Cancer Science
The official journal of the Japanese Cancer Association

TGF-β1 signal pathway may contribute to rhabdomyosarcoma development by inhibiting differentiation

Shouli Wang,[1,3] Lingchuan Guo,[1] Liang Dong,[1] Lingling Guo,[1] Shigang Li,[1] Jiajia Zhang[1] and Maomin Sun[2,3]

Departments of [1]Pathology and [2]Anatomy, Soochow University School of Medicine, Suzhou, China

(Received July 3, 2009/Revised December 25, 2009/Accepted January 19, 2010/Online publication March 7, 2010)

Overexpression of transforming growth factor-β1 (TGF-β1) and its downstream molecules in the rhabdomyosarcoma (RMS) RD cell line has been reported previously, but the regulatory role of TGF-β1 on RMS has not been studied extensively. In the present study, we showed that expression of TGF-β1 and its downstream molecules type II TGF-β receptor (TβRII) and Smad4 was significantly higher in RMS than in normal skeletal muscle, and there was a significant relationship between TGF-β1 expression and histological grade. Gene silencing with TGF-β1 short-hairpin RNA (shRNA)-expressing vectors significantly decreased the growth of RD cells, which was confirmed by caspase-3 (*in vitro*) and TUNEL (*in vivo*) assays. Moreover, a proportion of treated rhabdomyosarcoma (RD) cells changed to a round shape from the normal fusiform or polygonal shape and expressed myofilaments. Myogenin is one of the myogenic differentiation genes (MyoD) family of myogenic regulators, and was obviously higher in TGF-β1-shRNA-treated tumors than it in control at the mRNA and protein level. Immunohistochemical staining with myogenic differentiation markers such as myosin and desmin in subcutaneous RMS tissue showed that TGF-β1 shRNA increased staining for myosin. These results provide new insight into the biological function of TGF-β1 in malignant tumors, and imply that the TGF-β1 signal pathway is a potential therapeutic target for drugs that induce differentiation of RMS. (*Cancer Sci* 2010; 101: 1108–1116)

Most tumor cells secrete transforming growth factor-β1 (TGF-β1), which may affect their growth and the surrounding environment, and lead to tumor progression and metastasis.[1,2] However, the intriguing feature of TGF-β biology is that its effects are cell-type dependent. TGF-β1 inhibits proliferation of many epithelial cell types, and the accumulation of loss-of-function mutations in the TGF-β1/Smad signaling pathway contributes to the development of carcinoma.[1,3] TGF-β1 also acts as a growth stimulator for some cells of mesenchymal origin such as fibroblasts, chondrocytes, and osteoblasts, as well as several tumor cell lines,[4] and inhibits myogenic differentiation through Smad3-mediated transcriptional repression.[5]

Rhabdomyosarcoma (RMS) is the commonest malignant soft-tissue tumor in childhood and adolescence. Although most RMS tumor cells are characterized by expression of myogenic-promoting transcription factor, they fail to undergo terminal differentiation into skeletal muscle.[6,7] Several studies have indicated that increased levels of insulin-like growth factor II,[8] basic fibroblast growth factor,[9] epidermal growth factor,[10] and TGF-β1[11] in RMS are associated with tumorigenesis and tumor progression, by regulating growth and differentiation of RMS. We have shown previously that the RD embryonal RMS tumor cell line autocrines TGF-β1 and overexpression of its receptors and their downstream molecules.[12] Interruption of endogenous TGF-β/Smad signaling by RNAi-mediated Smad4 gene silencing may induce the growth inhibition and apoptosis of rhabdomyosarcoma (RD) cells.[13] Therefore, in the light of evidence of interplay between proliferation and differentiation within the myogenic lineage,[14] it is of interest to explore the possibility that TGF-β1 induces steps towards RMS tumorigenesis or blocks differentiation of RMS.

The aims of the present study were to examine expression of TGF-β1 and its downstream molecules in human RMS tissue and their relationship with clinicopathological parameters, and to demonstrate the effects of TGF-β1 silencing on differentiation and growth of RMS by using short-hairpin RNA interference (shRNAi) technology.

Materials and Methods

Tissue sampling. Sixty-eight RMS and 22 normal skeletal muscle samples were obtained from the West China Hospital of Sichuan University. All samples were confirmed histologically. The histological material consisted of initial biopsy or resection specimens and of post-chemotherapy biopsy specimens from residual, recurrent, or metastatic tumor sites. All specimens were embedded in paraffin and stained with hematoxylin–eosin. Forty-two of the patients were male, and 28 were female. Their ages were 1–30 years (median, 15 years). All RMSs were classified histologically as grade I (n = 25), grade II (n = 22), or grade III (n = 21), according to the most differentiated areas in the tissue.[15] The growth patterns were designated as solid, alveolar, or botryoid, but were not taken into consideration in the classification.

Immunohistochemistry. Paraffin-embedded tissue sections (3–5 μm thick) were immunostained. The slides were subjected to antigen retrieval as follows. After boiling in 10 mM citrate buffer for 10 min in a microwave oven, the tissue sections were incubated with 10% normal rabbit serum in buffer for 45 min to block nonspecific binding sites. Then, consecutive sections were incubated with antibodies (Table 1) at 4°C overnight. The slides were rinsed with washing buffer and incubated with peroxidase-conjugated IgG (Shanghai Genomics, Shanghai, China) for 45 min at room temperature. After washing, each section was stained with diaminobenzidine–chromogen substrate mixture (Dako, Carpinteria, CA, USA), and then counterstained with hematoxylin. Slides were examined under a light microscope. The expression of TGF-β1 signaling pathway proteins were classified according to the following grading system: staining extensity was categorized as 0 (no positive cells), 1 (≤25% positive cells), 2 (>25% and ≤50% positive cells), or 3 (>50% positive cells); and staining intensity was categorized as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The individual categories were added to give a total immunoreactive score (IRS).

[3]To whom correspondence should be addressed.
E-mail: wangsoly112@hotmail.com; sunmaomin@suda.edu.cn

doi: 10.1111/j.1349-7006.2010.01512.x
© 2010 Japanese Cancer Association

Table 1. Characteristics of the primary antibodies used

Antibody	Manufacturer	Catalog	Isotype	Applications	Dilution
TGFβ1	Santa Cruz Biotechnology, Santa Cruz, CA, USA	sc-52893	Mouse monoclonal IgG	IHC, WB	1:100, 1:1000
TβRII	Santa Cruz Biotechnology, Santa Cruz, CA, USA	sc-220	Rabbit polyclonal IgG	IHC	1:100
Smad4	Santa Cruz Biotechnology, Santa Cruz, CA, USA	sc-7966	Mouse monoclonal IgG	IHC	1:50
Caspase3	Santa Cruz Biotechnology, Santa Cruz, CA, USA	sc-7148	Rabbit polyclonal IgG	IF	1: 50
Ki-67	Boster, Wuhan, China	BA1508	Rabbit polyclonal IgG	IHC	1:100
Myosin	Invitrogen, Carlsbad, CA, USA	18-0105	Mouse monoclonal IgG	IHC	1:100
Desmin	Invitrogen, Carlsbad, CA, USA	18-0016	Mouse monoclonal IgG	IHC	1:100
Myogenin	Abcam, Cambridge, UK	Ab734	Mouse monoclonal IgG	IHC	1:50
β-Actin	Santa Cruz Biotechnology, Santa Cruz, CA, USA	sc-47778	Mouse monoclonal IgG	WB	1:1000

IHC, immunohistochemistry; TGF-β1, transforming growth factor-β1; TβRII, type II TGF-β receptor; WB, western blotting.

