J Physiol Sci (2010) 60:35–41
DOI 10.1007/s12576-009-0063-5

ORIGINAL PAPER

Endogenous transforming growth factor (TGF) beta1 promotes differentiation of smooth muscle cells from embryonic stem cells: stable plasmid-based siRNA silencing of TGF beta1 gene expression

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Received: 25 March 2009 / Accepted: 9 September 2009 / Published online: 29 September 2009

Abstract Transforming growth factor (TGF) beta1 has been shown to promote differentiation of smooth muscle cells (SMC) from some precursor cells. Whether endogenous TGF beta1 also contributes to SMC differentiation during embryogenesis, however, remains unclear. In this study, a plasmid-based TGF beta1 RNA interference embryonic stem (ES) cell line was constructed. Morphological observation showed that TGF beta1 knockdown significantly prevented differentiated cells from outgrowing from ES cells-derived embryoid bodies (EBs). Immunofluorescence staining indicated that SM alpha-actin-positive cells were confluent and dense in the control group but dispersed in the TGF beta1 knockdown group. RT-PCR and western blot suggested that TGF beta1 knockdown resulted in a decrease in the expression of early SMC markers SM alpha-actin and myocardin in EBs. Both the retarded extension of cell outgrowth and the decrease in SM alpha-actin and myocardin expression could not be rescued by addition of exogenous TGF beta1. These data suggest that endogenous TGF beta1 promotes differentiation of SMC from ES cells.

Keywords Cell differentiation · Embryonic stem cells · Small interfering RNA · Smooth muscle cells · Transforming growth factor beta1

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Introduction

Blood vessels constitute the first organ in the embryo and deliver blood cells, nutrients, and other molecules to all tissues of our body. Stabilization and maturation of nascent vasculature, formed by vasculogenesis or angiogenesis, requires recruitment of mural cells, i.e. smooth muscle cells (SMC) and pericytes, to the newly formed vessels and induction of their differentiation [1]. Transforming growth factor (TGF) beta1, a potent multifunctional cytokine, has been shown to promote differentiation of several kinds of precursor cells or differentiated cells into the cells that express one or more SMC markers [2–9]. For example, 10T1/2 cells express SM alpha-actin and SM22 alpha when treated with TGF beta1 [9]; neural crest-derived monc-1 cells express a variety of SMC-specific genes after TGF beta1 treatment [10]. However, loss of TGF beta1 function in vivo did not increase hematopoietic or endothelial cell proliferation, which might have been expected by deletion of a negative growth regulator, but rather resulted in defective hematopoiesis and endothelial differentiation. This suggests that the effects of exogenous TGF beta1 were not necessarily accordant with the function of the endogenous TGF beta1. Therefore, although TGF beta1 neutralization antibody could inhibit SMC-specific gene expression in embryonic stem (ES) cell-derived embryoid bodies (EBs) [11], the role of the endogenous TGF beta1 in the differentiation of SMC during embryonic development remains to be elucidated.

Knockout of TGF beta1 or components of its signaling pathway leads to embryonic death in mice before significant SMC maturation occurs [12], therefore, we use ES cells-derived EBs to observe the effects of endogenous TGF beta1 on SMC differentiation. ES cells-derived EBs go through many of the events of early embryonic development and have been highlighted as a more physiological

context in which to study SMC development than most other in-vitro systems [11]. Although studies on EBs provided evidence that addition of TGF beta1 neutralization antibody induced reduction of the expression of SMC markers [11], no loss-of-function studies have been performed to determine whether endogenous TGF beta1 contributes to SMC differentiation during early embryonic development. In this study, we took advantage of plasmid-based RNA interference technology to generate TGF beta1 stable silencing ES cell line to observe the effect of endogenous TGF beta1 on SMC differentiation during early embryonic development. To the best of our knowledge, this is the first time that RNAi-mediated TGF beta1 stable silencing ES cell line has been established.

