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Journal of Cellular Biochemistry 115:359–367 (2014)

Journal of Cellular Biochemistry

Sphingosine 1-Phosphate Receptors Negatively Regulate Collagen Type I/III Expression in Human Bone Marrow-derived Mesenchymal Stem Cell

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ABSTRACT

Collagen is the most abundant structural protein in mammals and is expressed in various tissues. In recent years, sphingosine 1-phosphate receptors (S1PRs) have been proven to play an important role in the regulation of collagen expression. Our previous studies reported that S1PRs are involved in TGF-β1-induced collagen expression via up-regulating S1PR1/3 in mouse bone marrow-derived mesenchymal stem cells (BMSCs), and result in experimental mouse liver fibrogenesis. But it remains unclear whether this process happens in human bone marrow-derived mesenchymal stem cells (hMSCs). In this study, we provide evidences that S1PR1/3, but not S1PR2, negatively regulate the expression of collagen in hMSCs using cellular and molecular approaches in vitro. We find that treatment of hMSCs with TGF-β1 up-regulated collagen expression in a dose- and time-dependent manner. Meanwhile, TGF-β1 inhibited the expression of S1PR1/3, but not S1PR2, in hMSCs in a time-dependent manner. Furthermore, either selective knock-down of S1PR1 or silencing S1PR3 induced collagen α1(I) and collagen α1(III) expression in hMSCs. In contrast, inhibition of S1PR2 by siRNA had no effects on the expression of collagen. Altogether, all these findings demonstrated that collagen expression was negatively regulated by S1PR1 and S1PR3 in hMSCs. This study highlights the differences between hMSCs and mouse BMSCs, provides a new regulation mechanism for collagen expression, and points out the risk of utilizing hMSCs in clinical applications. J. Cell. Biochem. 115: 359–367, 2014.

KEY WORDS: SPHINGOSINE 1-PHOSPHATE RECEPTOR; COLLAGEN; HUMAN MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are multipotent cells with self-renewal capacity and are present in various tissues, including blood, adipose tissue, trabecular bone, muscle, and dermis [Caplan, 1991]. MSCs have the potential to differentiate into distinct mesenchymal tissues, such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma [Pittenger et al., 1999]. Their pluripotent differentiation capacities make MSCs particularly suitable for cell transplantation or tissue engineering therapies. Recent studies have suggested that MSCs can be activated and specifically migrate to tissue injure sites, where they then perform repair functions [Pelttari et al., 2008; Uccelli et al., 2008; Horie et al., 2012]. It has been reported that MSCs can highly express collagen in some pathological processes and promote tissue repair [Pelttari et al., 2008; Horie et al., 2012]. Meanwhile, collagen secreted by MSCs can also contribute to liver fibrogenesis in liver injury [Russo et al., 2006; di Bonzo et al., 2008]. Our previous studies have demonstrated that mouse bone marrow-derived mesenchymal stem cells (BMSCs) can migrate to damaged liver and express collagen in mouse fibrotic liver [Li et al., 2009b; Yang et al., 2012]. Thus, it is desirable to identify the regulatory mechanisms underlying the collagen expression in MSCs.

Collagen is the most abundant structural protein in mammals. In human, collagen comprises ~30% of the total protein and forms the most prevalent component of the extracellular matrix [Shoulders and Raines, 2009; Ricard-Blum, 2011]. In fibrogenesis of cardiac, liver, renal, lung, and systemic sclerosis, the injured tissue is characterized by an accumulation of extracellular matrix proteins, especially

Disclosure statement: The authors disclose no conflicts of interest.
Grant sponsor: National Natural and Science Foundation of China; Grant numbers: 81030009, 81170407, 31301154.
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Manuscript Received: 15 May 2013; Manuscript Accepted: 6 September 2013
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 12 September 2013
DOI 10.1002/jcb.24670 • © 2013 Wiley Periodicals, Inc.

collagen α1(I) (Col α1(I)) and collagen α1(III) (Col α1(III)) [Bataller and Brenner, 2005; Cutroneo et al., 2007; Lin et al., 2008; Krenning et al., 2010; Peng et al., 2012]. The synthesis of collagen is highly regulated by different factors and cytokines at various levels [Ghosh, 2002]. In cardiac fibrosis, a transcription factor, scleraxis, appears to play a key role in the regulation of collagen synthesis [Bagchi and Czubryt, 2012]. TGF-β1 is a member of a large family of growth factors involved in numerous biological processes [Massague, 1998; Blobe et al., 2000; Jeon et al., 2006]. It also has been reported that TGF-β1 is a central mediator of fibrogenesis and up-regulates collagen expression in various tissues [Ghosh, 2002; Matsuzaki, 2009; Biernacka et al., 2011; Lan, 2011; Fernandez and Eickelberg, 2012]. In recent years, sphingosine 1-phosphate (S1P) receptors (S1PRs) have been proved to be an important molecule in the regulation of collagen expression and are involved in TGF-β1-induced differentiation and activation of many kinds of cells [Gellings et al., 2009; Li et al., 2009a; Liu et al., 2011]. In previous studies, we have reported that TGF-β1 can increase collagen levels and modulate the expression of S1PRs, resulting in liver fibrosis in mouse [Yang et al., 2012]. However, whether this process works in human bone marrow-derived mesenchymal stem cells (hMSCs) is unclear.

