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Expression of TGF-β Signaling Genes in the Normal, Premalignant, and Malignant Human Trophoblast: Loss of Smad3 in Choriocarcinoma Cells

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We had earlier shown that TGF-β controls proliferation, migration, and invasiveness of normal human trophoblast cells, whereas premalignant and malignant trophoblast cells are resistant to TGF-β. To identify signaling defects responsible for TGF-β resistance in premalignant and malignant trophoblasts, we have compared the expression of TGF-β signaling molecules in a normal trophoblast cell line (HTR-8), its premalignant derivative (RSVT2/C), and two choriocarcinoma cell lines (JAR and JEG-3). RT-PCR analysis revealed that all these cell lines expressed the mRNA of TGF-β1, -β2, and -β3, TGF-β receptors type I, II, and III, and post-receptor signaling genes smad2, smad3, smad4, smad6, and smad7 with the exception that TGF-β2 and smad3 were undetectable in JAR and JEG-3 cells. Immunoblot analysis confirmed the absence of smad3 protein in choriocarcinoma cells. Treatment with TGF-β1 induced smad3 phosphorylation and smad3 translocation to the nucleus in the normal and premalignant trophoblast cells. These results suggest that loss of smad3 may account for a functional disruption in the TGF-β signaling pathway in choriocarcinomas, but not in the premalignant trophoblast.

Key Words: **trophoblast; choriocarcinoma; tumor progression; TGF-β receptors; smad proteins.**

Transforming growth factor-β (TGF-β), existing as three isoforms (TGF-β1, -β2, and -β3) in the human, is a multifunctional polypeptide which regulates a variety of cellular functions including cell proliferation, differentiation, migration and apoptosis under physiological and pathological conditions. It is the most potent autocrine negative regulator of proliferation in most epithelial tissues (1). Ligand-induced signaling is mediated by two serine/threonine kinase receptors on the cell surface: TGF-β receptor type I (TβR-I) and type II (TβR-II). TGF-β receptor type III (TβR-III), which has no signaling capacity, localizes TGF-β on the cell surface, thereby facilitating TGF-β binding to the signaling receptors. Binding of TGF-β to TβR-II causes phosphorylation of this receptor which then heterotetramerizes with and phosphorylates the TβR-I, thereby activating downstream signaling events including the participation of the cytoplasmic smad proteins (2). To date, 10 different smads (smad1–smad10) have been identified in vertebrates (3, 4) and can be divided into three general groups: the receptor-activated smads including smad2 and smad3, the common-mediator smads including smad4 and the inhibitory smads including smad6 and smad7 in the human. Following receptor-induced activation, phosphorylated smad2 and smad3 (at their conserved SSXS motif contained at the C-terminus) oligomerize with smad4 to form the smad complex and then translocate to the nucleus where this complex binds to the nuclear DNA, leading to transcriptional activation of the target gene. These nuclear events are regulated by certain DNA-binding cofactors and corepressors (5, 6).

Loss of TGF-β responsiveness has been identified as a crucial event in the development and progression of epithelial malignancies (7, 8). This may result from deletion or mutation of TβR-I (9), TβR-II (10), smad2 and smad4 (11–13). However, smad3 has so far not been reported to be mutated or deleted in any human cancer.

The human placenta is a highly invasive "tumor-like" structure which invades the uterine endometrium and its vasculature during normal pregnancy in order to establish an exchange of nutrients and other molecules between the fetal and the maternal blood. However, placental invasion of the uterus is exquisitely regulated *in situ* (14). The invasive function is mediated by a subpopulation of the placental trophoblast known as the "extravillous trophoblast" (EVT) which

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proliferate, migrate out of chorionic villi and invade the uterine decidua (15). Our laboratory has succeeded in propagating EVT cells from first trimester human chorionic villus explant cultures (16) which simulate EVT cells *in situ* in their proliferative, migratory and invasive functions as well as the expression of EVT cell associated markers (17, 18). Utilizing these cell lines in functional assays *in vitro*, it was established that molecular mechanisms responsible for normal EVT cell invasiveness are identical to those of cancer cells (19, 20). However, unlike cancer cells, normal EVT cell lines are short-lived (living for 12–15 passages), and nontumorigenic in nude mice (21). We have shown that normal EVT cell functions such as proliferation (17), migration (18) and invasiveness (22) are controlled by decidua-derived TGF-β, whereas choriocarcinoma (malignant EVT) cell lines are resistant to anti-proliferative as well as anti-invasive effect of TGF-β (23).

By stable transfection with pRSV-T (SV40 Tag plasmid) into a normal short-lived EVT cell line HTR-8, we selected a long-lived cell line RSVT-2 which was further subjected to a forced crisis regimen in culture to generate an immortal cell line RSVT2/C (24). The latter cell line exhibits a premalignant phenotype as indicated by hyperproliferative and hyperinvasive behavior, unresponsiveness to anti-proliferative and anti-invasive actions of TGF-β, deficiency in gap junctional intercellular communication (GJIC), but inability for anchorage independent growth (as agar colonies) or tumorigenicity in nude mice (24, 25). Thus, it appears that resistance to negative regulation by TGF-β is common to both premalignant and malignant trophoblast. However, it remains unknown whether the TGF-β signaling defects underlying the resistance are identical or different in the two cell types.

