Abstract. Transforming growth factor-ß (TGF-ß) signals through membrane-bound heteromeric serine/threonine kinase receptors. Upon ligand binding, TGF-ß activates intracellular Smad proteins and regulates proliferation and apoptosis in various cell types. To demonstrate the effects of TGF-ß/Smad signal on growth and apoptosis of human embryonal rhabdomyosarcoma (RMS) cells, a strategy of RNAi-mediated ‘gene silencing’ of Smad4 was used to interrupt endogenous TGF-ß/Smad signaling in an RMS cell line, RD, and the regulation of exogenous TGF-ß1 to growth and apoptosis of the cells was also determined. Physiologically, TGF-ß/Smad signaling was essential for the normal growth of RD. The interruption of endogenous TGF-ß/Smad signaling by RNAi significantly suppressed the growth of RD cells and dramatically induced apoptosis of RD cells. Exogenous TGF-ß1 also inhibited the growth of RD cells, but had no effect on apoptosis. It also partially counteracted the growth inhibition and apoptosis induced by Smad4 silencing in RD cells. These findings provide a new insight into how TGF-ß/Smad signaling regulates the growth and apoptosis of cancer cells. Moreover, as a powerful tool, shRNA interference suppresses endogenous Smad4 gene expression and subsequently modulates cell growth and apoptosis, which may provide a novel basis for the development of rational intervention strategies in RMS therapy.

Introduction

Transforming growth factor-ß (TGF-ß) belongs to a large family of multifunctional polypeptides that are involved in the regulation of growth, differentiation, apoptosis, migration and adhesion of various cell types (1,2). It has been shown to possess potent antiproliferative activity for many cell types including hepatocytes, epithelial cells, and hematopoietic cells, and it has also been implicated in the induction of apoptosis in hepatocytes, prostatic epithelial cells, and myeloid cells. TGF-ß binds to TGF-ß receptor type II (TßR-II) and stabilizes the heteromeric complex with TGF-ß receptor type I (TßR-I) resulting in the transphosphorylation and activation of TßR-I by constitutively active TßR-II. The activated TßR-I then phosphorylates and activates receptor-regulated Smad2 and Smad3. Phosphorylated receptor-regulated Smads associate with the common Smad4, translocate to the nucleus and then modulate transcription of TGF-ß target genes (3-5).

Rhabdomyosarcoma (RMS) is a common malignant tumor in children under 15 years. Despite aggressive approaches including surgery, dose-intensive combined chemotherapy and radiation therapy, the outcome for patients with metastatic RMS remains poor (6,7). RMS can express various growth factor loops, including insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and TGF-ß, which regulate growth and differentiation of RMS and are likely involved in RMS tumorigenesis and tumor progression (8-11). Better understanding of how these growth factors modulate proliferation and apoptosis of cancer cells may ultimately have implications for the treatment of RMS.

RNA interference (RNAi) is a powerful technique to silence genes post-transcriptionally, in which double-stranded RNA (dsRNA) inhibits gene expression in a sequence-specific manner via degradation of the corresponding mRNA (12-15). RNAi is initiated by an event where dsRNAs are recognized by Dicer, a member of the RNase III protein family (16). The Dicer enzyme cleaves dsRNAs into 21- or 23-nucleotide (nt) short interfering RNAs (siRNA). These siRNA duplexes are incorporated into a protein complex called the RNA-induced silencing complex (RISC), which recognizes and cleaves the cognate mRNA. The RNAi technique is not only a routine tool for studies of gene function, but also represents a promising therapeutic strategy. Two approaches, i.e. siRNA and short hairpin RNA (shRNA), have been developed for the application of RNAi technology in mammalian cells (17,18).

Our previous study demonstrated that cells of a human embryonal RMS cell line, RD, autocrined TGF-ß1, overexpressed TßR-I, TßR-II and downstream molecules-Smads 2, 3, and 4, and had functional TGF-ß1/Smad signaling (19). The aim of this study was to determine how TGF-ß/Smad signaling regulates the growth and apoptosis of RD cells by using highly potent shRNA interference to suppress Smad4
gene expression, and the regulatory effect of exogenous TGF-ß1 on the growth and apoptosis of the cells.

Materials and methods

**Cell culture.** RD cells were obtained from American Type Culture Collection (ATCC, Manassas, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in 5% CO2 humidified atmosphere at 37°C.