S1PRs are members of a G protein-coupled receptor family, including five receptors named S1PR1, S1PR2, S1PR3, S1PR4, and S1PR5, which were formerly termed endothelial differentiation gene (EDG) receptor-1, -5, -3, -6, and -8, respectively [Sanchez and Hla, 2004]. S1PR1–3 are widely expressed in different tissues, including brain, lung, heart, spleen, kidney, intestine, diaphragm, and liver. S1PR4 is specifically expressed in the lymphoid tissue, and S1PR5 is mainly expressed in the central nervous system [Hla and Brinkmann, 2011]. After binding with S1P, S1PRs active downstream signaling pathways and mediate a wide variety of cellular responses in different cells, including proliferation, migration, adhesion, and morphogenesis [Brinkmann, 2007]. It has been reported that activated S1PRs affect the expression of various genes. During differentiation of human lung fibroblasts, transfection of S1PR3 siRNA reduces TGF-β1-induced fibronectin protein expression [Kono et al., 2007]. In the process of smooth muscle cell differentiation, S1PR2 is the major S1PR sub-type that regulates the expression of differentiation marker genes [Medlin et al., 2010]. The specific Gi/Go inhibitor inhibits S1P-induced RANKL mRNA expression in MH7A cells [Takeshita et al., 2012]. In lung adenocarcinoma cells, S1PR3 signaling increases epidermal growth factor receptor expression via the Rho kinase pathway [Hsu et al., 2012]. S1PRs also regulate collagen level in TGF-β-stimulated cardiac fibroblasts [Gellings et al., 2009]. Moreover, we have reported that S1PR1/3 are involved in collagen expression during the activation of human hepatic stellate cell line, LX-2 cells [Liu et al., 2011], and in the differentiation of BMSCs during experimental mouse liver injury [Yang et al., 2012].

In this study, the effect of S1PRs on collagen expression in hMSCs was investigated. We detected for the first time that S1PRs influenced collagen expression in hMSCs in a negative manner. Importantly, S1PR1 and S1PR3, which were repressed by TGF-β1, seemed to be critically implicated in the process. These results serve as a warning about the differences in collagen regulation between human and mouse BMSCs. We should be more careful when we use the conclusions from experimental animal models to explain the mechanisms of human diseases. We present a new mechanism for the regulation of collagen expression in hMSCs, and point out the risks of using hMSCs in clinical application.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT

Human mesenchymal stem cells (Cyagen, Sunnyvale, CA) were cultured in Dulbecco's modified Eagle's medium (Cyagen) supplemented with 10% hMSC-qualified fetal bovine serum (Cyagen), 1% Penicillin–Streptomycin solution and 1% glutamine, at 37°C in 5% CO_2. As recommended by the manufacturer, hMSCs derived from bone marrow of healthy 18–45 years old with informed consent were cultured as a monolayer and were then cryopreserved at the second passage. These cells were tested for the multipotent differentiation ability along the osteogenic, chondrogenic, and adipogenic lineages. The characterization of the cells was also explored. These cells were positive for CD29, CD44, and CD105 (>70%), and negative for CD34 and CD45 (<5%) in flow cytometry assays. After purchased, the cells were thawed and maintained properly. hMSCs of passage 6 to passage 10 were used in the experiments as the user manual recommended. Unless otherwise indicated, cells treated with chemicals and reagents were cultured in medium without serum for 24 h, and treated with TGF-β1 (PeproTech, London, UK) at different concentrations for another 24 h.

RNA INTERFERENCE

The siRNA sequences specifically targeting human S1PR1, S1PR2, or S1PR3 were synthesized by Ambion (Austin, TX; Cat#: 4390824). Transient transfection of siRNAs (40 nmol/L) was performed using Invitrogen Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA), as recommended by the manufacturer. Control cells were treated with 40 nmol/L RNAi negative control duplexes (scrambled siRNA). After 48 h, cells were used to perform further assay.

REAL-TIME RT-PCR

Total RNA was extracted from hMSCs with or without treatments, using RNeasy kit (Qiagen, Hilden, Germany). Real-time RT-PCR was performed with an ABI Prism 7300 sequence-detecting system (Life Technologies, Foster City, CA), as described previously [Li et al., 2011]. Primers (MWG Biotech, Ebersberg, Germany) used for real-time RT-PCR were as follows: 18S rRNA, sense, 5′-GTAACCCGTTGAACCCCATT-3′, and anti-sense, 5′-CCATCCAATCGGTAGTAGCG-3′; procollagen α1(I) (Col α1(I)), sense, 5′-AGGTCCCCCTGGAAAGAA-3′, and anti-sense, 5′-AATCCTCGAGCACCCTGA-3′; procollagen α1(III) (Col α1(III)), sense, 5′-AGCTGGAAAGAGTGGTGACAG-3′, and anti-sense, 5′-CCTTGAGGACCAGGAGCAC-3′; S1PR1, sense, 5′-TCTGCGGGAAGGGAGTATGT-3′, and anti-sense, 5′-CGATGGCGAGGAGACTGAA-3′; S1PR2, sense, 5′-TGCCCGCCTTCAGCAT-3′, and anti-sense, 5′-AAAAAGTAGTGGGCTTTGTAGAGGAT-3′; S1PR3, sense, 5′-TCTCAGCCTTCATCCATTAACTCTAC-3′, and anti-sense, 5′-AGGGAGCCTTATGTCATACCACAA-3′. Probe (Applied Biosystems) used for real-time RT-PCR was as follows: SphK1: Hs00184211_m1.