Disruption of TGF-β signaling appears to be an important event in epithelial tumor progression. Specific defects in the TGF-β signaling pathway have been identified in many of these cancers, including prostate (9), gastric (10), colorectal (11), lung (13), breast (26), liver (27), and pancreatic cancer (28). However, the nature of TGF-β signaling defects in the premalignant and malignant trophoblast remains unexplored. As a first step in identifying changes in the genes responsible for TGF-β resistance in the premalignant and malignant trophoblasts, we have compared the expression of mRNAs and proteins of TGF-β signaling molecules in the normal EVT cell line (HTR-8), its premalignant derivative (RSVT2/C) and two malignant EVT (choriocarcinoma) cell lines (JAR and JEG-3). We could not detect any major difference in the expression of TGF-β signaling molecules between the normal and premalignant trophoblast cells, suggesting an alternate mechanism for cellular TGF-β insensitivity. However, choriocarcinoma cells have lost the expression of smad3 and endogenous TGF-β2.

MATERIALS AND METHODS

Cell lines and cell culture. The normal EVT cell line HTR-8 was established from a first trimester human placenta by the villus explant culture method (17, 21). This cell line senesces after 12–15 passages *in vitro*. The immortal and premalignant RSVT2/C cell line was produced by a forced crisis regimen from the long-lived RSVT-2 cells derived by transfecting HTR-8 cells with pRSV-T (24). Choriocarcinoma cell lines, JAR and JEG-3 were obtained from the American Type Culture Collection (Rockville, MD) and used as representatives of malignant EVT.

All cell lines were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco) in the presence of 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified atmosphere of 5% CO_2. The passages of HTR-8 at 7–10 and of RSVT2/C at 60–66 were used for the present experiments.

TGF-β treatment. Cells were plated into 10-cm petri dishes (Falcon, Becton Dickinson) or chamber slides (Lab-Tek II, Nalge Nunc, Naperville, IL) and cultured in the presence of 10% FBS for 2 days. Cells were washed and replaced with serum-free medium for 1 h prior to treatment with TGF-β1 (Sigma, St. Louis, MO). For immunofluorescence staining to identify the location of smad3 protein, cells were treated with 10 ng/ml TGF-β1 in serum-free medium for 15 min. For the analysis of phosphorylation of smad3 protein, cells were treated with 10 ng/ml TGF-β1 in serum-free medium for 1 h.

RNA extraction and reverse transcription-polymerase chain reaction. Total RNA was extracted from cultured cells using cell lysates in TRIZOL Reagent (Gibco) as described previously (29) and stored at −80°C until RT-PCR analyses. Two μg of total RNA was denatured at 70°C for 10 min in the presence of 100 pmol of random primers p(dN)6 (Boehringer Mannheim, Indianapolis, IN) and then reverse transcribed in a final volume of 20 μl at 42°C for 50 min in 1× reaction buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) containing 200 units of reverse transcriptase (SuperScript II, Life Technologies), 0.5 mM each of dNTP and 40 units of RNase inhibitor (RNAguard, Pharmacia, Madison, WI). The reaction was terminated by heating the mixture at 95°C for 5 min.

Amplification was carried out in a volume of 20 μl containing 1× reaction buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 25 pmol of sense and antisense primers (Table 1; synthesized locally at the UWO Oligo Factory), and 1 unit of *Taq*–DNA polymerase (PLATINUN, Life Technologies). Amplification was performed at 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, for the following cycles: 30 for TGF-β1, TGF-β2, TGF-β3, TβR-I, TβR-II, TβR-III, smad2, smad3, smad4, and smad7; 40 for smad6; 28 for β-actin. In all experiments, a possible contamination was checked by reverse transcription reactions in which reverse transcriptase was omitted and by amplifications in which water was used instead of cDNA. PCR products were analyzed on a 2.5% agarose gel containing ethidium bromide.

Immunoprecipitation of smad3 protein. At the end of the incubation period, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) containing 1 mM DTT, 1 mM $NaVO_4$, 5 mM NaF, protease inhibitor mix (Complete Mini tablet, Boehringer Mannheim, Germany). After a 30-min incubation on ice, the whole cell lysates were centrifuged at 13,000*g* for 20 min at 4°C and the supernatant was collected. The protein content was detected by using a BCA Protein Assay kit (Pierce, Rockford, IL) and subjected to immunoprecipitation or Western blot.

