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Histone deacetylase 4 is required for TGFβ1-induced myofibroblastic differentiation

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Abstract

Transforming Growth Factor β1 (TGFβ1) is a crucial cytokine triggering myofibroblastic (MF) differentiation, a process involved in tissue healing as well as in pathologic conditions such as fibrosis and cancer. Together with cell shape modifications, TGFβ1-mediated differentiation of fibroblasts into myofibroblasts is characteristically associated with the neo-expression of smooth muscle α-actin (α-SMA), a cytoskeletal protein that enhances their contractile activity. Several cellular differentiation programs have been linked to epigenetic regulation of gene expression, including gene methylation and histone acetylation. Herein, we sought to investigate the role of histone deacetylases (HDAC) in TGFβ1-induced MF differentiation. We found that TSA, a global inhibitor of class I and class II HDACs, prevented α-SMA transcript and protein expression and morphological changes mediated by TGFβ1 in cultured human skin fibroblasts. In order to identify the HDAC(s) participating in MF differentiation, the impact of specific HDAC silencing (HDAC1 through HDAC8) using RNA interference was investigated in fibroblasts exposed to TGFβ1. Among the eight HDACs tested, silencing of HDAC4, HDAC6, and HDAC8 expression impaired TGFβ1-induced α-SMA expression. HDAC4 silencing most efficiently abrogated α-SMA expression and also prevented TGFß1-mediated morphological changes. Forced down-regulation of HDAC4 stimulated the expression of 5′-TG-3′-Interacting Factor (TGIF) and TGIF2 homeoproteins, two known endogenous repressors of the TGFβ signaling pathway, but not of the inhibitory Smad7. Collectively, these data suggest that HDAC4 is an essential epigenetic regulator of MF differentiation and unveil HDAC4 as a potential target for treating MF-related disorders.

Keywords: Histone deacetylase; TGFβ1; TSA; Myofibroblast; α-SMA; TGIF; Smad7

1. Introduction

Transforming growth factor β1 (TGFβ1) is the prototype of a large family of growth factors that regulate a wide range of biological processes, exerting their functions both during embryogenesis, in terms of organogenesis and morphogenesis, and in the adult organism, where they orchestrate complex phenomena such as inflammation, tissue repair and neoplastic transformation/progression [1]. TGFβ1 broad activities include, among others, context-specific inhibition or stimulation of cell proliferation, control of extracellular matrix (ECM) synthesis and degradation, control of epithelial-to-mesenchymal transition, mediation of cell and tissue responses to injury, control of carcinogenesis, and modulation of immune functions. Remarkably, TGFβ1 is sufficient to induce the generation of granulation tissue and angiogenesis *in vivo* and is the prominent growth factor able to stimulate the differentiation of fibroblasts into myofibroblasts, a process termed myofibroblastic (MF) differentiation that contributes to the pathobiology of chronic fibrotic disease [2–4].

The observation that mesenchymal cells are capable of transforming into myofibroblasts during tissue repair and under several pathologic conditions, including fibrocontractive diseases (e.g. Dupuytren's disease or hypertrophic scars), fibrosis (e.g. cirrhosis), and stromal reactions to tumor growth and invasion, has been extensively documented [5,6]. One of the main features of myofibroblasts is represented by an important contractile apparatus [7], and in particular by the neo-expression of the smooth muscle α-actin (α-SMA) isoform [8]. Myofibro-

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blasts are indeed morphologically and biochemically intermediate between fibroblasts and smooth muscle cells and are recognized to play a central role in closing the wound tissue, through their capacity to produce a strong contractile force [6,9–11]. In fact, in addition to being a well-accepted marker of myofibroblast differentiation, α-SMA has been shown to be sufficient to enhance fibroblast contractile activity [12,13].