Methods

Construction of TGF beta1 RNAi vectors

Three mouse TGF beta1 mRNA (Gene bank accession number NM_011577) target sequences were designed using a short interference RNA (siRNA) template design tool (http://www.dharmacon.com): 1, 5′-CAGATCCTGT CCAAACTAA-3′; 2, 5′-GCAACAACGCCATCTATG A-3′; 3, 5′-GAACCAAGGAGACGGAATA-3′. One non-sense oligonucleotide RNA fragment containing a 14 bp-nucleotide 5′-TCGAGGCGGCCGCG-3′ was used as control. The three target sequence-based oligonucleotide and one non-sense sequence-based double-strained oligonucleotide were synthesized and cloned into the entry vector pEN_mH1c (10326369; ATCC, Manassas, VA, USA) by BamHI and XhoI. Subsequently, the recombinant vectors were subcloned into short hairpin RNA (shRNA) eukaryonic expression vector pDS_hpEY (10326384, ATCC) to construct the TGF beta1 shRNA eukaryotic expression vector pDS_shTGF beta1 and the control vector pDS_control, respectively, by using the LR recombinant kit (Invitrogen, Carlsbad, CA, USA). All the recombinant plasmids were confirmed by DNA sequencing. Three TGF beta1 shRNA vectors and a pDS_control vector were transfected into NIH3T3 fibroblasts by using Lipofectamine 2000 reagent (Invitrogen) and positive clones were selected with 500 mg/L neomycin. Silencing efficiency of the three TGF beta1 shRNA vectors was assessed by RT-PCR and western blot analysis and the most effective one (containing target sequence 3) was chosen as the targeting construct and used for subsequent transfection of ES cells.

ES cell culture and generation of EBs

Embryonic stem cell culture and generation of EBs were based on the protocol described by Li and Yurchenco [13]. In brief, mouse ES cell line R1 was maintained in an undifferentiated state on a feeder layer of mitotically inactivated primary mouse embryonic fibroblasts or STO (mouse embryonic fibroblast line) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA), supplemented with 15% fetal calf serum (FCS; Gibco), 2 mM L-glutamine (Gibco), 5×10^{-5} M beta-mercaptoethanol (Serva, Heidelberg, Germany), non-essential amino acids (Gibco; stock solution diluted 1:100), 1 mmol/L pyruvate (Invitrogen), 100 U/mL penicillin (Sigma–Aldrich, St Louis, MO, USA), 100 mg/L streptomycin (Sigma–Aldrich), and 1000 U/mL leukemia inhibitory factor (LIF; Chemicon, Billerica, MA, USA). To generate EBs, ES cells were trypsinized and then cultured in suspension for 5 days in EB medium (similar to ES cell medium but with 20% serum and without LIF and beta-mercaptoethanol). Then EBs were plated on to 0.1% porcine gelatin (Sigma–Aldrich)-coated plates or cultured in three-dimensional Matrigel (BD Biosciences, San Jose, CA, USA) on day 6. The medium was changed daily. The ES cells or EBs were observed under both phase contrast and fluorescence microscopes (Olympus IX70). RNA/protein extraction was performed at desired time points. For SMC differentiation analysis, EBs that attached to gelatin-coated plates for 5 days underwent morphological observation, immunofluorescence staining, reverse transcription (RT)-PCR or western blot.

Transfection of mouse ES cells and silencing efficiency assessment

Prior to transfection, ES cell line R1 was cultured on 0.1% gelatin-coated dishes without feeder layers. LIF supplementation of the ES cell-culture medium was at twice the normal concentration (2000 U/mL) to maintain the undifferentiated state of ES cells. TGF beta1 shRNA targeting vector or pDS_control vectors were transfected into R1 by Lipofectamine 2000 in accordance with the manufacturer's instructions. After 6 days of 500 mg/L neomycin selection, cell clones constantly expressing yellow fluorescent protein (YFP) were selected under a fluorescence microscope, expanded, and analyzed for TGF beta1 expression by western blot. RNA and total protein were collected from EBs for expression analysis of SMC markers (SM alpha-actin and myocardin). For the exogenous TGF beta1 rescue experiment, 2, 5, or 10 ng/mL recombinant TGF beta1 was included in the medium of TGF beta1 knockdown ES cells-EBs on day 0. The medium was changed daily to ensure the validity. Cell outgrowth was observed under a phase-contrast microscope. Expression of SM alpha-actin and myocardin were detected by RT-PCR and western blot analysis.