Tumor cell line and cell culture. The human embryonal RMS RD cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA).[16] Normal human primary skeletal myoblasts, obtained from passage culture,[12] were maintained in complete Dulbecco's modified Eagle's medium (DMEM) that contained 10% fetal calf serum (FCS) and 100 μg/mL doxycycline, and then incubated at 37°C in a 5% CO_2 humidified atmosphere. The differentiation medium comprised serum-free DMEM with 10 mg/mL insulin and transferrin.[17,18]

Construction and transfection of TGF-β1-shRNA-pSUPER vector. TGF-β1-specific shRNAs were designed with the Target Finder of Ambion (http://www.ambion.com/techlib/misc/siRNA_finder.html). The humanTGF-β1-specific oligonucleotide sequences 5′-GATCCCCCAAUUCCUGGCGAUACCUC-ttcaagagaGAGGUAUCGCCAGGAAUUGUUUttcaagagaCAG-CTCCATGTCGATAGTCTTTTTGGAAA-3′ and 5′-AGCTTTT-CCA AAAAGACTATCGAC ATGGAGCTGtctcttgaaCAGCT-CCATGTCGATAGTCGGG-3′ were synthesized according to the method of Sangon (Shanghai, China). The TGF-β1-specific parts of the sequence are underlined, and this is the optimal site for TGF-β1 gene silencing, as described previously.[19] These oligonucleotides were ligated into the mammalian expression vector pSUPER gfp-neo (catalog: VEC-PBS -0006; OligoEngine, Seattle, WA, USA) at the *Bgl*II and *Hin*dIII cloning sites. The H1-RNA promoter was cloned in front of the gene-specific targeting sequence and five thymidines (T5) as a termination signal, as it produces a small RNA transcript, as reported previously.[20] A neomycin resistance cassette was cloned into the pSUPER vector to select for stable transfectants. The vectors were transfected into RD cells using Effectent Transfection Reagent (Qiagen, Hilden, Germany). Cells were selected with neomycin to establish a stable cell line for TGF-β1-siRNA expression.

Western blotting. A total of 3×10^5 cells were plated in 60-mm dishes and allowed to adhere for 24 h. On day 6 after selecting the stable cell line for TGF-β1-siRNA expression with neomycin, cells were harvested and lysed in TNES buffer. Equal amounts of total protein were electrophoresed on 8% SDS polyacrylamide gels and electrotransferred onto polyvinyl dine difluoride membranes. The membranes were blocked with 1× TBS that contained 5% nonfat powdered milk (w/v), 0.02% sodium azide, and 0.02% Tween-20, and were then incubated with anti-TGF-β1 monoclonal antibody (Table 1), diluted at 1:1000. After washing, primary antibodies were detected with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted at 1:2000, using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology). Equality of sample loading was demonstrated by anti-β-actin immunoblotting (Santa Cruz Biotechnology).

Enzyme-linked immunosorbent assay (ELISA). Six days after selecting the stable cell line for TGF-β1-siRNA expression with neomycin, the selected RD cells were plated in 96-well plates at 4×10^4 cells per well for 6 days, and some untreated RD cells acted as a control. TGF-β1 protein levels in culture medium were determined by ELISA (Jingmei Company, Guangzhou, China) according to the manufacturer's instructions. TGF-β1 antibody was coated onto a 96-well plate. Biotinylated secondary antibody and streptavidin–horseradish peroxidase (HRP) were used for detection of binding of TGF-β1. Absorbance was determined at 570 nm with an ELISA reader (Model 550; Bio-Rad, Hercules, CA, USA).

Immunofluorescence staining. After cultivating on coverslips for 24 h in DMEM that contained 10% FCS, RD cells were treated with TGF-β1 shRNA. One, 2, 4, and 6 days after transient transfection with TGF-β1 shRNA in DMEM that contained 1% FCS, caspase-3-positive cells were analyzed with immunofluorescence staining. Briefly, the coverslips were washed with iced PBS and fixed with 4% paraformaldehyde for 15 min at 4°C. After washing with PBS, the cells were blocked with 5% goat serum in PBS that contained 1% BSA at 37°C for 30 min. The cells were incubated at 4°C overnight with antibodies (Table 1) in PBS that contained 1% BSA. After the cells were washed with PBS, a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated IgG was applied as the secondary antibody for 1 h at room temperature. The slides were washed, mounted, and photographed under a fluorescence microscope (BX2-FLB3-000; Olympus, Tokyo, Japan). At least 200 cells/slide in random fields were counted with a ×20 objective to determine the percentage of FITC-positive cells. Each experiment was repeated at least three times.

Cell growth assays. RD cells were plated at 4×10^4 cells per well in 96-well plates in the absence or presence of TGF-β1-shRNA-pSUPER for 1, 2, 4 and 6 days. During the last 4 h of each treatment, cells were pulsed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) 10 μL/well or with 3.7×10^4 [^{3}H]thymidine (1 μCi)/well. Subsequently, cells were incubated at 37°C for 4 h to allow MTT formazan formation. The medium and MTT were replaced by 100 μL DMSO to dissolve the formazan crystals. After 30 min, the optical density (OD) at 557 nm was determined using a microplate reader (Model 550; Bio-Rad). For [^{3}H]thymidine incorporation assay, after cells were pulsed

© 2010 Japanese Cancer Association

Table 2. Primer sequences from Cui Chang-Hao [21]

Targets	Sense primer 5′–3′	Antisense primer 5′–3′	Annealing temperature (°C)	Product size (bp)
Myogenin	GCCACAGATGCCACTACTTC	CAACTTCAGCACAGGAGACC	62	519
Myosin	CAGTAGCCCCATCACATTTG	ATAACGCAATGGACAAGTGG	65	566
Desmin	CCTACTCTGCCCTCAACTTC	AGTATCCCAACACCCTGCTC	62	565
GAPDH	CCACCCATGGCAAATTCCAT	TCAAGACGGCAGGTCAGGTC	70	597

with [^{3}H]thymidine, the cells were rinsed with PBS and DNA were precipitated with 10% (wt/vol) trichloroacetic acid for 30 min at 4°C. The amount of [^{3}H]thymidine incorporated was measured with a liquid scintillation counter. The mean values from triplicate wells for each treatment were determined.

