. Nakamura, H. Nishi, Y. Suzuki, Y. F. Li & M. Takayama: Expression and activity of matrix metalloproteinase 2 and 9 in human trophoblasts. *Placenta* 24, 53-64 (2003)
25. Staun-Ram E, S. Goldman, D. Gabarin & E. Shalev: Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. *Reprod Biol Endocrinol* 2, 59 (2004)
26. Bischof P: Endocrine, paracrine and autocrine regulation of trophoblastic metalloproteinases. *Early Pregnancy* 5, 30-31 (2001)
27. Karmakar S & C. Das: Regulation of trophoblast invasion by IL-1beta and TGF-beta1. *Am J Reprod Immunol* 48, 210-219 (2002)
28. Hills F. A, M. G. Elder, T. Chard & M. H. Sullivan: Regulation of human villous trophoblast by insulin-like growth factors and insulin-like growth factor-binding protein-1. *J Endocrinol* 183, 487-496 (2004)
29. Yang C, L. Dai, X. G. Liu, Q. Chen, X. R. Zhang & M. Y. Guo: Effect of transforming growth factor beta1/Smad signaling pathway on the expression and enzymatic activity of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 in cultured rat mesangial cells. *Zhonghua Bing Li Xue Za Zhi* 32, 553-557 (2003)
30. Yuan W & J. Varga: Transforming growth factor-beta repression of matrix metalloproteinase-1 in dermal fibroblasts involves Smad3. *J Biol Chem* 276, 38502-38510 (2001)
31. Leivonen S. K, A. Chantry, L. Hakkinen, J. Han & V. M. Kahari: Smad3 mediates transforming growth factor-beta-induced collagenase-3 (matrix metalloproteinase-13) expression in human gingival fibroblasts. Evidence for cross-talk between Smad3 and p38 signaling pathways. *J Biol Chem* 277, 46338-46346 (2002)
32. Mix K. S, C. I. Coon, E. D. Rosen, N. Suh, M. B. Sporn & C. E. Brinckerhoff: Peroxisome proliferator-activated receptor-γ-independent repression of collagenase gene expression by 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid and prostaglandin 15-deoxy-delta(12,14) J2: a role for Smad signaling. *Mol Pharmacol* 65, 309-318 (2004)
33. Lee E. O, J. L. Kang & Y. H. Chong: The amyloid-beta peptide suppresses transforming growth factor-beta1-induced matrix metalloproteinase-2 production via Smad7 expression in human monocytic THP-1 cells. *J Biol Chem* 280, 7845-7853 (2005)
34. Ogawa K, F. Chen, C. Kuang & Y. Chen: Suppression of matrix metalloproteinase-9 transcription by transforming growth factor-beta is mediated by a nuclear factor-kappaB site. *Biochem J* 381, 413-422 (2004)
35. Chen X, E. Weisberg, V. Fridmacher, M. Watanabe, G. Naco & M. Whitman: Smad4 and FAST-1 in the assembly of activin-responsive factor. *Nature* 389, 85-89 (1997)
36. de Caestecker M. P, T. Yahata, D. Wang, W. T. Parks, S. Huang, C. S. Hill, T. Shioda, A. B. Roberts & R. J. Lechleider: The Smad4 activation domain (SAD) is a proline-rich, p300-dependent transcriptional activation domain. *J Biol Chem* 275, 2115-2122 (2000)
37. Wong C, E. M. Rougier-Chapman, J. P. Frederick, M. B. Datto, N. T. Liberati, J. M. Li & X. F. Wang: Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor beta. *Mol Cell Biol* 19, 1821-1830 (1999)
38. Yingling J. M, M. B. Datto, C. Wong, J. P. Frederick, N. T. Liberati & X. F. Wang: Tumor suppressor Smad4 is a transforming growth factor beta-inducible DNA binding protein. *Mol Cell Biol* 17, 7019-7028 (1997)
39. Liberati N. T, M. B. Datto, J. P. Frederick, X. Shen, C. Wong, E. M. Rougier-Chapman & X. F. Wang: Smads bind directly to the Jun family of AP-1 transcription factors. *Proc Natl Acad Sci U S A* 96, 4844-4849 (1999)
40. Verrecchia F, L. Vindevoghel, R. J. Lechleider, J. Uitto, A. B. Roberts & A. Mauviel: Smad3/AP-1 interactions control transcriptional responses to TGF-beta in a promoter-specific manner. *Oncogene* 20, 3332-3340 (2001)
41. Bergman M. R, S. Cheng, N. Honbo, L. Piacentini, J. S. Karliner & D. H. Lovett: A functional activating protein 1 (AP-1) site regulates matrix metalloproteinase 2 (MMP-2) transcription by cardiac cells through interactions with JunB-Fra1 and JunB-FosB heterodimers. *Biochem J* 369, 485-496 (2003)
42. Bachmeier B. E, A. Albini, R. Vene, R. Benelli, D. Noonan, C. Weigert, C. Weiler, R. Lichtinghagen, M. Jochum & A. G. Nerlich: Cell density-dependent regulation of matrix metalloproteinase and TIMP expression in differently tumorigenic breast cancer cell lines. *Exp Cell Res* 305, 83-98 (2005)
43. Piek E, W. J. Ju, J. Heyer, D. Escalante-Alcalde, C. L. Stewart, M. Weinstein, C. Deng, R. Kucherlapati, E. P. Bottinger & A. B. Roberts: Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. *J Biol Chem* 276, 19945-199953 (2001)
44. Yagi K, D. Goto, T. Hamamoto, S. Takenoshita, M. Kato & K. Miyazono: Alternatively spliced variant of Smad2 lacking exon 3. Comparison with wild-type Smad2 and Smad3. *J Biol Chem* 274, 703-709 (1999)
45. Dennler S, S. Huet & J. M. Gauthier: A short amino-acid sequence in MH1 domain is responsible for functional

differences between Smad2 and Smad3. *Oncogene* 18, 1643-1648 (1999)
46. Shi Y, Y. F. Wang, L. Jayaraman, H. Yang, J. Massague & N. P. Pavletich: Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. *Cell* 94, 585-594 (1998)
47. Nagarajan R. P & Y. Chen: Structural basis for the functional difference between Smad2 and Smad3 in FAST-2 (forkhead activin signal transducer-2)-mediated transcription. *Biochem J* 350, 253-259 (2000)

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