IMMUNOFLUORESCENCE AND HIGH CONTENT ANALYSIS

hMSCs (5,000) were plated in the wells of 96-well plates (Corning, NY) and treated with chemicals after overnight attachment. The cells with or without treatments were fixed in 4% paraformaldehyde in PBS for 30 min. Cells were then washed twice with PBS, permeabilized in 0.5% Triton X-100 in PBS for 15 min, blocked with 2% BSA for 1 h, and incubated with antibodies recognizing S1PRs, Col α1(I) or Col α1 (III) (Santa Cruz, CA) diluted in PBS (1:100), Cy3-conjugated AffiniPure goat anti-rabbit IgG antibody or Cy3-conjugated AffiniPure donkey anti-goat IgG antibody (1:500) was used as secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). The antibodies recognizing Col α1(I) and Col α1(III) were raised against amino acids 1,021–1,217 mapping within an internal region of Collagen α1 type I and a peptide mapping near the C-terminus of Collagen α1 type III, respectively. After incubation with PBS containing 10 μg/ml DAPI for 5 min, cells were washed twice with PBS, and 100 μl PBS were left in each well. For negative controls, cells were processed the same way, except that incubation with the primary antibody was omitted. The plates were imaged on a Thermo Scientific CellInsight personal cell imaging (PCI) platform (Cellomics, Inc., Thermo Fisher Scientific Inc., Waltham, MA), with a ×10 objective using the Thermo Scientific Cellomics iDEV Software. Thirty-six fields were automatically acquired by the software, corresponding to at least 3,000 cells. The total Cy3 fluorescence intensity of each well was analyzed by Cellomics Cell Health Profiling BioApplication software.

WESTERN BLOTS

hMSCs were seeded in 10-cm tissue culture plates, and treated with siRNA or chemicals. Lysates were prepared with lysis buffer (RIPA, Solarbio Science & Tecnology Co., Ltd., China) as recommended by the manufacturer. Fifty micrograms of protein extracts were electrophoresed in 7.5% (for collagen) or 10% (for S1PRs) SDS/polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. The blots were blocked with 1% bovine serum albumin (BSA) at room temperature for 1 h and incubated with primary antibody described (1:500) overnight at 4°C. Non-specific IgG served as the negative control. After 1 h incubation at room temperature with appropriate IRDyeTM 800-conjugated secondary antibody (1:10,000), immunoreactive bands were detected using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE) and analyzed with Odyssey software.

STATISTICAL ANALYSIS

Data were expressed as means ± SEM and were analyzed by Student's *t*-test or analysis of variance when appropriate. $P < 0.05$ was considered significant.

RESULTS

TGF-β1 INDUCES COLLAGEN EXPRESSION IN hMSCs

We first examined the effects of TGF-β1 on the expression of collagen in hMSCs. hMSCs were treated with 10 ng/ml TGF-β1 and collected at 0, 4, 12, and 24 h. Real-time RT-PCR analysis showed that the mRNAs of Col α1(I) and Col α1(III) were up-regulated from 12 h with a maximal increase (~3.4-fold and ~2.8-fold, respectively) at 24 h (Fig. 1A,B). We then detected their mRNA levels in hMSCs treated with 0, 1, 2, 5, 7.5, or 10 ng/ml TGF-β1 for 24 h. The results showed that the mRNA of Col α1(I) was markedly up-regulated in 1 ng/ml TGF-β1, and continued to increase in a dose-dependent way (Fig. 1C). At the same time, the mRNA of Col α1(III) was induced in hMSCs treated with 5 ng/ml TGF-β1, with a maximal increase at a concentration of 10 ng/ml. Western blots were also employed to detect collagen protein expressions. Col α1(I) and Col α1(III) protein levels were obviously induced by TGF-β1 in hMSCs (Fig. 1D). After confirming the specificity of Col α1(I) and Col α1(III) antibodies by Western blot, we performed immunocytochemistry assays to measure the expression of collagen protein in hMSCs. Immunofluorescent staining showed that TGF-β1 up-regulated collagen protein level in hMSCs (Fig. 1E). In addition, mature collagen secreted into the medium was also detected by immunofluorescence, especially Col α1 (III) (Fig. 1E, arrowhead). We then examined the total fluorescence intensity of collagen protein expressions with High Content Analysis in cells with or without TGF-β1. High Content Analysis is an application of automated microscopy and image analysis to cell biology, which calculates population statistics for all the cells selected for analysis and is more accurate than Western blot when antibodies are specific. In hMSCs treated with 2 ng/ml and 10 ng/ml TGF-β1, collagen protein expression was markedly up-regulated (Fig. 1F). These results suggest that TGF-β1 induces collagen expression in hMSCs.

S1PR1, S1PR2, AND S1PR3 ARE EXPRESSED IN hMSCs

We next asked whether S1PRs participated in TGF-β1-induced collagen expression in hMSCs. To answer this question, we first examined the expression of S1PRs in hMSCs. The results of RT-PCR and Western blots indicated that these three receptors were detectable as mRNAs and proteins in hMSCs (Fig. 2A,B). To further confirm it, immunocytochemistry assays were used to determine the expression of these receptors in hMSCs. As expected, the immunostaining for S1PR1-3 was clearly detected (Fig. 2C). Meanwhile, the images of Western blots and immunostaining for S1PRs revealed clear and specific signals, as no signals were detected when Western blots or immunostaining was performed in the absence of primary antibodies (Fig. 2B,C). These results show that S1PR1-3 are expressed in hMSCs.