Western blot analysis

Western blot was performed as previously described [14]. In brief, cell lysates of all groups of ES cells and EBs at different time points were centrifuged and clear supernatants were collected. Equal amounts of proteins were resolved by 8% (myocardin), 10% (SM alpha-actin), or 15% (TGF beta1) SDS–polyacrylamide gel electrophoresis, transferred to PVDF membranes, and incubated with rabbit anti-mouse SM alpha-actin polyclonal antibody (1:1000; Lab Vision and Neomarkers, Fremont, CA, USA), rabbit anti-mouse myocardin polyclonal antibody (1:1000; Abcam, Cambridge, MA, USA), or rabbit anti-mouse TGF beta1 polyclonal antibody (1:1000; R&D Systems, Minneapolis, MN, USA) followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2500; Sigma–Aldrich). Membranes were processed with an enhanced chemiluminescence reagent (ECL; Amersham Biosciences, Sweden) and exposed to film. The image of the blots were captured by use of the Bio-Rad gel documentation system and quantified by use of Quantity One V4.52 software.

Migration assay of EB outgrowth

After 3 days culture on gelatin-coated dishes, the migration distance of the outgrowth for each day 5 (D5) EB was measured in ten locations by use of Image-Pro Plus 5.1 software and the average migration distance used as one data point. A minimum of ten EBs from each group were used to quantify migration distance.

Reverse transcription-PCR analysis

Total RNA was extracted from EBs at different time points with Trizol (Gibco) in accordance with the manufacturer's instructions. The cDNA was synthesized from 1 μg total RNA and subjected to PCR amplification by using the PrimeScript RT-PCR Kit (Takara Bio, Otsu, Shiga, Japan) with DNA primers selective for the mouse genes: TGF beta1, forward 5′-GGCGGTGCTCGCTTTG-3′, reverse 5′-GCTGATCCCGTTGATTTCC-3′; SM alpha-actin, forward 5′-CTGACGCTGAAGTATCC-3′, reverse 5′-GAC TCCATCCCAATGAAAG-3′; myocardin, forward 5′-CA GTGAAGCAGCAAATGACTCG-3′, reverse 5′-TCCCAA GGAGATTCCGTGAAG-3′. For each gene, the DNA primers were derived from adjacent exons to ensure that the PCR product represents the specific mRNA species without genomic DNA contamination. GAPDH, forward 5′-TCTTCACCACCATGGAGAAGG-3′, reverse 5′-GTT GTCATGGATGACCTTGGCC-3′ was used as control. The following PCR program was used: 30 cycles of denaturation at 94°C for 30 s, annealing at 54.6°C (TGF beta1), 57.6°C (SM alpha-actin), 60°C (myocardin) or 55°C (GAPDH) for 30 s, and extension at 72°C for 1 min. RT-PCR products were electrophoresed in 1.8% agarose gel containing 0.1% ethidium bromide. Images of the fluorescent bands were captured by use of the Bio-Rad gel documentation system and quantified by use of Quantity One V4.52 software.

Immunofluorescence staining

For Immunofluorescence staining, EBs and the differentiated outgrowing cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 5% goat serum at room temperature followed by overnight incubation with anti-mouse SM alpha-actin polyclonal antibody (1:100; Lab Vision and Neomarkers). The cells were then incubated with CY5-conjugated goat anti-rabbit IgG (1:100; Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature. After being mounted with DABCO–glycerol (2% DABCO in 80% glycerol in PBS) anti-fading media, coverslips were sealed and examined by use of an Olympus FV-500 laser scanning confocal microscope.

