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
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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 morphogenetic proteins (BMPs) whereas Smad2 and Smad3 mediate TGF-beta/activins signals. R-Smads then form heteromeric complexes with Co-Smads (common-partner Smads) (Smad4 and Smad6) 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 decidua-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 decidua-derived TGF-beta. This anti-invasive effect of TGF-beta is mediated by a down-regulation of uPA and an up-regulation of TIMP-1 (14, 15) and PAI-1 (16). 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™ 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 EcoRI-HindIII 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 Clal-Xbal fragment of pRK5F-N-Flag-Smad2 into the blunt-ended Xhol-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™ 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.
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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 transfecteds 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-quantitatively 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 ACG 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 PT67 cell clones with highest titers were selected for 2 to 3 weeks, and the resultant cell populations were transferred to 24-well plates and were maintained in G418-containing medium after growing to a large scale.

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 transduced 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-mercaptoethanol, 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 GTG 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,
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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. Semi-quantitative 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, 50 mM Tris, pH7.5), 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 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.
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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).

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 its proenzyme (compared with parental HTR-8/SVneo cells, con-HTR, or pLXSN-HTR cells; Figure 4A and B).
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.
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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 promoters 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-beta1 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 retroviral expression of Smad3, but not Smad2, augments TGF-beta1-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.

6. ACKNOWLEDGMENTS

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7. REFERENCES

differences between Smad2 and Smad3. *Oncogene* 18, 1643-1648 (1999)


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