INDUCTION OF COL α1(I) AND COL α1(III) EXPRESSION BY TGF-β1 DEPENDS ON DOWN-REGULATION OF S1PR1/3, BUT NOT S1PR2

We next examined whether TGF-β1 affected the expressions of S1PRs in hMSCs. In cells treated with 0, 1, 2, 5, 7.5, or 10 ng/ml TGF-β1, S1PR1 and S1PR3 mRNAs, but not S1PR2, were observably decreased, even at the minimal TGF-β1 concentration (1 ng/ml). The inhibition of S1PR1/3 did not depend on the concentration of TGF-β1 (Fig. 3A). hMSCs were treated with 10 ng/ml TGF-β1, and collected at different times. TGF-β1 repressed S1PR1 and S1PR3 mRNA expression in a time-dependent manner (Fig. 3C, left and right). We also employed Western blots and immunofluorescent staining to determine the expressions of S1PR proteins. As shown in Figure 3B, the protein levels of S1PR1 and S1PR3 were decreased, while the S1PR2 protein level did not change in hMSCs treated with 10 ng/ml TGF-β1. Immunostaining showed that TGF-β1 down-regulated S1PR1 in hMSCs, and the total fluorescence intensity counted by

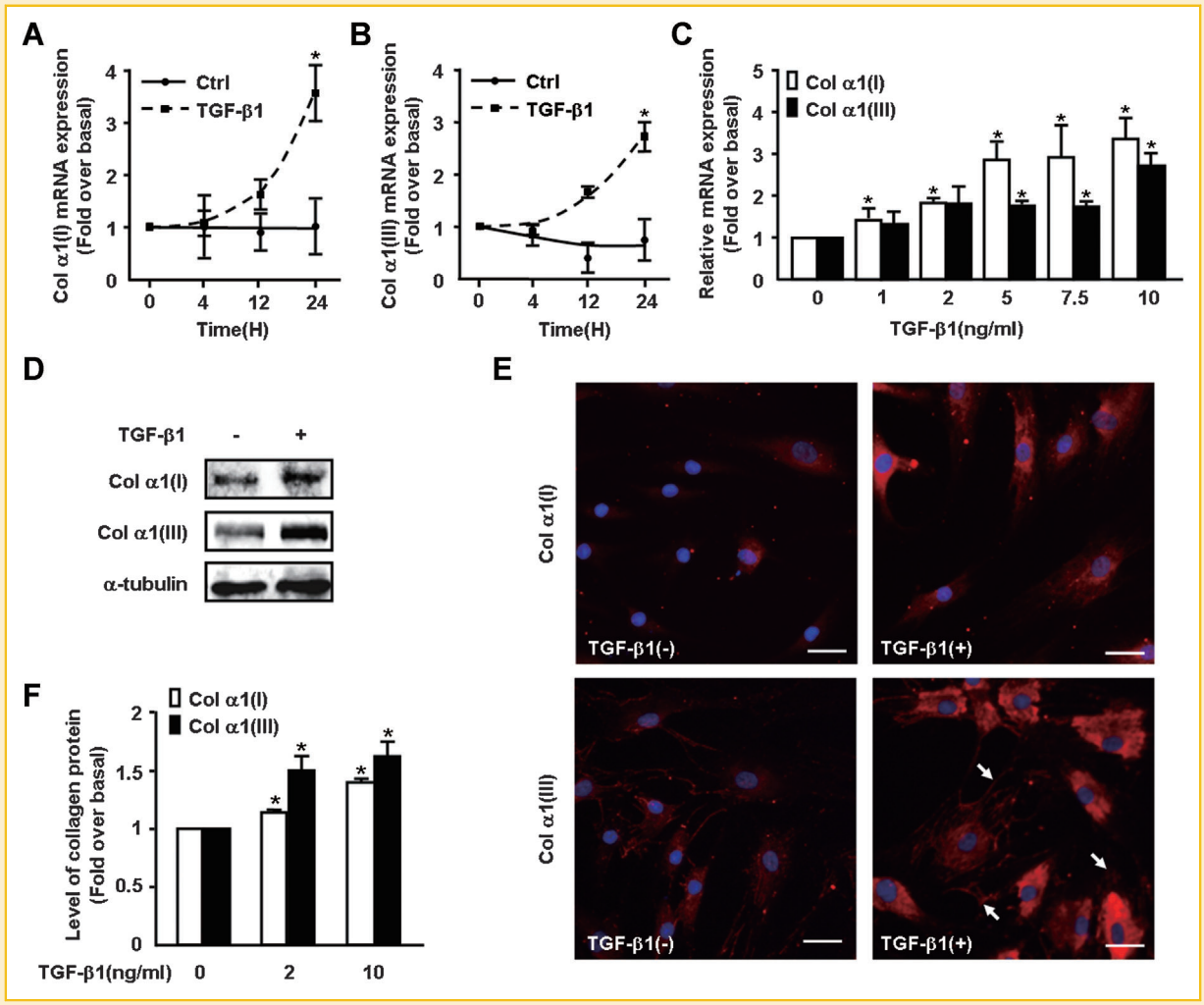

Fig. 1. TGF-β1 induces collagen expression in hMSCs. mRNA and protein levels of Col α1(I) and Col α1(III) were measured in hMSCs with or without TGF-β1. A,B: hMSCs were treated with 10 ng/ml TGF-β1 and collected at different times, the relative mRNA expression of Col α1(I) and Col α1(III) were examined by real-time RT-PCR. C: hMSCs were treated with different concentrations of TGF-β1 and collagen mRNAs were detected 24 h later. D: hMSCs were treated with TGF-β1 (10 ng/ml) for 24 h, and collagen protein expressions were detected by Western blot as described in methods. E: Representative images of immunofluorescence analysis to track the expression of Col α1(I) and Col α1(III) in cells with TGF-β1. F: Total fluorescence intensity of collagen proteins counted with High Content Analysis as described in the Methods Section. Scale bars, 25 μm. All results were confirmed in at least three independent experiments. Arrows in (E) indicated collagen protein secreted into the medium. Data were presented as the mean ± SEM. $^{*}P < 0.05$, compared with control.

High Content Analysis also indicated that TGF-β1 reduced S1PR1 protein by ~35% at a concentration of 2 ng/ml and ~40% at 10 ng/ml (Fig. 3D). Similar to the mRNA expression, S1PR2 protein level did not change in hMSCs with either 2 or 10 ng/ml TGF-β1, compared with control (Fig. 3E). TGF-β1 reduced the S1PR3 protein expression by ~45% at 2 ng/ml and ~50% at 10 ng/ml (Fig. 3F). These results indicate that TGF-β1 inhibits S1PR1 and S1PR3 expression in hMSCs.