**RNA interference.** shRNAs targeting human Smad4 RNA were designed according to the standard selection criteria (20) and biologically synthesized by Wuhan GenSil Co., Ltd. (GenSil, Wuhan, China). All designed shRNA sequences were checked by BLAST search to preclude sequences with significant similarity to other genes in the human genome. The synthetic shRNA sequences are summarized in Table I. RD cells were seeded in 12-well plates at a density of 8x10⁴ cells/well and cultured for 24 h. Cells were washed with phosphate-buffered saline (PBS) and transfected with 1 μl of DMEM containing 1 μg shRNA using 12 μl metafectenetm (Biontex, Munich, Germany), according to the manufacturer's instructions.

**Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR).** RD cells were harvested for total RNA isolation with TRIzol reagent (Gibco-BRL, Grand Island, USA) according to the manufacturer's recommended protocol. Total RNA (2 μg) was reversely transcribed into cDNA for PCR amplification with an RT-PCR kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The sequences of the primers for human Smad4 were 5'-AAAG GTGAAGGTGATGTTTGGGTC-3' (sense) and 5'-CTGCG CAGCTATTCCACCTACTGATCC-3' (antisense). Thesequences of primers for human ß-actin, an internal control, were 5'-ACC CCCACTGAAAGAAGATGA-3' (sense) and 5'-ACTTTCA AACCTCCATGATG-3' (antisense). The reaction conditions were 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by a 7-min incubation at 72°C in a Hotstart PCR system. PCR products were separated electrophoretically on a 1.8% agarose gel stained with ethidium bromide for visualization. The electrophoresis band of Smad4 mRNA was quantified by Image Quant (Version 5.2).

**Western blot analysis.** RD cells were harvested and lysed in TNES buffer [50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate, and 1% NP-40 containing protease inhibitors (20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride)]. The amount of protein was quantitated by a colorimetric assay (Bio-Rad, Hercules, USA). Equal amounts of total proteins from cell extracts were electrophoresed on a 12% SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was blotted in a blocking buffer and probed with antibodies against Smad4 or ß-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Detection was performed using an enhanced chemiluminescence system (Santa Cruz Biotechnology).

**Immunofluorescence staining.** RD cells on the coverslips were washed with PBS and fixed with 4% paraformaldehyde for 15 min at 4°C. After washing with PBS, the cells were blocked with 5% rabbit normal serum in PBS containing 1% bovine serum albumin (BSA) at 37°C for 30 min. The cells were incubated at 4°C overnight with the Smad2/3 primary antibodies (Dako, Glostrup, Denmark) in PBS containing 1% BSA. Cells were washed with PBS again, and incubated with 1:400 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody for 30 min at room temperature. The slides were then washed, air-dried, mounted and photographed under a laser scanning confocal microscope (LSCM, Bio-Rad).

**[3H]thymidine incorporation assay.** RD cells were plated at 4x10⁴ cells per well in 96-well plates, consisting of a
control group, an shRNA group, a TGF-ß1 group, and an shRNA + TGF-ß1 group. Cells were cultured for 24, 48, and 72 h. During the last 4 h of each treatment, cells were pulsed with 3.7x10^4 Bq (1 μCi)/well [3H]thymidine. Then the cells were rinsed with PBS and DNA was precipitated with 10% (wt/vol) trichloroacetic acid for 30 min at 4°C. The amount of [3H]thymidine incorporated was determined by liquid scintillation counting. The mean values from triplicate wells for each treatment were determined.

**MTT assay.** RD cells were plated at 4x10^4 cells per well in 96-well plates, consisting of a control group, an shRNA group, a TGF-ß1 group, and an shRNA + TGF-ß1 group. Cells were cultured for 24, 48, and 72 h. During the last 4 h of each treatment, MTT (Sigma, St. Louis, MO, USA) was added to the medium to a final concentration of 50 μg/ml, which was converted to an insoluble purple formazan by cleaving the tetrazolium ring with dehydrogenase enzymes. Then, the medium was removed and the formazan product was dissolved with 150 μl DMSO (Sigma) per well for 10 min. Absorbance was determined at 570 nm. The mean values from triplicate wells for each treatment were determined.

**Flow cytometry analysis.** RD cells in various groups were fixed in 70% ethanol and stained with 100 μg/ml propidium iodide at 4°C for 1 h. DNA content was monitored by FACScan (COULTER, Elite, USA).