Statistical analysis

All experiments were performed in hexaplicate and repeated at least three times. Data analyses were performed using SPSS 11.1 software. Quantitative data are presented as mean ± standard deviation (SD). Differences between two groups were analyzed by use of Student's two-tailed t test and were considered significant when $p < 0.05$ or $p < 0.01$.

Results

Identification of TGF beta1 knockdown ES cell clones

To assess the effect of endogenous TGF beta1 signaling on SMC differentiation during early embryonic development, we first generated the stable TGF beta1 knockdown ES cell line by transfection of R1 with pDS_shTGF beta1 vectors. We picked up a stable cell clone with the highest TGF beta1 silencing efficiency (named TIR1) for the following experiments. Another stable cell clone that transfected with pDS_control vector was also generated and named LIR1. Undifferentiated TIR1 and LIR1 showed typical ES cell morphology, large and round colonies with clear boundaries, similar to R1 under a phase-contrast microscope and strong fluorescence under 514 nm excitation (Fig. 1a).

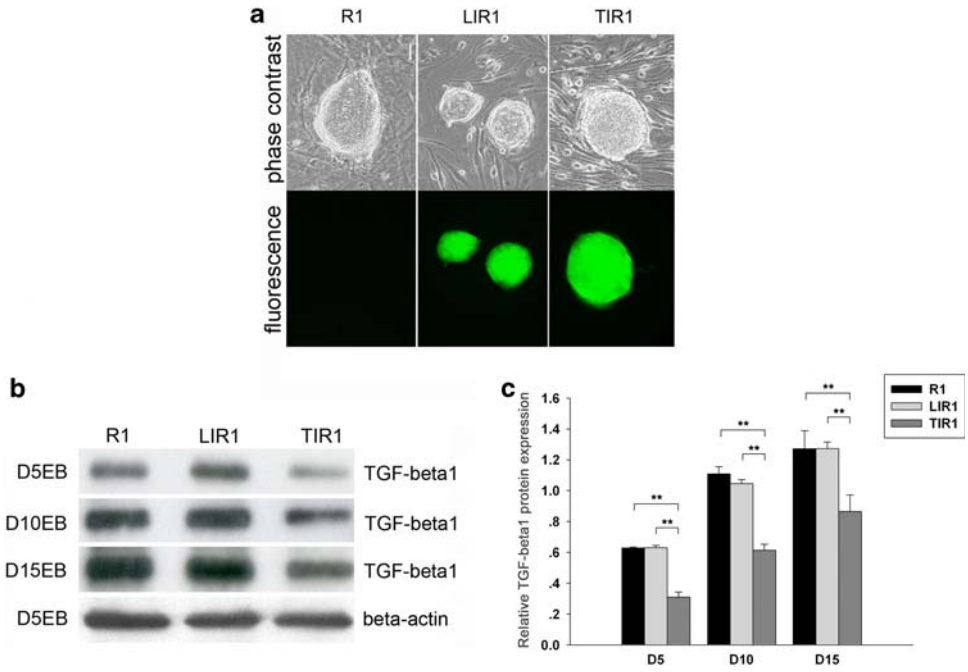

Fig. 1 Identification of TGF-beta1 knockdown ES cell clones. **a** Phase contrast and fluorescence microscopy of ES cell clones of R1 (wild type), TIR1 (TGF-beta1 knockdown), and LIR1 (pDS_control vector). *Scale bar* 100 μm. **b** Western blot analysis for TGF-beta1 expression in ES cells and EBs at three stages of differentiation (D5, D10, and D15) of R1, TIR1, and LIR1 with beta-actin as loading control. **c** Quantification of TGF-beta1 expression in EBs of R1, LIR1, and TIR1 with beta-actin as loading control. $^{**}p < 0.01$

Western blot analysis showed that TGF beta1 expression significantly decreased on D5, D10, and D15 EBs of TIR1 compared with that of the wild-type R1 group and the LIR1 group, while no significant decrease in TGF beta1 expression could be seen between the wild-type R1 group and the LIR1 group (Fig. 1b, c).