Equal quantities of protein (500 μg) were precleared with GammaBind G Sepharose beads (Amersham, Piscataway, NJ) at 4°C for 1 h. After centrifugation, the supernatant was collected and incubated with 2 μg rabbit anti-smad3 antibody (Zymed Laboratories Inc., South San Francisco, CA) at 4°C for 1 h, followed by

TABLE 1

Sense and Antisense PCR Primers Used in Experiments

Target mRNA	Primer sequence 5′ → 3′	Position in sequence	Product size (bp)	GenBank Accession No.
TGF-β1				
Sense	acc aac tat tgc ttc agc tc	nt 1688–1707	197	X02812
Antisense	tta tgc tgg ttg tac agg g	nt 1884–1866		
TGF-β2				
Sense	ctc gat atg gac cag ttc at	nt 546–565	a: 344	M19154
Antisense	gca ttc ttc tcc att gct ga	nt 973–954	b: 428	
TGF-β3				
Sense	cct ttc agc cca atg gag at	nt 945–964	259	X14149
Antisense	aca cag cag ttc tcc tcc aa	nt 1203–1184		
TβR-I				
Sense	tcg tct gca tct cac tca t	nt 483–501	342	NM_004612
Antisense	gat aaa tct ctg cct cac g	nt 824–806		
TβR-II				
Sense	gca cgt tca gaa gtc ggt ta	nt 1650–1669	493	D50683
Antisense	gcg gta gca gta gaa gat ga	nt 2142–2123		
TβR-III				
Sense	aat ctg ggc cat gat gca g	nt 2553–2571	286	NM_003243
Antisense	act gct gtt ttc cga ggc t	nt 2838–2820		
Smad2				
Sense	aag aag tca gct ggt ggg t	nt 161–179	246	AF027964
Antisense	gcc tgt tgt atc cca ctg a	nt 406–388		
Smad3				
Sense	cag aac gtc aac acc aag t	nt 238–256	308	NM_005902
Antisense	atg gaa tgg ctg tag tcg t	nt 545–527		
Smad4				
Sense	cca gga tca gta ggt gga at	nt 1542–1561	243	U44378
Antisense	gtc taa agg ttg tgg gtc tg	nt 1784–1765		
Smad6				
Sense	tga att ctc aga cgc cag ca	nt 1878–1897	386	AF035528
Antisense	gct cga agt cga aca cct t	nt 2263–2245		
Smad7				
Sense	gcc ctc tct gga tat ctt ct	nt 843–862	320	AF015261
Antisense	gct gca taa act cgt ggt ca	nt 1162–1143		
β-Actin				
Sense	aca atg tgg ccg agg act tt	nt 3200–3219	260	M10277
Antisense	gca cga agg ctc atc att ca	nt 3459–3440		

incubation with GammaBind G Sepharose beads overnight at 4°C. Thereafter, the beads were washed four times with the lysis buffer. The immune complexes were eluted by boiling for 3 min in a 2× sample buffer (120 mM Tris–HCl, pH 6.8, 25% glycerol, 4% SDS, 0.01% bromophenol blue) containing 200 mM DTT and subjected to SDS–PAGE. Specific bands were detected by using a rabbit anti-phosphoserine antibody (Zymed).

Western blot of total and phosphorylated smad. Cell lysates or immunoprecipitated proteins were prepared as above. Equal quantities of protein were separated by SDS–PAGE gradient gel (5–20%) and transferred to PVDF membrane (Hybond-P, Amersham). After blocking with 5% nonfat milk in 10 mM TBS-T (Tris buffered saline with 0.05% Tween 20) for 1 h, membrane was incubated with the primary antibody for 2 h at room temperature or overnight at 4°C. Primary antibodies were used as follows: rabbit anti-smad2, rabbit anti-smad3 and rabbit anti-phosphoserine antibodies were purchased from Zymed; mouse anti-smad4 antibody was purchased from Santa Cruz Biotechnology Inc. Secondary antibodies were goat anti-rabbit IgG conjugated with horseradish peroxidase (Cedarlane Laboratories Ltd., Hornby, ON) or sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham) diluted 1:10,000. Specific signals were detected using a ECL+ Plus kit (Amersham) and visualized after exposure to a HyperfilmECL film (Amersham). Signals were scanned (EPSON TWAIN Pro) and quantitated (NIH Image).

Immunofluorescence staining. Immunofluorescence staining of smad3 in HTR-8 cells was performed in chamber slides (Lab-Tek, Nalge Nunc International, Rochester, NY). Cells were washed twice with ice-cold PBS and fixed with acetone/methanol at −20°C for 3 min. After 3 times PBS washes (each 10 min), cells were blocked in PBS containing 10% FBS for 30 min. Following 3 further washes, cells were incubated with rabbit polyclonal anti-smad3 antibody (Zymed, 5 μg/ml) in blocking buffer for 2 h at room temperature. Cells were washed again and incubated with FITC conjugated anti-rabbit IgG diluted 1:100. Control staining was carried out with nonimmune IgG used at the same concentration as the primary antibody. Cellular localization of fluorescence was examined by fluorescence microscopy. Photographs were taken with a Zeiss Axiphot microscope (Germany) with a Northern Eclipse Software (Empix Imaging Inc., Mississauga, ON).

Statistical analysis. All results are expressed as mean values ± SEM. "*n*" refers to the number of replicate experiments conducted with an individual cell preparation. Statistical comparisons were done with ANOVA or the Student's *t* test. Differences were considered significant at values of $P < 0.05$.