**DAPI staining.** RD cells on coverslips were washed with PBS, fixed with 4% paraformaldehyde for 15 min at 4°C, and then incubated in 5 μg/ml DAPI (Sigma) at room temperature for 5 min. The slides were then washed, air-dried, mounted and photographed under LSCM.

**Electron microscopy.** RD cells were harvested, fixed by 3% glutaraldehyde and osmium tetroxide, respectively, dehydrated, and embedded in Epon812. Thin sections were cut at a thickness of 50 nm, uranyl acetate and lead citrate stained, and examined under the H-600IV electron microscope.

**Statistical analysis.** All data were expressed as mean values ± standard deviation (SD). Analyses were carried out using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Statistical comparisons between two groups or among groups were determined using t-test or one-way ANOVA. A P-value (two sided) of <0.05 was considered statistically significant.

**Results**

**Positional effects of shRNAs against Smad4 mRNA.** To assess the positional effects, shRNAs were designed to target five different sites on the human Smad4 mRNA (Table I). The RNAi efficiency in RD cells was evaluated by RT-PCR and Western blotting. The human Smad4 shRNAs Y1 and Y3 (1 μg each), which targeted the 461-481 nt and 671-691 nt of the human Smad4 cDNA sequence, respectively, efficiently reduced the levels of Smad4 mRNA expression, with Y1 being more efficient than Y3 (Fig. 1A). Similarly, the expression of Smad4 was remarkably suppressed by Y1 and Y3 interference at the protein level (Fig. 1B). Since the RNAi effect by Y1 was found to be the most reproducible, Y1 was used for the subsequent RNAi experiments.

**Time-course analysis of Smad4 RNAi.** To obtain the maximum RNAi effect, we examined the time-course of Smad4 gene expression after shRNA transfection. Smad4 mRNA began to decrease 12 h after transfection with shRNA Y1, and the
expression of Smad4 mRNA was not detectable from 24 h to 72 h but was detected again 96 h following transfection (Fig. 2A). Smad4 shRNA Y1 transfection also resulted in marked reduction of Smad4 protein expression level, which was detected 24 h after shRNA transfection and lasted for >48 h (Fig. 2B). These results suggest that RNAi induced by shRNA Y1 effectively leads to silencing of the Smad4 gene in RD cells.

Effect of Smad4 RNAi on TGF-β/Smad signaling. To detect if Smad4 silencing abrogates TGF-β/Smad signaling, the distribution of Smad2/3 was determined after Y1 transfection.

Figure 2. Time-course of shRNA Y1 on Smad4 expression. RD cells were transfected with 1.0 μg Y1 and harvested at indicated times for RT-PCR (A) or Western blot (B) analyses. Results of RT-PCR were quantitated as described in Fig. 1. β-actin was used as loading control.

Figure 3. Effect of shRNA Y1 on TGF-β/Smad signaling. RD cells were treated with 5 ng/ml TGF-β for 12 h in the absence or presence of Y1 treatment. The expression of Smad2/3 in RD cells was then analyzed by immunofluorescent staining.
Figure 4. Effects of Smad4 shRNA on proliferation of RD cells. (A) RD cells were treated with 1.0 μg Y1 and/or 5 ng/ml TGF-β1 and incubated for the indicated times. During the last 4 h of each treatment, cells were pulsed with 3.7x10^4 Bq/well [3H]thymidine and then harvested for [3H]thymidine incorporation detection. (B) RD cells were treated with 1.0 μg Y1 and/or 5 ng/ml TGF-β1 and incubated for the indicated times. During the last 4 h of each treatment, cells were treated with 50 μg/ml MTT and then dissolved in DMSO for absorbance detection at 570 nm. (C) RD cells were treated with 1.0 μg Y1 and/or 5 ng/ml TGF-β1 and incubated for 72 h. Cells were then fixed for flow cytometry analysis of cell cycle. The bars represented the means ± SD of the data obtained in three independent experiments.

The growth viability of RD greatly decreased in a time-dependent manner in Y1-group, Y1 + TGF-β1-group, and TGF-β1-group, compared with control group. The bars represent the means ± SD of the data obtained in three independent experiments.