To further determine the role of S1PRs on TGF-β1-induced collagen expression, we employed selective S1PR siRNAs and measured the expression of collagen with real-time RT-PCR and Western blots. After transfection of S1PR1-siRNA or scramble siRNA (SCR siRNA), real-time RT-PCR was performed after 48 h to assess the levels of S1PRs, Col α1(I) and Col α1(III) mRNA. In hMSCs treated with S1PR1-siRNA, S1PR1 mRNA expression was down-regulated by ~85% (Fig. 4A), and this knock-down of S1PR1 increased the expression of Col α1(I) and Col α1(III) by ~2.4-fold and ~1.9-fold, respectively (Fig. 4B). Western blots also showed similar results, that S1PR1 knockdown increased collagen protein expression (Fig. 4C). We also used a potent, selective S1PR2 siRNA to analyze the role of S1PR2 in the expression of collagen. After siRNA transfection, the expression of S1PR2 mRNA reduced by ~65% compared to the SCR siRNA (Fig. 4D), but neither mRNA nor protein levels of Col α1(I) and Col α1(III) were changed after S1PR2 knockdown (Fig. 4E,F). Finally, we detected the effect of S1PR3 on collagen expression in hMSCs. Transfection of S1PR3 siRNA in hMSCs decreased S1PR3 expression by ~95% at the mRNA level (Fig. 4G), and increased the expression of Col α1(I) and Col α1(III) in hMSCs (Fig. 4H,I). In all cells treated with S1PR1-, S1PR2-, or S1PR3-targeted siRNA, we also detected other

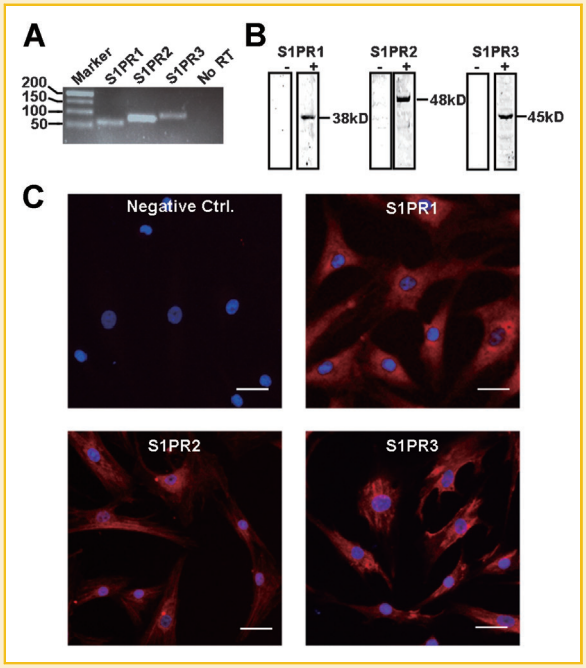

Fig. 2. Expression of S1PRs in hMSCs. A: Expression of S1PR1–3 mRNA in hMSCs was measured by RT-PCR, the PCR products were size-fractionated in a 2% agarose gel. No-RT means RT-PCR performed in the absence of M-MLV reverse transcriptase. B: S1PR protein expressions in hMSCs were detected by Western blot as described in the Methods Section. C: The representative images of immunostaining for S1PR1, S1PR2, or S1PR3 (red) in hMSCs, as visualized by immunocytochemical analysis. Cells were co-stained with DAPI to identify nuclei (blue). Scale bars, 25 μm.

S1PRs to confirm the specificity of these siRNAs, revealing that the specific siRNAs did not alter the expressions of other S1PRs (data not shown).