RNA isolation and RT-PCR. After 1 week of maintaining the stably transfected cell line for TGF-β1-siRNA expression with differentiation medium (described above), total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RT-PCR of myogenin, myosin, desmin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed with 2 μg total RNA. RNA for RT-PCR was converted to cDNA with a first-stand cDNA synthesis kit (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA), according to the manufacturer's recommendations. The sequences of PCR primers that amplify human but not mouse genes are listed in Table 2 (taken from reference[21]). PCR was performed with Takara recombinant Taq (Takara Shuzo, Kyoto, Japan) for 30 cycles, with each cycle consisting of 94°C for 30 s, 62 or 65°C for 30 s, and 72°C for 20 s, with an additional 10-min incubation at 72°C after completion of the last cycle. The PCR products were electrophoresed on 1.8% agarose gel and stained with ethidium bromide for visualization.

Electron microscopy. After the stably transfected cell line was maintained in differentiation medium for 6 days, the cells were harvested, fixed with 3% glutaraldehyde and osmium tetroxide, dehydrated, and embedded in Epon812. Thin sections were cut at a thickness of 50 nm, stained with uranyl acetate and lead citrate, and examined under an H-600IV electron microscope (Hitachi, Tokyo, Japan).

Xenografts of RD cells in nude mice. The experimental protocol was approved by the China Institutional Ethics Review Committee for Animal Experimentation. A total of 30 male BALB/c nude mice (5–6 weeks old and 18 g in weight) were purchased form SLAC Laboratory Animal (Shanghai, China). The mice were divided into three groups of 10 mice each. All 30 mice were injected subcutaneously in the right flank with 100 μL RD cell suspension (1×10^7). Once tumors reached approximately 0.5 cm^3, animals in the treated group were injected intratumorally with 200 μL TGF-β1-shRNA-pSUPER and those in the control group with 200 μL pSUPER, once weekly for 3 weeks. Tumor size was measured blindly twice weekly with calipers, and tumor volume was calculated. One week after the end of treatment, the tumors were harvested. TGF-β1, Ki-67, and TUNEL staining were examined. Histological differentiation was evaluated by observing changes in morphology and expression of myogenic differentiation markers.

Apoptosis assay. To determine whether the change in proliferation was a consequence of RD cell apoptosis, TUNEL staining was examined *in vivo*. The tissue sections were prepared as described above. Briefly, the treated cells were permeabilized with 0.1% Triton X-100 for FITC end-labeling of the fragmented DNA of the apoptotic RD cells, using a TUNEL cell apoptosis detection kit (Beyotime, Shanghai, China). Primary antibody diluted in PBS/BSA was incubated for 1 h at 37°C, and then washed in PBS, followed by 30 min incubation with FITC-conjugated antirabbit antibody. The slides were then washed, air dried, mounted, and photographed under fluorescence microscopy as mentioned above.

Statistical analysis. All data were presented as mean and standard deviation. Analysis was carried out using SPSS 10.0 (Chicago, IL, USA). Differences between the control samples and TGF-β1 shRNA-treated samples were compared using a paired-samples *t*-test, and *P*-values reported were two sided. $P < 0.05$ was considered statistically significant.

Results

Expression of TGF-β1, type II TGF-β receptor (TβRII), and Smad4 in RMS. Moderate and strong immunoreactivity for TGF-β1,

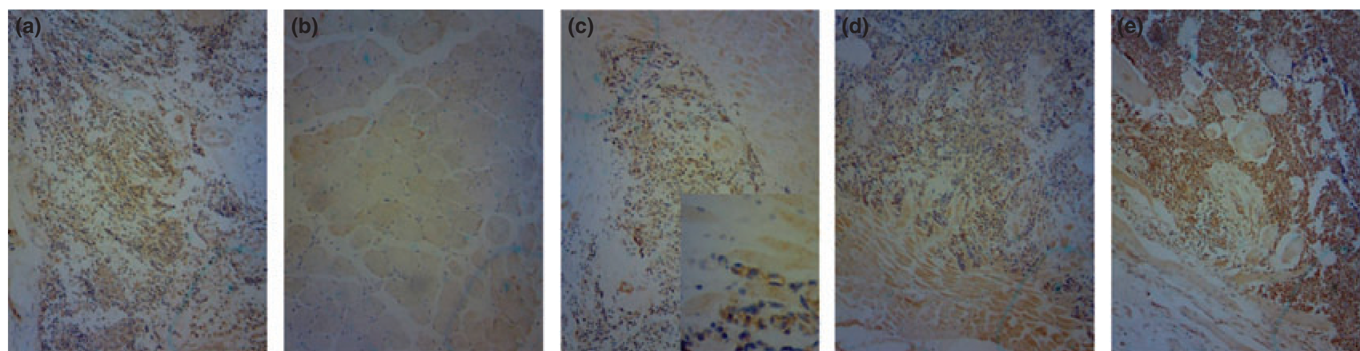

Fig. 1. Immunodetection of transforming growth factor-β1 (TGF-β1) (a–c), TGF-β1 type II receptor (TβRII), (d) and Smad4 (e) in rhabdomyosarcomas. (a) TGF-β1 labeling of rhabdomyosarcoma. Original magnification, ×100. (b) Reduced expression of TGF-β1 in normal skeletal muscle cells. Original magnification, ×100. (c) Distribution of TGF-β1 protein is shown in rhabdomyosarcoma cells and adjacent normal skeletal muscle tissue. Inset: there was higher expression of TGF-β1 in rhabdomyosarcoma tissue than that in normal skeletal muscle tissue. Original magnification, ×100; inset, ×400. (d) TβRII was observed in the rhabdomyosarcoma cells as well as normal skeletal muscle tissue. Original magnification, ×100. (e) Smad4 was observed in rhabdomyosarcoma cells and also found occasionally in adjacent normal skeletal muscle cells. Original magnification, ×100.

doi: 10.1111/j.1349-7006.2010.01512.x
© 2010 Japanese Cancer Association

TβRII, and Smad4 were observed in 70.6% (48/68), 55.9% (38/68), and 73.5% (50/68) of RMS samples (Fig. 1). TGF-β1 and Smad4 in the RMS was stained more intensely than that in normal skeletal muscles ($P < 0.01$). However, no significant difference was found in TβRII expression between RMS and normal skeletal muscles ($P > 0.05$) (Table 3). In addition, there was a significant relationship between TGF-β1 expression and tumor differentiation and histological grade ($P < 0.001$). However, there was no significant difference in TGF-β1 expression in RMS with relapse or metastasis (21/28, 75%) *versus* that without relapse or metastasis (27/40, 67.5%) ($P > 0.05$). There was no significant relationship between TβRII and Smad4 expression and histological grade ($P > 0.05$) (Table 4).