TGF beta1 knockdown resulted in morphological changes and downregulated SMC markers expression in differentiated EBs

Morphological observation and quantitative analysis for outgrowth distance of D5 EBs showed that, after 3 days of further differentiation on a gelatin-coated surface, an extensive outgrowth from the central cellular aggregates could be seen in the EBs of R1 and LIR1, but a significantly defective or delayed outgrowth existed in EBs of TIR1 (Fig. 2a, b). Immunofluorescence staining of attached EBs showed SM alpha-actin-positive cells located at the edge of the EBs in both the LIR1 and TIR1 groups. These cells were confluent and dense in the LIR1 group whereas they were dispersed in the TIR1 group (Fig. 2c). The similar defective outgrowth could be seen in the TIR1 group when EBs were cultured on three-dimensional Matrigel (Fig. 3). RT-PCR and western blot analysis for expression level of SMC markers showed that both mRNA (Fig. 4a) and protein (Fig. 4b) expression of SM alpha-actin and myocardin in the TIR1 group significantly decreased compared with the LIR1 group.

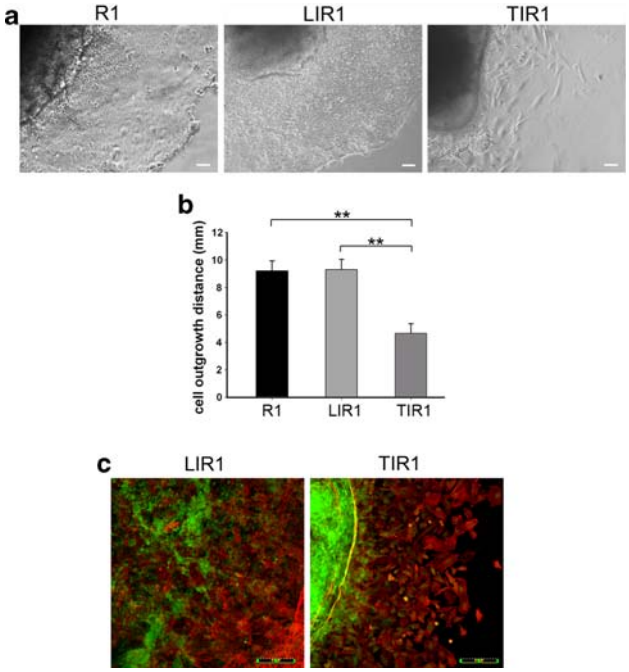

Fig. 2 Retarded extension of cell outgrowth from TGF-beta1 knockdown EBs on gelatin-coated surface. D5 EBs of the R1, LIR1 (pDS_control vector), and TIR1 (TGF-beta1 knockdown) groups were cultured on a gelatin-coated surface for 3 days and were analyzed. **a** Phase contrast microscopy of differentiated EBs of the three groups. **b** Quantitative analysis of the outgrowth in EBs of the three groups. **c** Fluorescence microscopy of yellow fluorescent protein (YFP) and SM alpha-actin of the same cell outgrowth from differentiated EBs of the LIR1 and TIR1 groups. *Scale bar* 100 μm

The rescue experiment showed that the defective or delayed cell outgrowth and the inhibited expression of SM alpha-actin and myocardin in the TGF beta1 knockdown

group were not significantly improved after addition of exogenous TGF beta1 (data not shown).