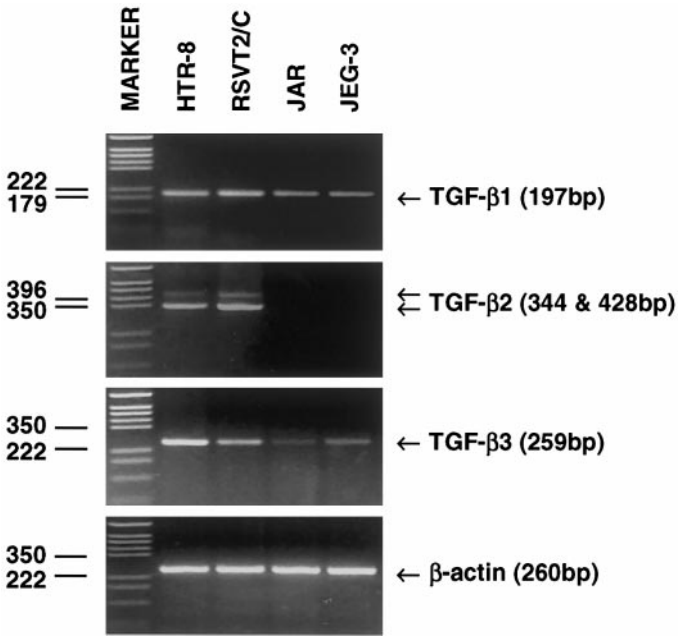

FIG. 1. mRNA expression of TGF-β isoforms in trophoblast cells. Total RNA was isolated and 2 μg was subjected to RT-PCR with the specific primers to detect TGF-β1, TGF-β2, and TGF-β3 isoforms. PCR products were separated by gel electrophoresis. A representative experiment is shown. First lane shows DNA ladder marker. Numbers on the left side of the gel images indicate molecular size (bp). RT-PCR for β-actin (lower panel) was performed as an internal control. TGF-β1 and TGF-β3 were expressed in all cell lines. TGF-β2 was expressed in HTR-8 and RSVT2/C cells, but not in JAR and JEG-3 cells. There were two bands in the second panel representing TGF-β2 precursors a (344 bp) and b (428 bp).

RESULTS

Gene Expression in Trophoblast Cell Lines

To compare the expression of genes involved in TGF-β signaling pathway in the normal (HTR-8), premalignant (RSVT2/C) and malignant (JAR and JEG-3) trophoblast cell lines, we designed specific primers utilizing sequences in the GenBank database to ascertain that target genes sharing sequences were not cross amplified. By using reverse transcriptase linked polymerase chain reaction (RT-PCR), we found that TGF-β1 and TGF-β3 isoforms were expressed in all of the trophoblast cell lines; TGF-β2 was expressed in the normal and premalignant trophoblast, but not in JAR and JEG-3 cells (Fig. 1). The PCR products of TGF-β2 in HTR-8 and RSVT2/C cells appeared as two bands that represented TGF-β2 precursor isoforms: TGF-β2a (344 bp) and TGF-β2b (428 bp), which are produced by alternative mRNA splicing (30). In addition, we observed that all the cell lines were positive for TGF-β receptors: TβR-I, TβR-II and TβR-III (Fig. 2). Since a defect in signaling can occur at the level of the receptors or intracellular signaling machinery, and since all the trophoblast cell lines expressed all the TGF-β receptors, we next examined the downstream molecules in the TGF-β signaling cascade, the smads. RT-PCR analysis revealed that smad2, smad4, smad6 and smad7 were expressed in all cell lines studied here (Fig. 3). Interestingly, smad3 was expressed exclusively in non-malignant cell lines, HTR-8 and RSVT2/C cells. There was no detectable level of smad3 transcript in choriocarcinoma cell lines, JAR and JEG-3 cells (Fig. 3).

Expression of smad Proteins

Loss of smad3 but not smad2 expression in choriocarcinomas as opposed to the normal and premalignant EVT was further examined at the protein level. Immunoblot analysis (Fig. 4) revealed that smad3 protein was absent in JAR and JEG-3 cells, but present in HTR-8 and RSVT2/C cells. This was consistent with the findings on mRNA expression. However, smad2 protein was detected in all investigated cell lines, albeit at the different levels. We further observed that smad4 protein was expressed at very high levels in premalignant RSVT2/C cells. In contrast, smad4 protein was expressed at very low levels in choriocarcinoma JAR and JEG-3 cells.