Suppression of TGF-β1 expression by RNAi. The transfection efficiency of the recombinant pSUPER vector system that contained TGF-β1 shRNA is shown in Figure 2. The pSUPER vector system directs the synthesis of shRNA, which is processed to form small-interfering RNA (siRNA), and persistently suppresses gene expression in mammalian cells.[22] We used the pSUPER vector system to express shRNA against TGF-β1. Green fluorescence protein (GFP) was incorporated as a reporter gene. Both pSUPER gfp-neo-control and pSUPER gfp-neo-TGF-β1 shRNA were transfected into RD cells. Twenty-four hours after transfection, the expression of GFP in cells was checked by fluorescence microscopy, and no difference was found between these two groups (Fig. 2a). Western blotting was performed to verify suppression of TGF-β1 in RD cells. As shown in Figure 2(b), TGF-β1 protein expression was detected after treatment of control pSUPER vector (lane 1). Conversely, TGF-β1 protein expression was virtually eliminated from RD cells that were stably transfected, as indicated in lane 2 (*$P < 0.05$). To explore whether active TGF-β1 proteins were secreted into the medium, ELISA was used to determine TGF-β1 proteins in the culture medium. As showed in Figure 2(c), there was a significant decrease in the level of TGF-β1 proteins in the shRNA-treated groups (*$P < 0.05$).

Changes in TGF-β1-shRNA-induced RD growth arrest. To investigate the effect of TGF-β1 shRNA on the growth of RD cells *in vitro*, MTT and [^{3}H]thymidine incorporation assays were used. As shown in Figure 3(a), the proliferation rate of RD cells stably expressing TGF-β1 shRNA was reduced, as demonstrated by a time-dependent decrease in OD. Similar results were found for [^{3}H]thymidine incorporation (Fig. 3b). Significant differences in viability were observed after 4 ($P < 0.05$) and 6 ($P < 0.01$) days.

To explore the effect of TGF-β1 shRNA on cell growth *in vivo*, tumorigenicity was quantified. All nude mice developed detectable tumors after tumor cell inoculation. On day 14 after intratumoral injection of TGF-β1-shRNA-pSUPER, tumors were dissected and weighed (mean ± SD, $n = 10$). There was a significant difference between group I (RD/TGF-β1shRNA-control) and group II (RD/TGF-β1shRNA) ($P < 0.05$) (Fig. 3d, asterisk). These findings suggested that TGF-β1 shRNA inhibited proliferation of RD cells, which was further confirmed by Ki-67 expression (Fig. 3e,f). Immunohistochemistry demonstrated that TGF-β1 shRNA induced RD growth arrest.

To determine whether growth arrest was a consequence of RD cell apoptosis, caspase-3 (*in vitro*) and TUNEL (*in vivo*) were examined. As shown in Figure 3(c,f), the number of caspase-3-positive cells increased significantly after 4 days of TGF-β1 shRNA treatment but not in the control cells ($P < 0.05$). Immunohistochemical staining with TUNEL in subcutaneous RMS tissue showed that TGF-β1 shRNA induced an increase in the percentage of TUNEL-positive cells ($P < 0.05$). These results indicated that knockdown of TGF-β1 in RD cells arrested their growth by inducing apoptosis.

Effects of TGF-β1 shRNA on RD cell differentiation. To examine the role of pSUPER-TGF-β1 shRNA on differentiation of RD cells, RT-PCR and immunohistochemistry were used to explore the expression of myogenin, myosin, and desmin. As shown in Figure 4(a), there was a significant difference ($P < 0.01$) in the ratio of myogenin mRNA/GAPDH between the treated (87%) and the control (33%) groups, but there was no significant difference in expression of myosin or desmin mRNA/GAPDH. In a parallel experiment, we investigated the effects of pSUPER-TGF-β1 shRNA on RD cell morphology. A proportion of RD cells changed to a round shape from the normal fusiform or polygonal shape, although no cell detachment

Table 3. Summary of IHC mean intensity scores for TGF-β1, TβRII, and Smad4 protein in RMS and normal tissue

	TGF-β1		*P*-value	TβRII		*P*-value	Smad4		*P*-value
	Low (0–4)	High (6–9)		Low (0–4)	High (6–9)		Low (0–4)	High (6–9)	
RMS	20 (29.4)	48 (70.6)	<0.001*	30 (41.1)	38 (55.9)	>0.05	18 (26.5)	50 (73.5)	<0.001*
Normal	15 (68.2)	7 (31.8)		12 (54.5)	10 (45.5)		14 (63.6)	8 (36.4)	

**P*-value is significant. IHC, immunohistochemistry; RMS, rhabdomyosarcoma; TGF-β1, transforming growth factor-β1; TβRII, type II TGF-β receptor.

Table 4. Relationship between the expression of TGF-β1, TβRII, Smad4 protein, and clinicopathologic parameters

	TGF-β1		*P*-values	TβRII		*P*-values	Smad4		*P*-values
	Low (0–4)	High (6–9)		Low (0–4)	High (6–9)		Low (0–4)	High (6–9)	
Histological grade									
I ($n = 25$)	15 (60)	10 (40)	<0.001*	11 (46)	14 (56)	>0.05	7 (38)	18 (72)	>0.05
II ($n = 22$)	4 (18.2)	18 (81.8)		10 (45.5)	12 (54.5)		6 (27.3)	16 (72.7)	
III ($n = 21$)	1 (4.8)	20 (95.2)		9 (42.9)	12 (57.1)		5 (23.8)	16 (76.2)	
Relapse or metastasis of RMS									
With 28	7 (25)	21 (75)	>0.05	12 (42.9)	16 (57.1)	>0.05	6 (21.4)	22 (78.6)	>0.05
Without 40	13 (32.5)	27 (67.5)		18 (45)	22 (55)		12 (30)	28 (70)	

**P*-value is significant. IHC, immunohistochemistry; RMS, rhabdomyosarcoma; TGF-β1, transforming growth factor-β1; TβRII, type II TGF-β receptor.