Discussion

Although a tremendous amount of work has been performed to elucidate the role of TGF beta1 on SMC differentiation, most studies have been confined to certain precursor cell models, for example neural crest stem cells, epicardial cells, or stromal cells, etc., in which cell fates are already predetermined. The mouse TGF beta1 gene knockout model has been used to detect the role of TGF beta1 signaling in vascular development. Unfortunately however, the embryos showed developmental retardation, edema, and necrosis before significant SMC development could be observed [12]. Recently, the role of TGF beta1 and its signaling pathway in the development of SMC from totipotential ES cells was investigated. Evidence was provided that TGF beta1 signaling plays an important role during the differentiation of SMC through the Smad2 and Smad3 pathways [11]. In that study, however, the role of endogenous TGF beta1 itself was not elucidated. Therefore, we successfully established a TGF beta1 SiRNA ES cell line to investigate the role of endogenous TGF beta1 on SMC differentiation during totipotential stem cell development. To the best of our knowledge, this is the first time that the TGF beta1 SiRNA ES cell line has been established.

We took advantage of the ES cell-EB system as the experimental model for two reasons. First, the developmental processes in the ES cell-EB system are not dependent on formation of a functional yolk sac vasculature as in the embryo; second, it could go through many of the events of early embryonic development and thus mimic the in-vivo physiological environment more than most other in vitro models [11]. Our study showed that extensive outgrowth from the central cellular aggregates could be markedly inhibited in TGF beta1 gene knockdown EBs. Further work needs to be done to determine

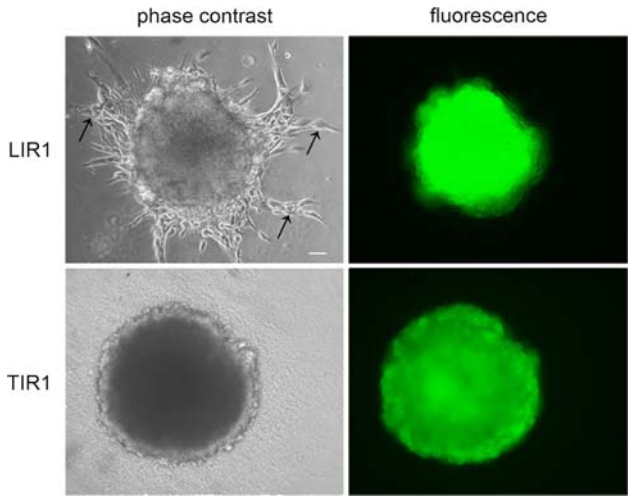

Fig. 3 Retarded extension of cell outgrowth from TGF-beta1 knockdown EBs in three-dimensional Matrigel. After differentiation for 2 days, D5 EBs cultured in Matrigel sprouted (*up arrow*) in EBs of the LIR1 group (pDS_control vector) whereas little sprouting could be seen in EBs of the TIR1 group (TGF-beta1 knockdown). *Scale bar* 100 μm