Taken together, our results indicate that TGF-β1 inhibits S1PR1 and S1PR3 expression in hMSCs. Using specific siRNAs of S1PRs, we find that blockage of S1PR1/3, but not S1PR2, can induce high expression of Col α1(I) and Col α1(III). We prove that collagen expression is negatively regulated by S1PR1/3 in hMSCs, and that TGF-β1 could increase collagen expression partially by repressing of S1PR1 and S1PR3 in hMSCs.

DISCUSSION

In the current study, we illustrate for the first time that TGF-β1 up-regulates collagen expression by repression of S1PR1/3, but not S1PR2, in hMSCs. We also provide evidence that S1PR1/3 play a crucial role in the expression of collagen in vitro. The specific siRNAs of S1PR1 or S1PR3 induces high levels of Col α1(I) and Col α1(III) in hMSCs. However, the signaling pathways which are involved in TGF-β1-induced collagen expression remain to be investigated.

It has been reported that collagen expression is up-regulated by TGF-β1 in various tissues [Matsuzaki, 2009; Biernacka et al., 2011; Lan, 2011; Fernandez and Eickelberg, 2012]. In the present study, we used High Content Analysis to detect the protein levels of collagen. In recent years, an increasing number of papers describe High Content Analysis as a novel application [Zock, 2009]. High Content Analysis provides population statistics for all the cells selected for analysis. In our research, 36 images were acquired by the software and the total fluorescence intensity of each well (at least 3,000 cells) was analyzed. We also found that the collagen protein expressions are changed after treatment with TGF-β1. Unlike the changes in mRNA levels, the protein level of Col α1(III) is increased more markedly than Col α1(I) in TGF-β1 treated cells. A reason for this discrepancy might be the different efficiencies of the real-time RT-PCR primers and the primary antibodies of Col α1(I) and Col α1(III). It also has been reported that TGF-β1 induces the migration of cells [Vo et al., 2013; Zhang et al., 2013], but TGF-β1 does not produce a marked effect on hMSCs migration in our study (Supplemental Fig. 1).

S1PRs are widely expressed in various cells, especially S1PR1-3. The distinct combinations of S1PR subtypes in different cell types produce an appropriate biological action. It is helpful to the understanding of the pathophysiological role of each S1PR to know the pattern of expression. For this purpose, we examine the expression of S1PRs in hMSCs. Using RT-PCR, Western blots and immunostaining analysis, S1PR1-3 are clearly detected in hMSCs. We also examine whether TGF-β1 influences the expression of S1PRs. TGF-β1 represses the mRNA and protein levels of S1PR1 and S1PR3, but not S1PR2. In several published reports, S1PRs have been implicated in supporting a key role in transmitting the effects of TGF-β1. For example, knock-down of S1PR2 completely suppresses TGF-β1-stimulated collagen production and differentiation of cardiac fibroblasts [Gellings et al., 2009]. S1PR2 and S1PR3 are previously identified as downstream of TGF-β1 in lung fibroblasts [Kono et al., 2007]. Furthermore, in the differentiation of myocytes caused by TGF-β1, S1PR3 plays a key role [Cencetti et al., 2010]. We therefore want to explore whether S1PRs are involved in TGF-β1-induced collagen expression in hMSCs. It must to be reminded that in all previous studies, the effects elicited by TGF-β1 are transmitted by the up-regulation of S1PRs. Here, we demonstrate for the first time that TGF-β1 induces collagen expression via down-regulation of S1PR1/3 in hMSCs. It has been reported that TGF-β1 up-regulates sphingosine kinase-1 in C2C12 myoblasts in a Smad-dependent manner, and concomitantly modifies the expression of S1PRs [Cencetti et al., 2010]. In another study, it was demonstrated that the TGF-β/ALK5/Smad2/3 signaling pathway is implicated in angiogenesis and that Smad2/3 signaling is indispensable for the maintenance of vascular integrity via the fine-tuning of S1PR1 expressions [Itoh et al., 2012]. However, the mechanisms by which TGF-β1 represses S1PRs in hMSCs remains to be investigated.

We employ specific S1PR siRNAs to determine the effect of S1PR1-3 on collagen expression in hMSCs. Using selective siRNAs of S1PR1-3, we find that S1PR1 and S1PR3 have a negative regulatory effect on collagen expression, while S1PR2 does not influence collagen levels in hMSCs. In previous studies, it was proven that S1PRs play an important role on the expression of collagen. In mouse cardiac fibroblasts, S1P increases collagen expression in a S1PR2- and Rho kinase-dependent manner [Gellings et al., 2009]. A research on mouse ventricular fibroblasts proved that S1P stimulates cell proliferation and decreases collagen secretions via S1PR3 [Benamer et al., 2011]. The sphingolipid agonist dihydrosphingosine 1-phosphate (dhS1P)