© 2010 Japanese Cancer Association

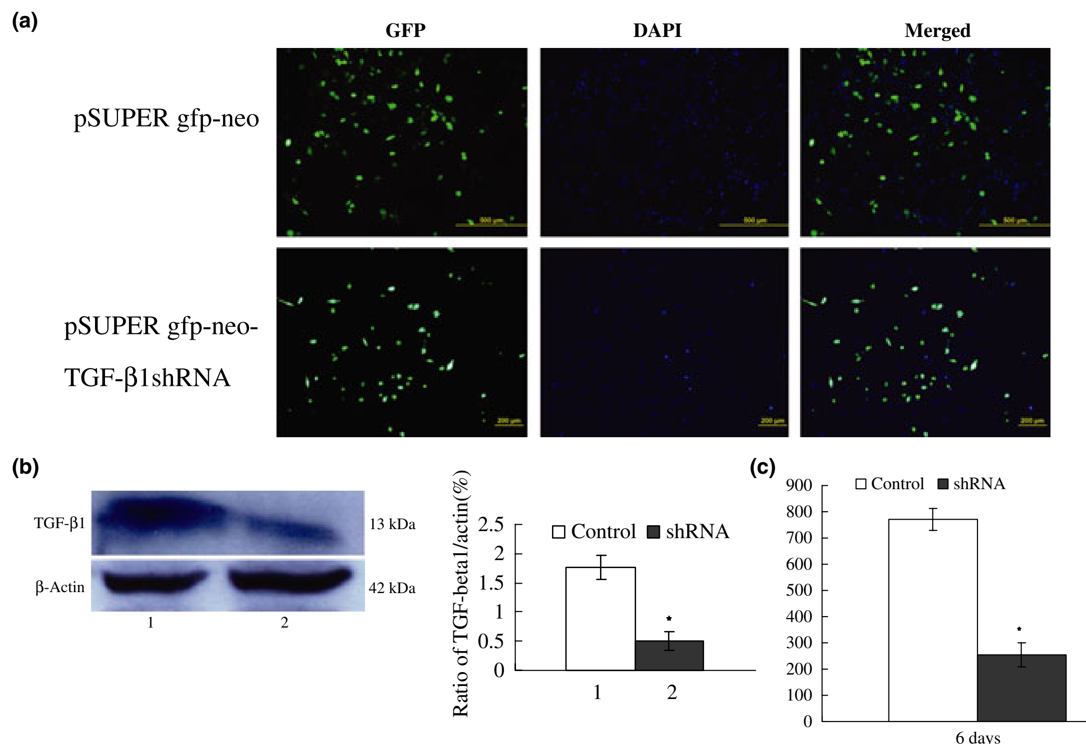

Fig. 2. Transfection of recombinant pSUPER vector system. (a) Fluorescence microscope images of RD cells 24 h after transfection with pSUPER gfp-neo or pSUPER gfp-neo- TGF-β1shRNA. Left row, green filter signals; middle row, DAPI staining for nuclei; and right row, merged. (b) Expression of transforming growth factor-β1 (TGF-β1) protein was explored with western blot analysis. After 6 days, cells that were transfected with control pSUPER vector expressed TGF-β1 protein (lane 1), whereas, cells that were stably transfected showed down-regulation of TGF-β1 expression (lane 2). (*$P < 0.05$). (c) ELISA was used to examine TGF-β1 protein in the culture medium. A decrease in TGF-β1 protein was observed in the shRNA-treated groups (*$P < 0.05$).

was observed, even after 2 weeks of treatment. This suggested growth arrest, which was further confirmed by the up-regulation of myofilaments (Fig. 4b). Immunohistochemical staining for myogenin, myosin, and desmin in subcutaneous RMS tissue showed that TGF-β1 shRNA induced an increase in staining for myogenin ($P < 0.01$) and myosin ($P < 0.05$) (Fig. 4c,d).

Discussion

Our previous studies have demonstrated that RD cells overexpress TGF-β1 and its receptors and downstream molecules, which was confirmed by the present study of RMS tumor tissue (Table 3). This is contrary to other studies, in which only 5% of colon carcinomas showed expression of TβRII and loss of nuclear expression of Smad4 in tumors with lymph node metastasis,[23] suggesting that the loss of function in the TGF-β1/Smad signaling pathway contributes to the development of carcinoma.[1,3] The present results suggest that the intact TGF-β1/Smad pathway in RMS cells performs a different biological function from that in other neoplasms, such as colon cancer, that originate from epithelia cells. Moreover, a significant relationship was found between TGF-β1 expression and tumor histological grade, according to differentiation,[15] and this may provide research opportunities for novel interventions of inducing differentiation in patients with RMS.

Previous studies have demonstrated that high levels of exogenous TGF-β1 inhibit growth of RD cells *in vitro*.[24] However, under physiological conditions, the autocrine function of TGF-β1 may not be concentration dependent. Another of our previous studies has shown that low levels of exogenous TGF-β1 can promote RD cell growth and antagonize the differentiation-inducing effect of 9-cis retinoic acid (9CRA).[25] Since these previous studies involved exogenous addition of different concentrations of TGF-β1, which may not reflect normal physiological conditions, whether or not endogenous TGF-β1 has a regulatory effect on RD cell growth and differentiation needs confirmation.

Currently, a significant research effort is being focused on the development of therapeutic approaches against cancer by targeting the TGF-β1 pathway.[26] Brain tumors are currently the major targets of anti-TGF-β therapy that utilizes antisense DNA or RNAi technology.[27,28] In the present study, we chose the pSUPER vector system, and the TGF-β1-specific parts of the sequence as the optimal site for TGF-β1 gene silencing, as described previously.[19] As shown in Figure 2, delivery of shRNA produces efficient TGF-β1 knockdown in RMS cells and may be a method of choice for shRNA delivery for gene function studies.

Treatment with TGF-β1 shRNA induced growth arrest of RD cells, as shown by MTT assay after 4 days, and confirmed by [3H]thymidine incorporation assay. To determine whether this phenomenon was a consequence of growth inhibition or cell apoptosis, expression of caspase-3, and TUNEL and Ki-67 assays were used. The results showed that TGF-β1 acted as a cell viability factor in RD cells via inhibition of apoptotic cell death. To study the tumorigenicity of TGF-β1, we and others have inhibited TGF-β1 expression with antisense technology, and have evaluated the consequences for tumor cell growth *in vitro* and tumor formation *in vivo*.[29,30] Consequent alterations in tumor growth have suggested that TGF-β1 functions as a growth regulator in a positive or negative way, depending on the study and model system used. In the present study, the significance of the biological effects of RD/TGF-β1 shRNA was corroborated by the observation of a reduction of tumorigenicity *in vivo* (Fig. 3). Thus, overexpression of TGF-β1 in RD