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<td>72h</td>
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72h Y1-group vs 72h TGF-β1-group, P=0.000

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<th>Time</th>
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<th>Y1-group</th>
<th>Y1+TGF-β1-group</th>
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72h Y1-group vs 72h TGF-β1-group, P=0.004

Figure 4. Effects of Smad4 shRNA on proliferation of RD cells. (A) RD cells were treated with 1.0 μg Y1 and/or 5 ng/ml TGF-β1 and incubated for the indicated times. During the last 4 h of each treatment, cells were pulsed with 3.7x10^4 Bq/well [3H]thymidine and then harvested for [3H]thymidine incorporation detection. (B) RD cells were treated with 1.0 μg Y1 and/or 5 ng/ml TGF-β1 and incubated for the indicated times. During the last 4 h of each treatment, cells were treated with 50 μg/ml MTT and then dissolved in DMSO for absorbance detection at 570 nm. (C) RD cells were treated with 1.0 μg Y1 and/or 5 ng/ml TGF-β1 and incubated for 72 h. Cells were then fixed for flow cytometry analysis of cell cycle. The bars represented the means ± SD of the data obtained in three independent experiments.
A

Control IF=600

Y1-group cultured 72h apoptosis bodies IF=600

Y1=TGF-β1-group

Y1 transfet 48h followed TGF-β1 24h Treatment, apoptosis bodies IF=600

TGF-β1-group IF=600

B

Control: normal nucleus of RD EM=×5000

Karyolemma shrinks; chromatin condense under karyollemma EM=×5000

Chromatin condense; karyolemma is integrated; manisicate nucleus EM=×5000
Smad2/3 redistributed into the nucleus of RD cells after TGF-ß1 treatment (Fig. 3). However, suppression of Smad4 expression by shRNA Y1 blocked the TGF-ß1-induced translocation of Smad2/3, suggesting that inhibition of the Smad4 function interrupts TGF-ß/Smad signaling in RD cells.

**Effect of Smad4 RNAi on the growth of RD cells.** Treatment with both TGF-ß1 and Smad4 shRNA Y1 inhibited [3H]thymidine incorporation into RD cells (Fig. 4A). However, there was no synergistic or additive effect when Smad4 shRNA Y1-treated RD cells were exposed to TGF-ß1. Instead, TGF-ß1 counteracted Y1-mediated growth inhibition (P<0.01). MTT assay showed similar results (Fig. 4B). Cell cycle analysis showed that interruption of endogenous TGF-ß/Smad signaling by Y1, as well as exogenous TGF-ß1 treatment, induced G1 arrest in RD cells, and similarly, TGF-ß1 treatment partially neutralized the effect of Y1 (Fig. 4C).

**Effect of Smad4 RNAi on apoptosis of RD cells.** A proportion of RD cells changed to a round shape from the normal fusiform or polygon shape, and some detached from the bottom 72 h after Y1 treatment (data not shown), suggesting the occurrence of apoptosis, which was further confirmed by DAPI staining and electron microscopy (Figs. 5A and B). Flow cytometry analysis demonstrated that Y1 treatment induced apoptosis in a time-dependent manner with a 4-fold induction at 72 h (Fig. 5C). TGF-ß1 did not induce apoptosis in RD cells, but treatment with TGF-ß1 significantly blocked Y1-induced apoptosis.

**Discussion**

Our previous study has demonstrated that exogenous TGF-ß1 inhibits the proliferation of human embryonal RMS cells and induces G1 cell cycle arrest (21). To further understand the effect of endogenous TGF-ß/Smad signaling on human embryonal RMS cell line RD, RNAi was employed to silence the Smad4 gene, the key mediator of TGF-ß/Smad signaling, in the present study. It was observed that knockdown of endogenous Smad4 gene expression blocked TGF-ß1/Smad signaling, inhibited the proliferation of RD cells and induced cell apoptosis, suggesting that endogenous TGF-ß/Smad signaling is essential for growth of RD cells.

It has been shown that siRNAs interfering with different sites on the same target mRNA exhibit remarkable differences in silencing efficiency, and only a few such siRNAs result in a significant reduction in the mRNA expression level (22), indicating that accessible RNAi target sites are rare in mRNAs. Similarly, in the present study, only two of the five designed shRNAs, especially Y1, potently suppressed Smad4 expression in a time-dependent manner. Immunofluorescent staining showed that TGF-ß1-induced translocation of Smad2/3 from the cytoplasm into the nucleus was blocked by Y1 treatment, confirming that Y1 successfully knocked down functional Smad4.