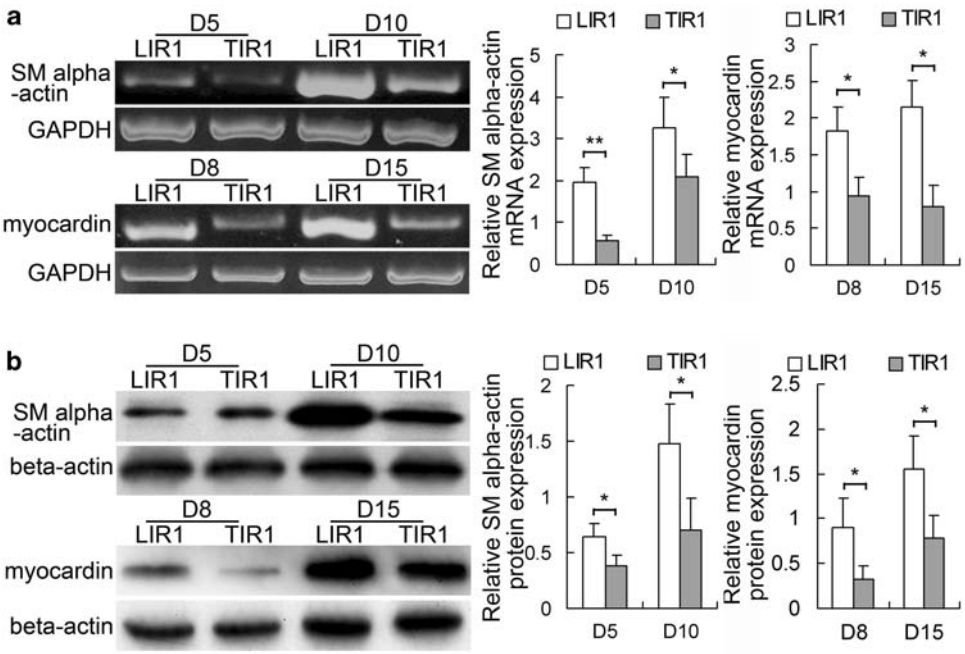

Fig. 4 TGF-beta1 knockdown resulted in downregulated expression of SMC markers (SM alpha-actin and myocardin) in differentiated EBs. **a** RT-PCR (*left panel*) and quantification (*right panel*) of SM alpha-actin and myocardin mRNA in EBs of the TIR1 (TGF-beta1 knockdown) and LIR1 (pDS_control vector) groups with GAPDH as loading control. **b** Western blot (*left panel*) and quantification (*right panel*) of SM alpha-actin and myocardin expression in EBs of the TIR1 and LIR1 groups with beta-actin as loading control. $^{*}p < 0.05$, $^{**}p < 0.01$

whether this is due to the inhibited differentiation, migration, or proliferation of these cells. Immunofluorescence indicated that reduced number of SM alpha-actin-positive cells may, at least partly, contribute to this inhibition of cell outgrowth. The model we have developed thus provides an ideal in-vitro system to understand the molecular mechanisms that induce SMC differentiation from totipotential stem cells.

SM alpha-actin is a desired SMC differentiation marker in that it is the first known SMC marker during development and that it is highly specific for SMC or SMC-like cells in adult animals in normal circumstances [15]. Myocardin is a potent SRF coactivator and an important component of a molecular switch for SMC development and differentiation [16, 17]. Thus we chose SM alpha-actin and myocardin as early SMC differentiation markers to detect the alteration related to TGF beta1 expression. Our previous study on ES cell differentiation indicated that SM alpha-actin could first be detected in ES cells, then downregulated as differentiation proceeded, and upregulated again at D5 and peaked around D10. Our previous study also showed that myocardin first expressed around D8 and peaked at D15. So we chose D5 and D10 as the time points to analyze the expression of SM alpha-actin and D8 and D15 to assess the expression of myocardin [18]. Accordant with the effect of neutralizing antibody [11], TGF beta1 knockdown resulted in significant down-regulation of SM alpha-actin and myocardin as assessed by RT-PCR and western blot. Addition of exogenous TGF beta1, however, failed to rescue the expression of SMC markers and the retarded cell outgrowth. This may suggest the difference between endogenous and exogenous TGF beta1 in regulating embryonic SMC development. Evidence has been provided that the first expression of TGF beta1 RNA is seen in the hemangioblast, and in both endothelial and hematopoietic progenitors. The TGF beta1$^{-/-}$ embryos have defective differentiation of both red blood cells and endothelial cells [12]. However, exogenous TGF beta1 is also a potent inhibitor of both endothelial and hematopoietic cell proliferation [19–21] Therefore, we suspect that addition of exogenous TGF beta1 could inhibit, or at least not increase, the expression of TGF beta1 RNA which leads to the failure of TGF beta1 addition in rescuing the expression of SMC markers and the retarded cell outgrowth.

In our study, we established a "loss of function" model based on ES cell-derived EBs and presented direct evidence that endogenous TGF beta1 promotes expression of early SMC markers during ES cells differentiation, although more needs to be done to understand the mechanism by which TGF beta1 knockdown resulted in the retarded extension of differentiated cell outgrowth from EBs.

Acknowledgments This work was supported by grants from the National Natural Science Foundation of China (30370526) to Yaling Han.

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