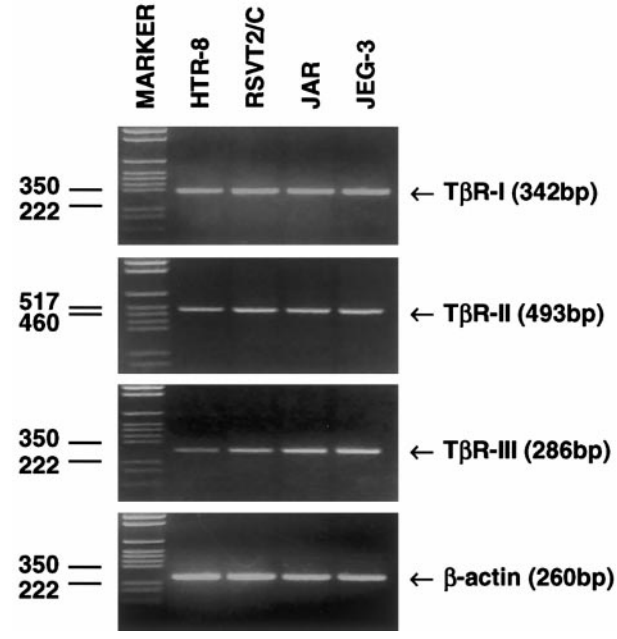

FIG. 2. mRNA expression of TGF-β receptors in trophoblast cells. Specific primers were used in RT-PCR to detect TβR-I, TβR-II and TβR-III mRNA. PCR products were separated by gel electrophoresis. A representative experiment is shown. First lane shows DNA ladder marker. Numbers on the left side of the gel images indicate molecular size (bp). RT-PCR for β-actin (lower panel) was performed as an internal control. TβR-I, TβR-II, and TβR-III mRNA were detected in all cell lines: HTR-8, RSVT2/C, JAR, and JEG-3.

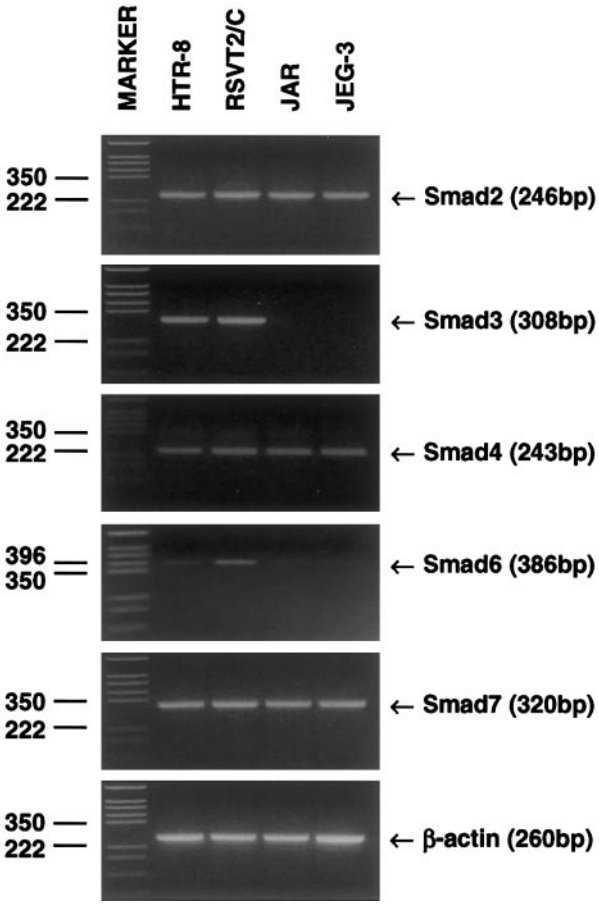

FIG. 3. Smad mRNA expression in trophoblast cells. Specific primers were used in RT-PCR to detect smad2, smad3, smad4, smad6, and smad7 mRNA. PCR products were separated by gel electrophoresis. A representative experiment is shown. First lane shows DNA ladder marker. Numbers on the left side of the gel images indicate molecular size (bp). RT-PCR for β-actin (lower panel) was performed as an internal control. The transcripts of smad2, smad4, smad6, and smad7 were detected in all cell lines: HTR-8, RSVT2/C, JAR, and JEG-3. Smad3 was expressed in HTR-8 and RSVT2/C cells, but not detectable in choriocarcinoma cells: JAR and JEG-3.

Phosphorylation of smad3 Induced by TGF-β in HTR-8 and RSVT2/C Cells

There are reports about the alteration of smad3 protein expression by TGF-β in some human mesangial and epithelial cells (31, 32), and therefore, we analyzed the protein expression and phosphorylation of smad3 in our trophoblast cell lines after addition of TGF-β1. Our results show that exposure of cells to TGF-β1 did not alter the level of total smad3 protein, but induced the phosphorylation of smad3 in HTR-8 and RSVT2/C cells (Fig. 5). The level of phosphorylated smad3 was very low in untreated HTR-8 cells. However, after TGF-β1 treatment for 1 h, there was about 55-fold increase in the level of phosphorylated smad3 in these cells (Figs. 5A and 5B). While untreated RSVT2/C cells exhibited no detectable level of phosphorylated smad3, TGF-β1 treatment of these cells for 1 h induced significant levels of the phosphorylated smad3 (Fig. 5C).