TGF-ß was originally identified as an important growth regulatory factor for its ability to stimulate the soft-agar growth of normal rat kidney fibroblasts (23). It was then shown that its function of growth regulation is also associated with the protective activity of sustaining cell survival via JNK cascade in rat-1 fibroblasts (24). Subsequently, TGF-ß was demonstrated to inhibit the proliferation of a wide variety of cell types including epithelial, endothelial, hematopoietic and certain cancer cells (25-28), which is mediated by inducing G1 arrest (29-31). Loss of responsiveness to growth inhibition by TGF-ß frequently accompanies the procession of malignancy and may contribute to the development of many types of human cancer. Our previous study demonstrated that RMS RD cells autocrine TGF-ß1 (at the highest concentration of 620 pg/ml in the medium without serum during 96-h culture) and express TGF-ß receptor and downstream molecular Smads (19). In the present study, although endogenous TGF-ß1 (at a concentration of 5 ng/ml) inhibited the growth of RD cells, RNAi-mediated Smad4 silencing resulted in increased proliferation of the cells and G1 cell cycle arrest, indicating that TGF-ß/Smad signaling plays a key role in the normal growth of RD cells.

TGF-ß also exerts apoptotic activity to some specific cell types such as hepatocytes, breast cancer cells, and epithelia cells (32-34). In certain cells, loss of ability of TGF-ß to induce apoptosis might participate in tumorigenesis and accelerate tumor progression (35,36). Serving as a common partner of receptor-regulated Smads, Smad4 is pivotal in...
the TGF-β/Smad signaling pathway. Smad4 is frequently inactive in pancreatic, biliary, and colorectal cancers (37-39). Expression of Smad4 in Smad-defective tumor cell lines has restored TGF-β signaling and induced cell arrest or apoptosis (40,41). Overexpression of Smad4 has been shown to induce apoptosis in MDCK canine epithelial cells (42). However, a completely contrary model of regulation of apoptosis by TGFβ signaling was observed in the present study. Loss of endogenous TGF-β signaling by interrupting Smad4 expression, but not exogenous TGF-β1, dramatically induced apoptosis of RD cells. Considering that Smad4 is overexpressed in RD cells (19), future investigation on the mutational analysis of Smad4 in RD cells will help to further clarify the function of TGF-β/Smad signaling.

Interestingly, although exogenous TGF-β1 had no effect on RD cell apoptosis, TGF-β1 treatment partially counteracted the effect of Smad4 shRNA in growth inhibition and apoptosis induction, as observed in the present study. One possible explanation is that once Smad4 is silenced by shRNA, TGF-β1 cannot function through Smad proteins and it may activate other pathways including survival pathways. Increasing evidence indicates that alternative, non-Smad pathways also participate in TGF-β signaling, including MAPK and PI3K pathways (43).

The present study demonstrates that RNAi technology represents a potent tool to inhibit endogenous Smad4 gene expression and to specifically modulate growth inhibition and apoptosis in RD cells. More importantly, the findings in the present study may have significant implications for tumor therapy. The present study shows that vector-borne expression of siRNAs can efficiently inhibit the function of target genes in vivo, and previous studies have demonstrated that siRNA can be directly applied to organs of postnatal mice by high-pressure injection into the tail vein, leading to the specific inhibition of target genes (44-46). However, despite the therapeutic potential, application of siRNA in gene therapy is challenging. The delivery efficiency into the target cells and the circumvention of immune response are major issues. Alternative delivery strategies such as various translocating peptides have been developed (47,48). Further development of these techniques may provide a new approach for the topical application of siRNA in gene therapy.

In conclusion, physiological TGF-β/Smad signaling is essential for the normal growth of RD cells. Interruption of endogenous TGF-β/Smad signaling by RNAi-mediated Smad4 gene silencing not only inhibits the growth but also induces apoptosis of RD cells. Exogenous TGF-β1 inhibits the growth of RD cells by inducing G1 arrest in the cell cycle, but has no effect on the apoptosis of RD cells. Our findings provide a new insight into how TGF-β/Smad signaling regulates the growth and apoptosis of RD cancer cells and an experimental basis for future investigations on RMS gene therapy.

Acknowledgements

This work was supported by the China Medical Board of New York, New York, USA (CMB 00-722).

References