doi: 10.1111/j.1349-7006.2010.01512.x
© 2010 Japanese Cancer Association

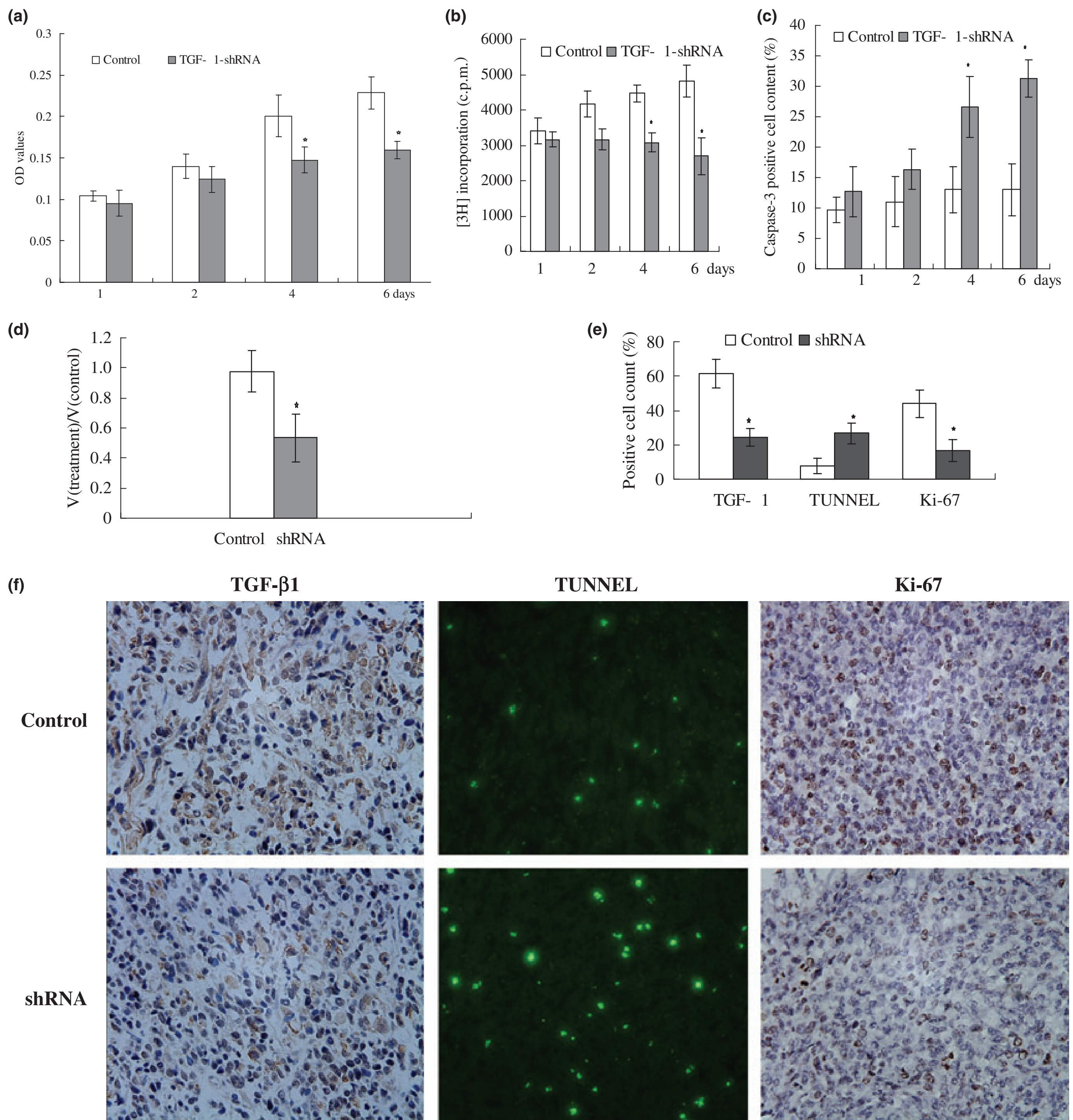

Fig. 3. The effect of transforming growth factor-β1 (TGF-β1) shRNA on growth of cells. Cells were seeded and treated with or without TGF-β1 shRNA. Cell growth was quantified by MTT assay (a), [3H]thymidine incorporation assay (b), and number of caspase-3-positive cells (c) *in vitro* with the transient transfection system, and measurement of tumor volume (d), TGF-β1, TUNEL, and Ki-67 expression *in vivo* (e,f) with intratumoral injection of TGF-β1-shRNA-pSUPER. Proliferation of rhabdomyosarcoma (RD) cells treated with TGF-β1 shRNA was reduced. To determine whether the change of viability was a consequence of RD cells apoptosis, TUNEL and caspase-3 was explored using immunofluorescence staining (c,e,f), and showed that injection of TGF-β1 shRNA actually inhibited the expression of TGF-β1 and induced RD cell apoptosis. Each experiment was performed three times. Photographs of TGF-β1 (×400), TUNEL (×400), and Ki-67 (×400).

cells conveyed an advantage presumably related to increased cell–matrix interactions and adhesiveness, and to a paracrine effect of TGF-β1 on the surrounding tissue that led to a more hospitable environment.

It is well known that the biological behavior of a tumor is related to the degree of differentiation of its cells, such that, usually the lower the degree of differentiation, the greater the aggressiveness expected from the tumor. Therefore, the present