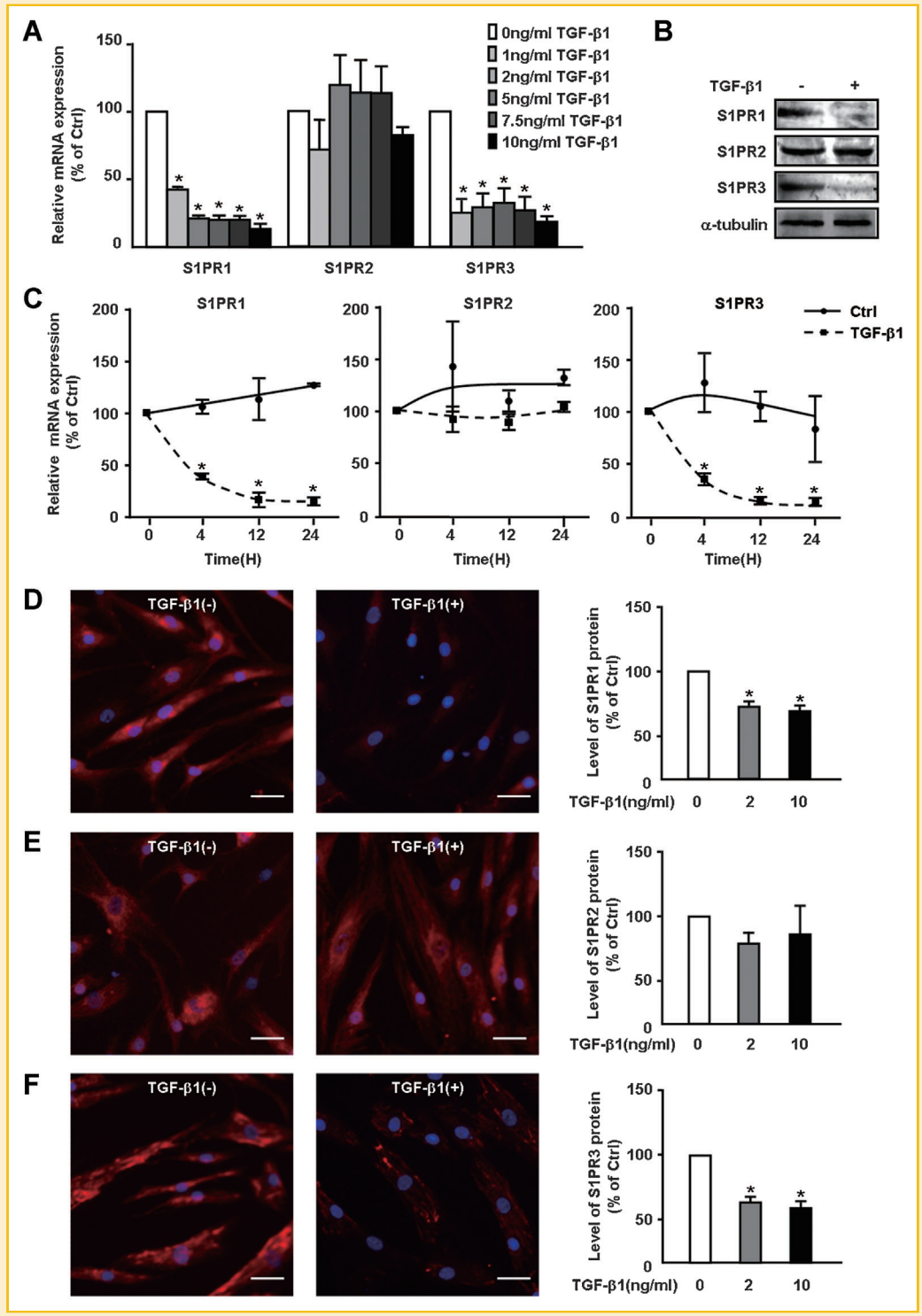

Fig. 3. TGF-β1 down-regulates expression of S1PR1/3, but not S1PR2, in hMSCs. The effect of TGF-β1 on the expressions of S1PR mRNAs was detected by real-time RT-PCR at different concentration of TGF-β1 (A) and time (C). Protein levels of S1PR1 (B,D), S1PR2 (B,E), and S1PR3 (B,F) in hMSCs with TGF-β1 were measured by Western blots, immunostaining and High Content Analysis. Scale bars, 25 μm. All results were confirmed in at least three independent experiments. Data were presented as the mean ± SEM. $^{*}P < 0.05$, compared with control.