Nuclear Localization of smad3 in normal Trophoblast and Premalignant Cells

We next analyzed subcellular localization of smad3 protein in HTR-8 and RSVT2/C cells and compared the fluorescence signals before and after TGF-β1 treatment. We found that smad3 was present predominantly in the cytoplasm of HTR-8 cells as well as RSVT2/C cells before TGF-β1 treatment (Figs. 6A and 6C). Upon 15 minutes treatment of TGF-β1, smad3 protein was translocated from the cytoplasmic to a nuclear location in both cells (Figs. 6B and 6D). However, no fluorescence signal was detected in JAR cells in the presence or absence of TGF-β1 (data not shown).

DISCUSSION

We have succeeded in propagating the highly proliferative, migratory and invasive extravillous subpopulation of first trimester human placentae to show that they utilize the same molecular mechanisms for invasion as cancer cells (19, 20), but their proliferation (17), migration (18) and invasion (22) *in situ* are stringently controlled by TGF-β, which is produced in abundance by the maternal decidua. These studies revealed that the TGF-β action on EVT cells was primarily paracrine (resulting from TGF-β produced by the decidua) and to

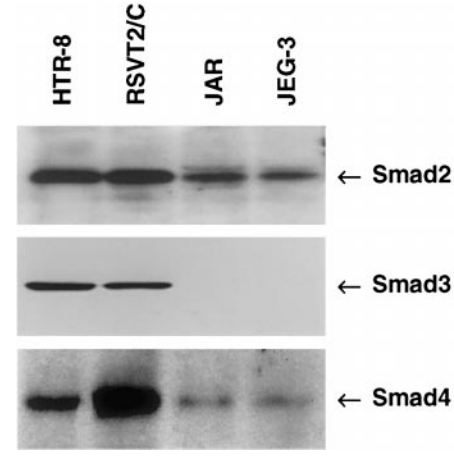

FIG. 4. Smad protein expression in trophoblast cells. HTR-8, RSVT2/C, JAR, and JEG-3 cells were isolated from petri dishes. Equal amounts of cellular proteins were subjected to SDS–PAGE and transferred to PVDF membrane. Specific signal was detected by using specific antibody against smad2, smad3, or smad4. A representative Western blot is shown. Smad2 (upper panel) and smad4 (lower panel) were expressed in all cell lines. There was very low levels of smad4 protein detected in JAR and JEG-3 cells. Smad3 protein (middle panel) was expressed in HTR-8 and RSVT2/C cells, but not detectable in JAR and JEG-3 cells.