TGFβ1-mediated MF differentiation is accompanied by changes in the expression of many genes, including cytoskeletal proteins (e.g. α-SMA), ECM proteins (e.g. collagens type I and III, fibronectin, and proteoglycans such as versican), and proteins inhibiting ECM degradation (e.g. PAI-1, Timp-1) [5,6]. TGFβ1 exerts its biological activities mainly through transcriptional regulation of diverse genes. Upon binding of this growth factor to its transmembrane serine/threonine kinase receptors, coregulatory proteins Sma- and Mad-related proteins Smad2 and Smad3 are phosphorylated by the receptors, form heterodimers with Smad4, and migrate into the nucleus to form multisubunit transcriptional complexes regulating target gene transcription [14–16]. TGFβ1 is able to mediate diverse responses because of the interaction of Smad complexes in the nucleus with different partner proteins that may be specific to a particular cell type and for a given set of environmental conditions [17]. Previously it has been suggested that the total cellular levels of Smad transcriptional co-activators and co-repressors factors play a critical role in controlling and fine-tuning the biological responses to TGFβ [16]. These regulatory co-factors may function by recruiting histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity to the target promoter [17–19]. Thus, some of TGFβ-driven MF differentiation-associated gene expression changes appear to be regulated epigenetically, through modifications of DNA methylation and histone acetylation levels. Recent data have also pointed at a role of HATs and HDACs in modulating the acetylation levels – and consequently the transcriptional activity – of Smad proteins [20].

In mammalian cells, HDACs constitute a family of 18 enzymes that regulate gene expression by modifying the acetylation level of nucleosomal histones and nonhistone proteins, including several transcription factors and tumor suppressors. These enzymes are usually separated into 3 classes on the basis of their similarity to various yeast histone deacetylases: (i) class I members (HDAC1, HDAC2, HDAC3, HDAC8, and HDAC11), which are homologous to the yeast RPD3 protein (ii) class II HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10), which have similarities to yeast HDA1 and, (iii) sirtuin (SIRT) proteins, which are homologous to the yeast SIR2 protein and require nicotinamide adenine dinucleotide (NAD) as a cofactor [21]. To date, 7 human SIRT homologues have been uncovered. Accumulating evidence suggests that histone deacetylase family members are involved in numerous important biological activities, ranging from hormone-induced gene regulation to cell proliferation, differentiation, apoptosis and senescence [21]. Previous reports have suggested that global suppression of HDAC activities by inhibitors targeting both class I and class II HDACs, inhibits both angiogenesis [22,23] and TGFβ1-induced fibrogenesis[24–26], two important processes occurring during wound healing.

The present study was aimed at (i) determining the influence of global HDAC inhibition on TGFβ1-mediated MF differentiation and (ii) identifying the HDAC(s) participating in this process. TSA was found to prevent α-SMA transcript and protein expression and morphological changes mediated by TGFβ1 in primary human skin fibroblasts. Among the eight first HDAC members tested, RNA interference-mediated silencing of HDAC4 most efficiently abrogated TGFβ1-induced phenotypic transformation. HDAC4 silencing was associated with an increased expression of 5′-TG-3′-Interacting Factor (TGIF) and TGIF2, two homeodomain proteins known to repress TGFβ activities. Altogether, these data indicate that HDAC4 is a required epigenetic mediator of MF differentiation driven by TGFβ1.

2. Materials and methods

2.1. Cell lines, reagents, and treatments

Primary human skin fibroblasts were established by outgrowth of normal skin biopsies as previously described [27]. Different batches of these cells were randomly selected for the experiments outlined below. Cells were maintained in Dubelcco's modified Eagle's medium (DMEM) supplemented with 10% decomplemented (heat-inactivated) fetal bovine serum (FBS; ICN Pharmaceuticals, Costa Mesa, CA, USA), penicillin (100 units/mL), streptomycin (100 μg/mL) and 2 mM L-glutamine at 37 °C in a humidified 95% air/5% CO_2 atmosphere. Cells were sub-cultured by trypsinization (0.05% trypsin, 0.53 mM EDTA) in phosphate-buffered saline (Dulbecco's PBS (DPBS) without calcium, magnesium, and sodium bicarbonate). Cells were reseeded at a ratio of 1:3 and used between passages 7 and 14. Absence of mycoplasma infection was routinely checked. All tissue culture reagents were obtained from Invitrogen (Merelbeke, Belgium) unless otherwise specified.