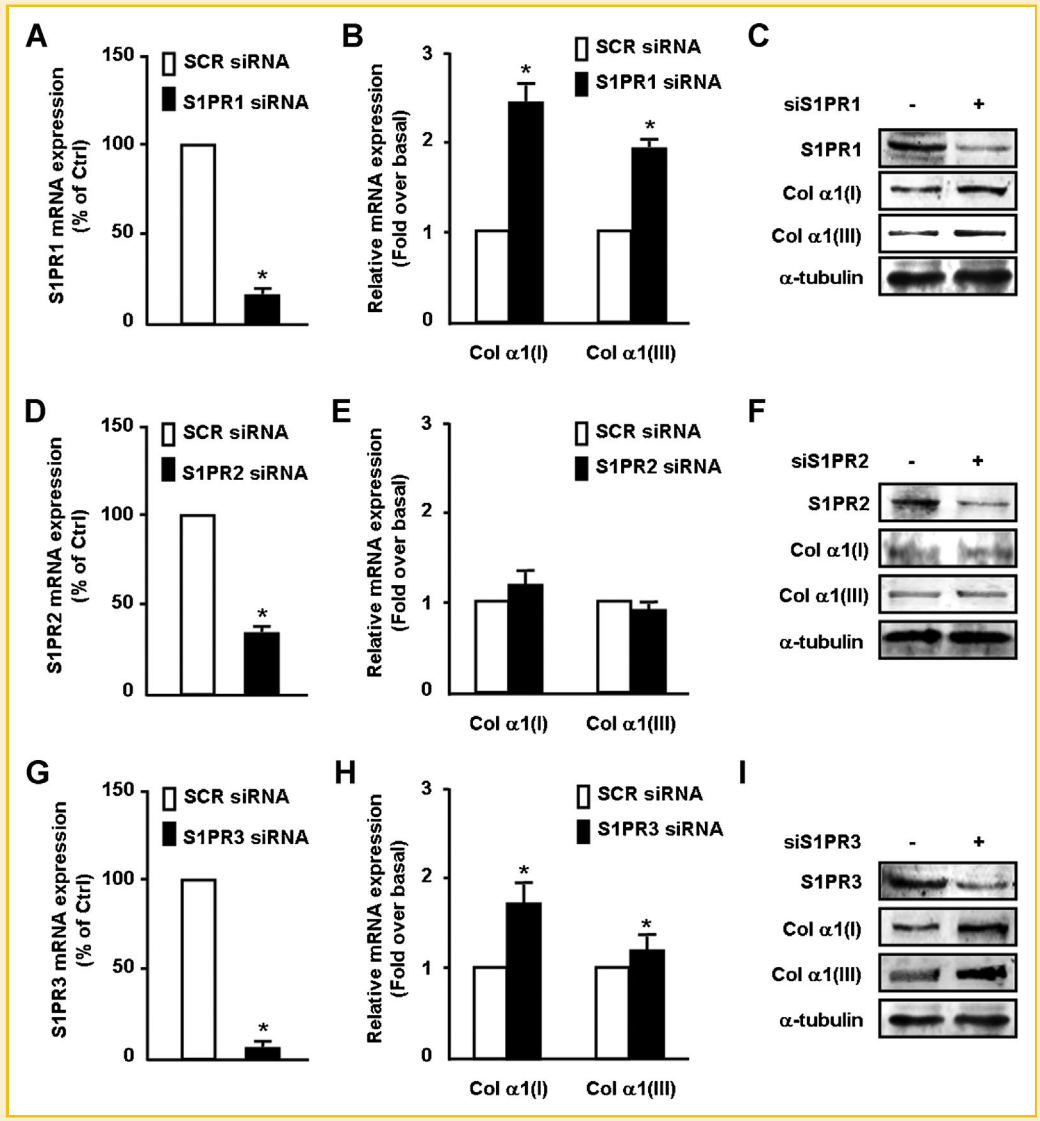

Fig. 4. Knock-down of S1PR1/3, but not S1PR2, increase Col α1(I) and Col α1(III) expression in hMSCs. hMSCs were transfected with S1PR1-, S1PR2-, or S1PR3-siRNA, and 48 h later, S1PR1 (A,C), S1PR2 (D,F), or S1PR3 (G,I) mRNA and protein expressions were detected. Collagen mRNA and protein expressions were also measured in hMSCs treated with S1PR1- (B,C), S1PR2- (E,F), or S1PR3- (H,I) siRNA. All results were confirmed in at least three independent experiments. Data were presented as the mean ± SEM. $^{*}P < 0.05$, compared with control.

normalized collagen expression via phosphorylation of Smad3 in systemic sclerosis fibroblasts, and the depletion of S1PR1 abrogates the effects of dhS1P and S1P on Smad phosphorylation levels in normal and systemic sclerosis fibroblasts [Bu et al., 2010]. Meanwhile, we have provided evidence that S1PRs, mainly S1PR1/3 are involved in liver fibrosis by action on hepatic myofibroblasts motility and activation of hepatic stellate cells, resulting in high expression of collagen [Li et al., 2009a,b, 2011; Liu et al., 2011; Yang et al., 2012]. It should be noted that S1PR1/3 also regulate collagen expression in mouse BMSCs, but in a positive-, not negative-regulation manner [Yang et al., 2012]. Due to species differences, experiments with animals are not always applicable to humans. It has been proven that the profile of cannabinoid receptor-1 expression in macrophages depends on species. Unlike murine macrophages, human macrophages display profound cannabinoid receptor-1 up-regulation in response to pro-inflammatory and pro-atherogenic stimuli [Han et al., 2009]. Also, unlike murine T regulatory cells, histone/protein deacetylases inhibitors do not enhance the transcription factor, FOXP3 mRNA or protein expression in purified human T regulatory cells [Akimova et al., 2010]. In our laboratory, we have identified an additional function regulated by S1PR1/3 involving the activation of human hepatic stellate cells [Liu et al., 2011], and this observation is in disagreement with studies performed by Brunati et al. in rat hepatic stellate cells, in which S1PR1 and S1PR2, but not S1PR3, were detectable [Ikeda et al., 2000; Brunati et al., 2008]. In previous studies, we have proven that mouse BMSCs can highly express collagen via

activating S1PR1/3 [Yang et al., 2012]. However, in the current study, we proved that collagen expression was negatively regulated by S1PR1 and S1PR3 in hMSCs.

In conclusion, we identify a key ability of S1PR1/3 to affect the regulation of collagen expression in hMSCs in a negative manner. This work provides new clues to the molecular mechanisms underlying the production of collagen elicited by the cytokine. Researchers should take note of which molecular mechanisms are different between human and mouse cells. These results expand our understanding of S1PRs and the regulation of collagen expression. Recently, MSCs have been used as a therapeutic tool for liver fibrosis treatment. Our results reveal the possibility that MSCs can secrete collagen during pathological processes. Care should therefore be taken when utilizing MSCs in clinical applications.

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