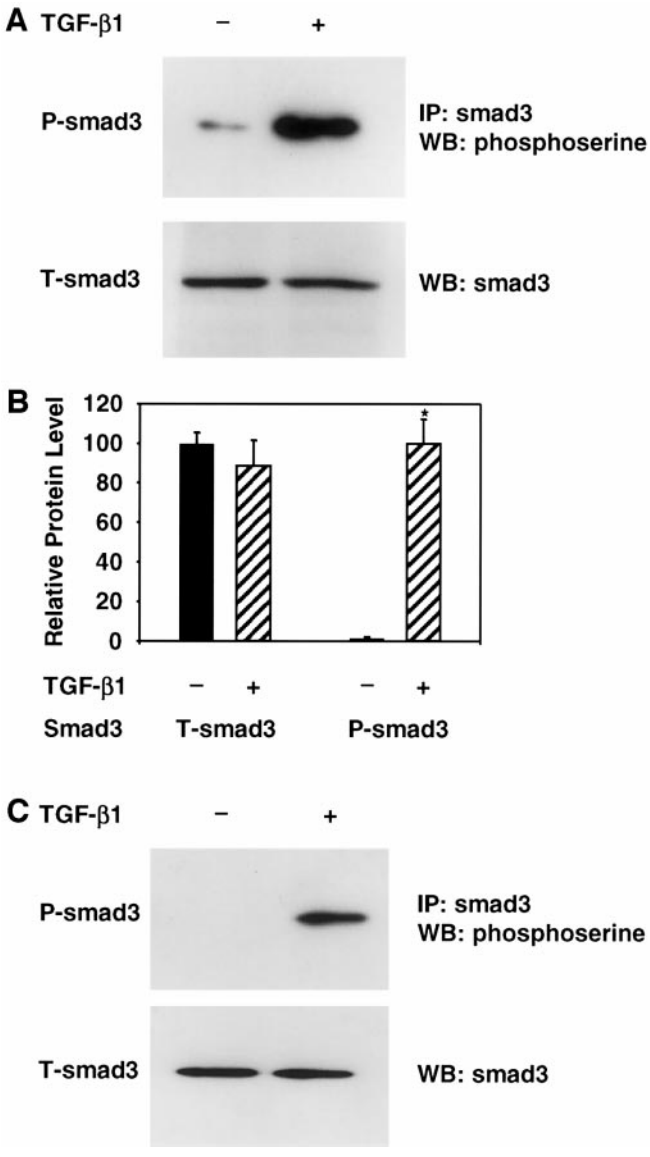

FIG. 5. Phosphorylation of Smad3 protein in HTR-8 and RSVT2/C cells. HTR-8 and RSVT2/C cells were cultured in the serum-free medium in the presence or absence of TGF-β1 (10 ng/ml) for 1 h. In A and C, whole cell lysates were immunoprecipitated with smad3 antibody and subjected to SDS–PAGE. The phosphorylated smad3 (P-smad3) was detected by using an antibody against phosphoserine (upper panel). The total smad3 (T-smad3) was detected by Western blot (lower panel). TGF-β1 treatment increased levels of P-smad3, but did not alter T-smad3 in HTR-8 cells (A). There was no detectable level of P-smad3 in RSVT2/C cells, whereas P-smad3 was detected in TGF-β-treated RSVT2/C cells (C, upper panel). TGF-β did not alter the T-smad3 in RSVT2/C cells (C, lower panel). In B, a histogram shows the relative levels of smad protein in HTR-8 cells determined by densitometric scanning. Specific bands corresponding to T-smad3 and P-smad3 were quantitated and data represent mean ± SEM ($n = 3$, $^*P < 0.001$ vs untreated).

a minor extent autocrine (due to endogenous TGF-β). Malignant (choriocarcinoma) as well as premalignant EVT cells were found to be resistant to antiproliferative and anti-invasive effects of TGF-β (23, 24). The anti-invasive effect of TGF-β on normal EVT cells was shown to be mediated by multiple mechanisms: a reduced matrix degrading ability of the cells due to a downregulation of urokinase-type plasminogen activator (uPA) required for activation of the matrix metalloproteases (MMPs), an upregulation of tissue inhibitor of metalloprotease (TIMP)-1 (21, 22) and plasminogen activator inhibitor (PAI)-1 (33), as well as a reduced migratory ability due to an upregulation of integrins (18) making the cells more adhesive to the extracellular matrix. Increased invasiveness of choriocarcinoma cells was explained by a marked downregulation of the expression of TIMP-1 gene (23), whereas both TIMP-1 and PAI-1 were shown to be downregulated in the premalignant RSVT/C cells (24). Both cell types failed to upregulate these molecules in response to TGF-β (23, 24).

In the present study, we have discovered that both normal and premalignant EVT cells express smad3 transcript and protein, and the protein is phosphorylated and translocated to the nuclei of these cells in response to TGF-β. In contrast, choriocarcinoma cells have no detectable level of smad3 transcript or protein. It has been shown that TGF-β upregulates TIMP-1 expression through the smad3 pathway in human dermal fibroblasts (34). Similarly, PAI-1 upregulation by TGF-β is also smad3-mediated. Smad3 is essential for an activation of the PAI-1 promoter by TGF-β through the Sp1 binding site (35). There are three smad3/smad4 binding sequences, termed as CAGA boxes, within the promoter region of the human PAI-1 gene, and mutation of these CAGA boxes was found to abolish TGF-β responsiveness (36). Targeted disruption of smad3 in fibroblast cells results in failure to activate the TGF-β-responsive promoter of the PAI-1 gene (37). On the other hand, dominant-negative smad2 or smad4 has no effect on this activation (38), indicating the selectivity of smad3 action. Conversely, cooverexpression of smad3/4 but not smad2/4 induces transcriptional activation of PAI-1 promoter (39). Thus, although choriocarcinoma cells contain TIMP-1 and PAI-1 genes, lack of smad3 transcript and protein in these cells can prevent TGF-β-mediated upregulation of the above molecules, and may account for the loss of anti-invasive effect of TGF-β. Furthermore, very low level of smad4 protein expression in choriocarcinoma cells may additionally contribute to TGF-β resistance in these cells.

As reviewed earlier, mutational events involving TGF-β signaling genes have been identified in many cancers. However, smad3 mutation or loss has so far not been reported in any human cancer. Loss of smad3 but not smad2 protein may be functionally important in the acquisition of TGF-β resistance by choriocarcinomas. This is in view of distinctive roles of smad2 vs smad3. Although they share 92% homology in their