Cells were treated with trichostatin A (TSA) (Sigma Chemical Company, St. Louis, MO, USA) at a final concentration of 50, 200, or 500 nM in DMSO every 24 h and/or TGFβ1 (Roche Diagnostics, Indianapolis, IN, USA) at a final concentration of 2.5 ng/mL in PBS every 24 h. Control conditions consisted of cells incubated with vehicles alone. Twenty-four hours after initial plating, cells were incubated with DMEM containing 1% FBS and the appropriate drug or vehicle upon start of the experiment.

2.2. Small interfering RNA (siRNA) transfection

The siRNAs used in this study were synthesized by Eurogentec (Seraing, Belgium) and Dharmacon (Lafayette, Colorado, USA) and were all made of 2 complementary nucleotide strands containing 21 bases. The sequences of the oligonucleotides are provided in Table 1; most of them have been utilized in previous studies [27–29]. Each pair of oligonucleotides was received annealed at a concentration of 100 μM in 50 mM Tris pH 7.5–8.0, 100 mM NaCl. Cells were transfected at a starting confluence between 40% and 50%. Calcium phosphate-mediated transfection was performed in 6-well plates with a final concentration of 40 nM of each siRNA, unless otherwise specified.

After 16 h of transfection, cells were rinsed twice with PBS, incubated in 10% FBS medium with or without TGFβ1 (2.5 ng/mL in PBS). Medium was changed every 24 h and cells were collected following 48 h of treatment. Mock transfections with transfection reagent alone served as negative control. Controls also included three different RNA duplexes: a scrambled HDAC4 siRNA (scHDAC4 siRNA) whose sequence corresponded to the reverse, and subsequently modified (inversion of 4 bases), sequence of the HDAC4#2 siRNA, a siRNA directed against GL3 luciferase [30], and a negative control siRNA designed by, and purchased from, Eurogentec (OR-0030-neg05).

2.3. Antibodies

Anti-HDAC4 antibody was raised against a synthetic peptide corresponding to residues surrounding amino acid 10 of human HDAC4 (Cell Signaling #2072). Anti-smooth muscle α-actin (α-SMA, clone 1A4) antibody was from

Table 1
siRNA oligonucleotidic sequences used

siRNA designation	Target mRNA	siRNA oligonucleotide sequence
HDAC1	HDAC1	5′-AAGCCGGUCAUGUCCAAAGUA-3′ 5′-UACUUUGGACAUGACCGGCUU-3′
HDAC2	HDAC2	5′-GCCUCAUAGAAUCCGCAUGTT-3′ 5′-CAUGCGGAUUCUAUGAGGCTT-3′
HDAC3	HDAC3	5′-AAUCAGAACUCACGCCAGUAU-3′ 5′AUACUGGCGUGAGUUCUGAUU-3′
HDAC4#1	HDAC4	5′-GACGGGCCAGUGGUCACUGTT-3′ 5′-CAGUGACCACUGGCCCGUCTT-3′
HDAC4#2	HDAC4	5′-CGACAGGCCUCGUGUAUGAUU -3′ 5′-UCAUACACGAGGCCUGUCGUU -3′
HDAC4#3	HDAC4	5′-GAAUGUACGACGCCAAAGAUU-3′ 5′-UCUUUGGCGUCGUACAUUCUU-3′
scHDAC4#2	None	5′-AGUCUGGUCCCGUGAAAGCUU-3′ 5′-GCUUUCACGGGACCAGACUUU-3′
HDAC5	HDAC5	5′-AACAGCAUGACCACCUGACAA-3′ 5′-UUGUCAGGUGGUCAUGCUGUU-3′
HDAC6	HDAC6	5′-CUGCAAGGGAUGGAUCUGATT -3′ 5′-CUGCAAGGGAUGGAUCUGATT-3′
HDAC7	HDAC7	5′-GGACAAGAGCAAGCGAAGUTT-3′ 5′-ACUUCGCUUGCUCUUGUCCTT-3′
HDAC8	HDAC8	5′-UGAGCCCCACCGAAUCCAATT-3′ 5′-UUGGAUUCGGUGGGGCUCATT-3′
GL3 Luc	GL3 Luciferase	5′-CUUACGCUGAGUACUUCGAUU-3′ 5′-UCGAAGUACUCAGCGUAAGUU-3′
EGT	None	Eurogentec non disclosed sequence (Cat N° OR-0030-Neg05)