© 2010 Japanese Cancer Association

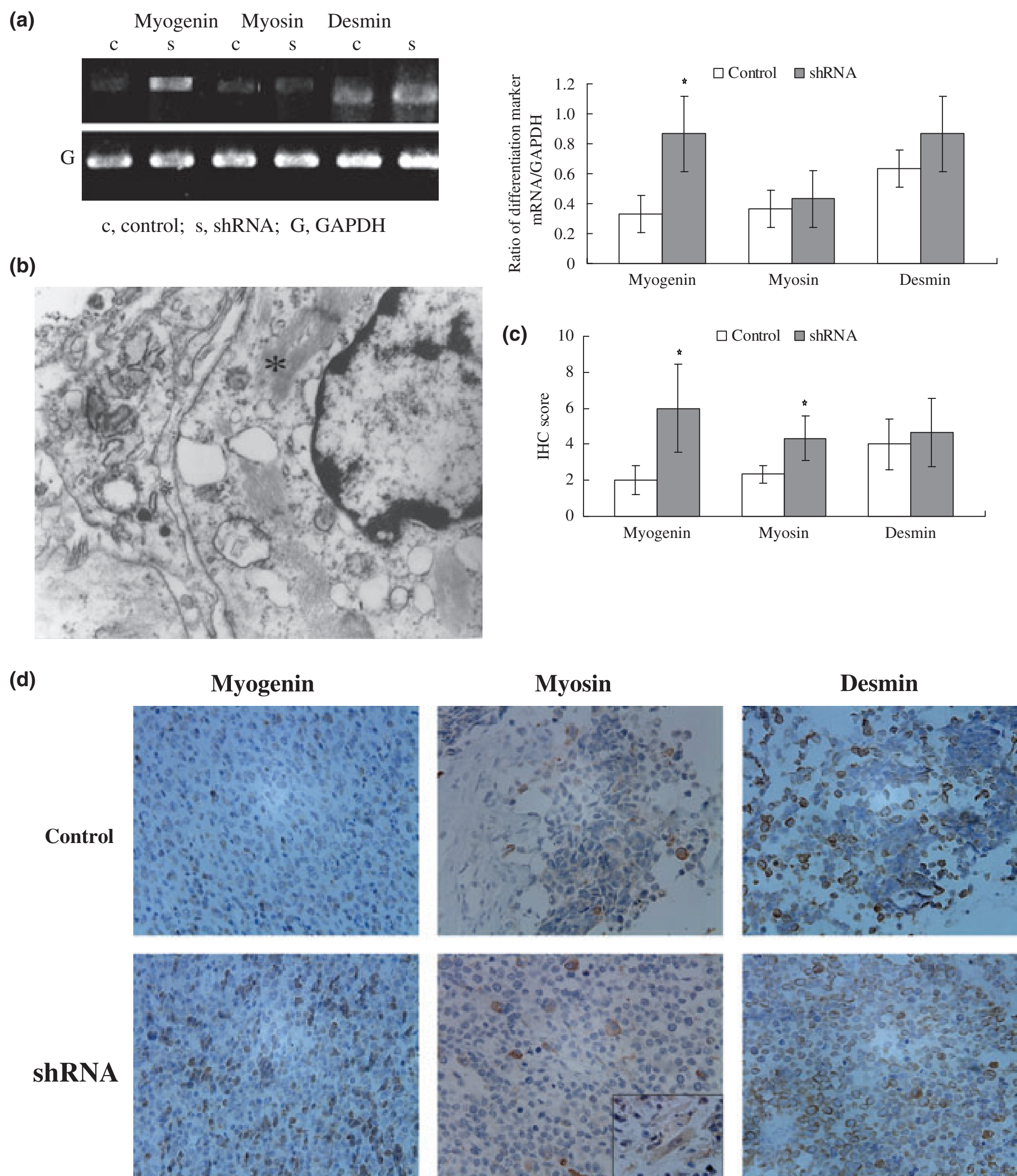

Fig. 4. Myogenic differentiation induced by transforming growth factor-β1 (TGF-β1) shRNA treatment in rhabdomyosarcoma (RD) cells. RT-PCR (a) and electron microscopy (b) were used to explore differentiation markers in RD cells with stable transfection system. Immunohistochemistry analysis (c,d, method *in vivo*) were used to explore differentiation markers in RD cells with intratumoral injection of TGF-β1-shRNA-pSUPER. There was a significant difference ($P < 0.01$) in the ratio of myogenin mRNA/GAPDH between the treated and control groups. More myofilaments were observed in RD cells treated with the stable transfection system than in the control group (b, increased myofilament marked with *; electron microscopy (EM), ×5000). Subcutaneous rhabdomyosarcoma (RMS) tissue showed that injection of TGF-β1-shRNA-pSUPER actually induced an increase in immunohistochemical staining for myogenin ($P < 0.01$) and myosin ($P < 0.05$) (c,d, ×400).

study investigated the effect of TGF-β1 shRNA on RD cells to explore the combination of differentiation-inducing treatment and surgery for preventing the development of the primary tumor and potential metastases. RD cells retain the rhabdomyoblast phenotype and undergo very limited myogenic differentiation, even though they express muscle regulatory factors (MRFs) MyoD and myogenin.[31] Our previous study has demonstrated that low concentrations of TGF-β1 can antagonize the differentiation-inducing effect of 9CRA.[25] Another study has also shown that TGF-β1 negatively regulates myogenic differentiation of muscle and RMS cells,[11] but little is known about the molecular mechanisms involved.

The MyoD family of myogenic regulators includes MyoD, myogenin, myf5, and MRF4/herulin/myf6, each of which can activate myogenesis when expressed in a variety of non-muscle cell types.[32] However, they show only limited expression of genes associated with terminal differentiation in RMS cells.[33] Among the myogenic regulators, myogenin is the most sensitive to repression by growth factor signals. This is in contrast to myf5 and MyoD, which are expressed in proliferating myoblasts prior to differentiation, and myogenin is not unregulated until cell proliferation has ceased.[34] In the present study, TGF-β1 shRNA induced expression of myogenin, which was increased at the mRNA and protein level, and induced myogenic differentiation, which was characterized by a marked increase in myosin expression. This led us to explore the possibility that changes in the amount and/or activity of myogenic regulators can determine RMS cell differentiation. These results suggest strongly

doi: 10.1111/j.1349-7006.2010.01512.x
© 2010 Japanese Cancer Association

that the TGF-β1 differentiation inhibitory effect depends on the level of muscle-specific gene activation in RMS. Myogenin, which functions within the regulatory pathway that leads to myogenesis, appears to serve as a target for negative regulation of myogenesis by TGF-β1 signals that control all downstream events within a differentiation regulatory cascade.

Some studies have demonstrated different mechanisms of differentiation in RMS. It has been shown that treatment of RD cells with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibits the activation of secreted latent TGF-β1, and induces growth arrest and myogenic differentiation in these cells.[11,35] Alterations in certain signaling pathways, for example IGFs and myostatin, exert similar differentiation effects on RMS cells.[36,37] Recent studies have shown that one intracellular signaling pathway activated during the differentiation of myogenic cell lines is p38 mitogen-activated protein kinase (MAPK), which has been shown to be necessary for the expression of several muscle structural genes.[38,39] Theses findings highlight the complexity of tumorigenicity in RMS. Our previous studies have shown that signaling of TGF-β1 from the cell surface to the nucleus can be directed through the MAPK (ERK) as well as the TGF-β1/Smad signaling pathway,[40] and that RNAi-mediated Smad4 gene silencing induces growth inhibition of RD cells.[13] Therefore, we can surmise that the differentiation inhibitory effect of TGF-β1 in RMS cells is related to modulation of the cellular response by the MAPK pathway.

In conclusion, we have shown that inhibition of TGF-β1 expression by shRNA-expressing vectors can reverse successfully the malignant behavior of RMS RD cells, by inhibiting growth and inducing differentiation. This suggests that RMS cells, which are derived from myogenic progenitors, are committed to a myogenic fate, but their differentiation is arrested by autocrine TGF-β1. This implies that TGF-β1 is a novel target for drugs that affect differentiation of RMS.

Acknowledgments

We would like to thank Professors G.H. Yang and H. Bu for their support of this work. We also thank the Department of Pathology, West China Hospital of Sichuan University, for providing us with the tumor specimens. We are equally indebted to Drs H. Wang and H. Guo for their useful preparatory work.

Disclosure Statement

The authors have no conflict of interest.