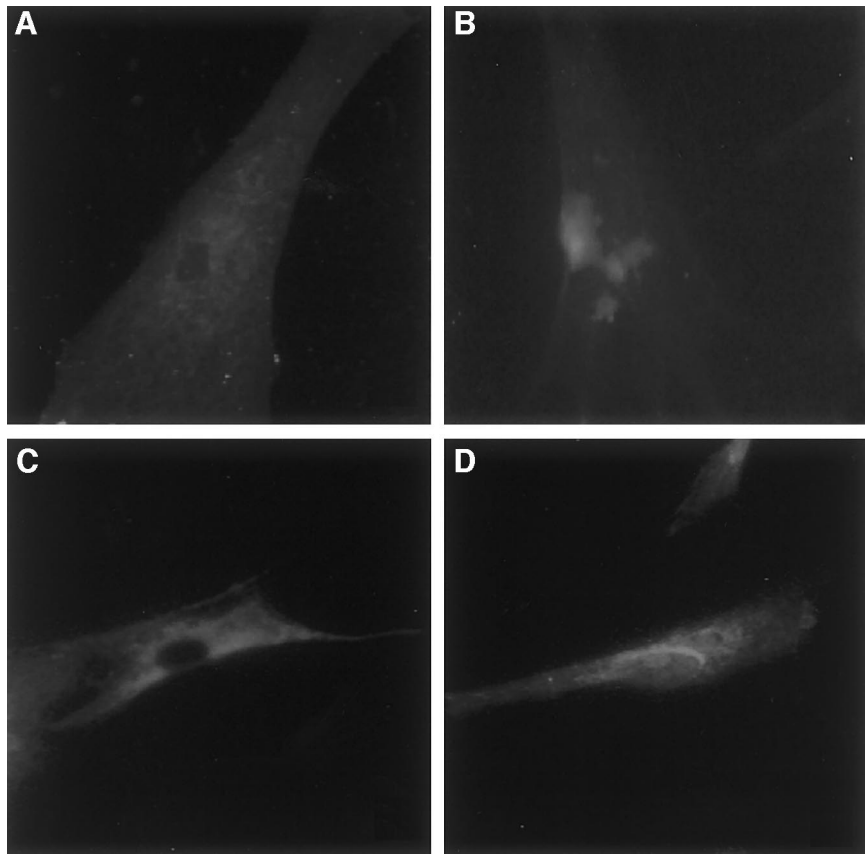

FIG. 6. Nuclear translocation of smad3 after TGF-β stimulation. HTR-8 or RSVT2/C cells were cultured in complete medium and then replaced in serum-free medium prior to treatment. Cells were exposed to TGF-β1 (10 ng/ml) for 15 min and then fixed in acetone/methanol for 3 min. Smad3 was localized in the cells by immunofluorescence staining using a rabbit anti-smad3 antibody. Smad3 staining was predominant in the cytoplasm of HTR-8 cell (A) and RSVT2/C cell (C) in the absence of TGF-β1, whereas nuclear staining for smad3 in HTR-8 cell (B) and RSVT2/C cell (D) was observed after TGF-β1 stimulation.

coding regions, they have divergent DNA binding activity and function (40, 41). Smad3 acts as a transcription factor by binding to an eight nucleotide TGF-β responsive sequence called CAGA box, whereas smad2 does not (41). TGF-β induced apoptotic death in human lung epithelial cells was higher with smad3 but not smad2 expression (32). Moreover, smad3 has a higher binding affinity than smad2 for a transcriptional coactivator p300 (42). Nuclear accumulation of smad3 is important for degradation of the corepressors Ski/SnoN (43). On the other hand, gene knockout studies of smad2, 3 and 4 in the mice have elucidated the roles of these genes in development and tumorigenesis. Targeted deletion of smad2 or smad4 each results in early embryonic lethality and in the inability to form mesoderm (44). Although smad3 null mice survive to adulthood, cells derived from these mice are hyperproliferative because of refractoriness to endogenous TGF-β (37). Furthermore, exon 2 targeted smad3 null mice can develop colorectal cancer (45).

Our findings of loss of smad3 expression in the human choriocarcinoma cells (JAR and JEG-3) are in conflict with a recent report of low but detectable levels of smad3 mRNA expression in JEG-3 cells (46). In the latter study, the authors utilized a rat smad3 cDNA probe to hybridize with human smad3 mRNA, raising the possibility of minor cross reactivity with smad2 which share 92% homology with smad3 in their coding region (41). In the present study, we have carefully designed our PCR primers from the human gene sequences to ensure specific amplification of either smad3 or smad2 gene. Furthermore, we verified our results at the protein level using specific antibodies.

We have excluded the role of smad3 signaling defect(s) in the acquisition of TGF-β resistance by the premalignant RSVT2/C cells. The role of mutational inactivation of other TGF-β signaling genes remains to be identified in these cells. TGF-β resistance in RSTV2/C cells was not due to SV40 Tag transformation per se, since another SV40 Tag immortalized EVT cell line HTR-8/SVneo derived from the same parental cell line HTR-8 but selected on the basis of neomycin resistance, has retained its full TGF-β sensitivity (21, 47). Premalignant RSVT2/C cells have been shown to have undergone additional genetic changes including the loss of IGF receptor type 2 which is retained by both

HTR-8 and HTR-8/SVneo cells (accepted for publication).

In vivo, the temporal and spatial synthesis of the three TGF-β isoforms is strictly regulated, as illustrated by their differential expression during embryogenesis (48) and wound healing (49). All the three TGF-β isoforms are expressed in variable amounts by the human placenta and the decidua *in situ* (17, 50–52). Small amount of TGF-β has also been shown to be produced by the extravillous trophoblast cells (17). These suggest its role as an autocrine and paracrine negative regulator of proliferation and invasiveness. It has been shown that loss of TGF-β expression in the skin and benign skin tumors is associated with a high risk of malignant change (53, 54). The present study shows that choriocarcinoma cells have no detectable level of TGF-β2 mRNA. It is possible that loss of TGF-β2 occurred prior to the loss of TGF-β sensitivity during the development of choriocarcinoma *in situ*, making the cells hyperproliferative and thus prone to further mutational events e.g., loss of smad3.

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