Dako (Glostrup, Denmark) and anti-α-tubulin (clone B512), anti-β-actin (clone AC15) monoclonal antibodies were from Sigma-Aldrich (Bornem, Belgium). Human osteosarcoma SaOS-2 cells transfected with a flag-tagged human HDAC4 expression vector (kindly provided by Dr. E. Verdin, Gladstone Institutes of Virology and Immunology, UCSF, CA, USA) using FUGENE 6™ transfection reagent were used as positive controls in the immunoblot experiments.

2.4. Western blot analysis

After rinses in PBS, *in vitro* grown subconfluent cells were scrapped in 1% SDS containing phosphatase inhibitors (1 mM Na_3VO_4) and Complete® protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Protein lysates were cleared by centrifugation at 10,000×*g* for 1 min in 20 μm columns (Machery-Nagel, Postfach, Düren, Germany). The shredded lysates were retrieved and frozen at −80 °C until use in immunoblot assays. The protein concentration was measured using a bicinchoninic acid determination kit (Pierce Chemical Co., Rockford, IL, USA). Equal amounts of protein lysates were resolved by size on 7.5%, 10% or 12% SDS-PAGE gels and transferred onto nitrocellulose (Biorad, Hercules, CA, USA) or polyvinylidene difluoride (Roche Diagnostics, Mannheim, Germany) membranes. Equal protein loading and transferring was examined by Coomassie gel staining (data not shown) and β-actin normalization. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (20 mM Tris base (pH 7.6), 150 mM NaCl) containing 0.1% or 0.05% Tween-20 (TBS-T), and probed with the following primary antibodies: anti-HDAC4, anti-α-SMA, anti-α-tubulin and anti-β-actin. After washing in TBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA) and developed using enhanced chemiluminescence detection system (ECL detection kit; Amersham Corp., Arlington Heights, IL), according to the instructions of the manufacturer. Membranes were exposed to Kodak X-Omat AR films.

2.5. Immunoperoxidase detection of smooth muscle α-actin (α-SMA)

Detection of smooth muscle α-actin (α-SMA) protein in human cells was performed with the use of an immunoperoxidase technique and the ABC Vectastain Elite kit (Vector Laboratories, Inc., Burlingame, CA, USA) according to the supplier's directions with some modifications. Primary human skin fibroblasts were seeded and grown onto poly-L-lysine-coated glass slides, washed with PBS, and then fixed with freshly prepared 3% paraformaldehyde for 10 min. Cells were permeabilized with 0.25% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA) in PBS for 10 min and washed with PBS again. After blocking of the endogenous peroxidase activity with 1.5% hydrogen peroxide in methanol for 30 min, the slides were heated for antigen retrieval in a water-bath at 95 °C in citrate buffer, allowed to cool down, and then incubated with 1% normal horse serum in PBS for 30 min. Mouse anti-α-SMA antibody at a dilution of 1:400 was incubated overnight at 4 °C, followed by biotinylated horse anti-mouse and the avidin–biotin–peroxidase complex. Washes were performed 3 times with PBS after each incubation step. Peroxidase activity was developed by a solution of 3–3′ diaminobenzidine tetrahydrochloride (DAB) (Vel, Leuven, Belgium) dissolved in PBS and 0.03% H_2O_2. The DAB solution was filtered and applied to the sections for 3 min. Finally, Carazzi's hematoxylin was used to counterstain the slides that were then dehydrated and mounted. Experiments in which the first antibody was omitted in the assay were used as controls. Photomicrographs of the slides were taken with a Zeiss microscope.