References

1 Leivonen SK, Kahari VM. Transforming growth factor-beta signaling in cancer invasion and metastasis. *Int J Cancer* 2007; **121**: 2119–24.
2 Stover DG, Bierie B, Moses HL. A delicate balance: TGF-beta and the tumor microenvironment. *J Cell Biochem* 2007; **101**: 851–61.
3 Levy L, Hill CS. Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev* 2006; **17**: 41–58.
4 Moses HL, Yang EY, Pietenpol JA. TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell* 1990; **63**: 245–7.
5 Liu D, Black BL, Derynck R. TGF-beta inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3. *Genes Dev* 2001; **15**: 2950–66.
6 Dias P, Parham DM, Shapiro DN, Webber BL, Houghton PJ. Myogenic regulatory protein (MyoD1) expression in childhood solid tumors: diagnostic utility in rhabdomyosarcoma. *Am J Pathol* 1990; **137**: 1283–91.
7 Parham DM. The molecular biology of childhood rhabdomyosarcoma. *Semin Diagn Pathol* 1994; **11**: 39–46.
8 El-Badry OM, Minniti C, Kohn EC, Houghton PJ, Daughaday WH, Helman LJ. Insulin-like growth factor II acts as an autocrine growth and motility factor in human rhabdomyosarcoma tumors. *Cell Growth Differ* 1990; **1**: 325–31.
9 Schweigerer L, Neufeld G, Mergia A, Abraham JA, Fiddes JC, Gospodarowicz D. Basic fibroblast growth factor in human rhabdomyosarcoma cells: implications for the proliferation and neovascularization of myoblast-derived tumors. *Proc Natl Acad Sci U S A* 1987; **84**: 842–6.
10 De Giovanni C, Landuzzi L, Frabetti F *et al.* Antisense epidermal growth factor receptor transfection impairs the proliferative ability of human rhabdomyosarcoma cells. *Cancer Res* 1996; **56**: 3898–901.
11 Bouche M, Canipari R, Melchionna R, Willems D, Senni MI, Molinaro M. TGF-beta autocrine loop regulates cell growth and myogenic differentiation in human rhabdomyosarcoma cells. *FASEB J* 2000; **14**: 1147–58.
12 Wang H, Yang GH, Bu H *et al.* Systematic analysis of the TGF-beta/Smad signalling pathway in the rhabdomyosarcoma cell line RD. *Int J Exp Pathol* 2003; **84**: 153–63.
13 Ye L, Zhang H, Zhang L *et al.* Effects of RNAi-mediated Smad4 silencing on growth and apoptosis of human rhabdomyosarcoma cells. *Int J Oncol* 2006; **29**: 1149–57.
14 Olson EN. Interplay between proliferation and differentiation within the myogenic lineage. *Dev Biol* 1992; **154**: 261–72.
15 Molenaar WM, Oosterhuis JW, Kamps WA. Cytologic ''differentiation'' in childhood rhabdomyosarcomas following polychemotherapy. *Hum Pathol* 1984; **15**: 973–9.
16 McAllister RM, Melnyk J, Finkelstein JZ, Adams EC Jr, Gardner MB. Cultivation in vitro of cells derived from a human rhabdomyosarcoma. *Cancer* 1969; **24**: 520–6.
17 Segev H, Fishman B, Ziskind A, Shulman M, Itskovitz-Eldor J. Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells* 2004; **22**: 265–74.
18 Yang Z, MacQuarrie KL, Analau E *et al.* MyoD and E-protein heterodimers switch rhabdomyosarcoma cells from an arrested myoblast phase to a differentiated state. *Genes Dev* 2009; **23**: 694–707.
19 Wang SL, Yao HH, Guo LL *et al.* Selection of optimal sites for TGFB1 gene silencing by chitosan-TPP nanoparticle-mediated delivery of shRNA. *Cancer Genet Cytogenet* 2009; **190**: 8–14.
20 Baer M, Nilsen TW, Costigan C, Altman S. Structure and transcription of a human gene for H1 RNA, the RNA component of human RNase P. *Nucleic Acids Res* 1990; **18**: 97–103.
21 Cui CH, Uyama T, Miyado K *et al.* Menstrual blood-derived cells confer human dystrophin expression in the murine model of Duchenne muscular dystrophy via cell fusion and myogenic transdifferentiation. *Mol Biol Cell* 2007; **18**: 1586–94.
22 Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002; **296**: 550–3.
23 Bacman D, Merkel S, Croner R, Papadopoulos T, Brueckl W, Dimmler A. TGF-beta receptor 2 downregulation in tumour-associated stroma worsens prognosis and high-grade tumours show more tumour-associated macrophages and lower TGF-beta1 expression in colon carcinoma: a retrospective study. *BMC Cancer* 2007; **7**: 10.
24 Ye L, Zhang HY, Wang H *et al.* Effects of transforming growth factor beta 1 on the growth of rhabdomyosarcoma cell line RD. *Chin Med J (Engl)* 2005; **118**: 678–86.
25 Wang SL, Yao HH, Qin ZH *et al.* Inhibition of differentiation by transforming growthFactor ß1 in rhabdomyosarcoma cells. *Chin J Clin Oncol* 2007; **4**: 327–32.
26 Biswas S, Criswell TL, Wang SE, Arteaga CL. Inhibition of transforming growth factor-beta signaling in human cancer: targeting a tumor suppressor network as a therapeutic strategy. *Clin Cancer Res* 2006; **12**: 4142–6.
27 Friese MA, Wischhusen J, Wick W *et al.* RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity in vivo. *Cancer Res* 2004; **64**: 7596–603.
28 Schlingensiepen KH, Schlingensiepen R, Steinbrecher A *et al.* Targeted tumor therapy with the TGF-beta2 antisense compound AP 12009. *Cytokine Growth Factor Rev* 2006; **17**: 129–39.
29 Wang SL, Yao HH, Guo LL *et al.* Antisenes oligonucleotide targeting TGF-β1 abrogates tumorigenicity of rhabdomyosarcoma in vivo. *Chin J Clin Oncol* 2008; **5**: 258–62.
30 Wu SP, Theodorescu D, Kerbel RS *et al.* TGF-beta 1 is an autocrine-negative growth regulator of human colon carcinoma FET cells in vivo as revealed by transfection of an antisense expression vector. *J Cell Biol* 1992; **116**: 187–96.
31 Bouche M, Senni MI, Grossi AM *et al.* TPA-induced differentiation of human rhabdomyosarcoma cells: expression of the myogenic regulatory factors. *Exp Cell Res* 1993; **208**: 209–17.
32 Braun T, Buschhausen-Denker G, Bober E, Tannich E, Arnold HH. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J* 1989; **8**: 701–9.
33 Malone M. Soft tissue tumours in childhood. *Histopathology* 1993; **23**: 203–16.

© 2010 Japanese Cancer Association

34 Wright WE, Sassoon DA, Lin VK. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* 1989; **56**: 607–17.

35 Andreasen PA, Georg B, Lund LR, Riccio A, Stacey SN. Plasminogen activator inhibitors: hormonally regulated serpins. *Mol Cell Endocrinol* 1990; **68**: 1–19.

36 Ricaud S, Vernus B, Duclos M *et al.* Inhibition of autocrine secretion of myostatin enhances terminal differentiation in human rhabdomyosarcoma cells. *Oncogene* 2003; **22**: 8221–32.

37 Xu Q, Wu Z. The insulin-like growth factor-phosphatidylinositol 3-kinase-Akt signaling pathway regulates myogenin expression in normal myogenic cells but not in rhabdomyosarcoma-derived RD cells. *J Biol Chem* 2000; **275**: 36750–7.

38 Keren A, Tamir Y, Bengal E. The p38 MAPK signaling pathway: a major regulator of skeletal muscle development. *Mol Cell Endocrinol* 2006; **252**: 224–30.

39 Perdiguero E, Ruiz-Bonilla V, Serrano AL, Munoz-Canoves P. Genetic deficiency of p38alpha reveals its critical role in myoblast cell cycle exit: the p38alpha-JNK connection. *Cell Cycle* 2007; **6**: 1298–303.

40 Guo H, Zhang HY, Wang SL, Ye L, Yang GH, Bu H. Smad4 and ERK2 stimulated by transforming growth factor beta1 in rhabdomyosarcoma. *Chin Med J (Engl)* 2007; **120**: 515–21.

doi: 10.1111/j.1349-7006.2010.01512.x
© 2010 Japanese Cancer Association