2.6. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the RNeasy mini kit (Qiagen, Inc., Valencia, CA), according to the manufacturer's protocol. For cDNA synthesis, 0.5 to 2 μg of total RNA were reverse transcribed in a 20-μL reaction mixture containing 0.2 μg of random hexamers (Amersham Biosciences, Little Chalfont, UK), 2 mM of each deoxynucleotide triphosphate (dNTP) (Eurogentec, Seraing, Belgium), 1× first-strand buffer (50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$) (Invitrogen), 10 mM dithiothreitol (Invitrogen), and 100 units of SuperScript™ II RNase H reverse transcriptase (Invitrogen). The RT reaction was performed at 42 °C for 50 min before a 15-min inactivation step at 70 °C. Reactions not containing the reverse transcriptase were used as controls. Quantitative real-time PCR was performed using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. PCR was performed using the TaqMan® PCR Universal Master Mix (Applied Biosystems). The sequences of the primers and probes for the human HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, and α-SMA genes were designed using the Primer Express software (Applied Biosystems)[31]. Taqman® primers and probes for the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, 18S RNA, cyclophilin A, 5′-TG-3′-interacting factor (TGIF, also termed TGF-ß-interacting factor), 5′-TG-3′-interacting factor 2 (TGIF2), and Sma and Mad-related protein 7 (SMAD7) genes were purchased from Applied Biosystems. All sets of primers and probes were selected to work under similar cycling conditions. cDNA samples (100 ng each) were mixed with 100 nM of each primer and TaqMan® Universal PCR Master Mix containing 1× PCR buffer, 5.5 mM $MgCl_2$, 0.8 mM dNTPs mix, 100 nM probe and 1 unit of AmpliTaq Gold® thermostable DNA polymerase (Applied Biosystems) in a total volume of 25 μL. Taqman® real-time PCR was performed for each target cDNA and transcript expression levels were normalized to those of GAPDH, 18S RNA, or cyclophilin A. All samples were run as triplicates. Acquired data were analyzed using the Sequence Detector software (Applied Biosystems).

2.7. Lactate dehydrogenase (LDH) release assay

In order to assess any potential cytotoxicity associated with TSA treatment or siRNA transfection of the cells, LDH activity in culture supernatants (triplicate per each condition) was determined using the fluorescent-base CytoTox-One homogenous membrane integrity assay (Promega Corp., Madison, WI, USA), according to the manufacturer's instructions.

2.8. Statistical analysis

Expression ratios were associated with their standard errors. An asymptotically Normal test was used to test for differences in expression ratios between two different experimental conditions. Unless otherwise specified, expression

ratios were compared with those of TSA-untreated cells or mock-transfected cells. Differences were considered significant when $p<0.05$.

3. Results

3.1. Trichostatin A, a global class I and class II HDAC inhibitor, prevents TGFβ1-mediated MF differentiation

To first examine the potential implication of HDACs in the process of TGFβ1-induced fibroblast-to-myofibroblast differentiation, human skin fibroblasts were exposed to TGFβ1 and/or trichostatin A (TSA) for 24 and 48 h. Morphological changes were then assessed and relative amounts of α-SMA transcript and protein levels were evaluated by real time Taqman® RT-PCR and Western blot/immunocytochemistry, respectively. As shown in Fig. 1A, TGFß1 treatment resulted expectedly in a markedly increased production of α-SMA by fibroblasts, as determined by Western blot analysis. In contrast, TSA down-regulated the abundance of α-SMA in cells either exposed to TGFβ1 or not, and completely suppressed the TGFβ1-induced overexpression of this cytoskeletal protein. The efficiency of HDAC inhibition by TSA was verified by analyzing the amount of acetylated α-tubulin, which was clearly augmented in the TSA-treated cells, in agreement with previous studies [28,32].

TGFβ1 not only can trigger the expression of α-SMA, one of the major markers of MF differentiation, but also can induce morphological changes that are intermediate between those featured by fibroblasts and smooth muscle cells. With the use of immunocytochemistry to detect α-SMA in fibroblasts treated with or without TGFβ1 for 48 h, we observed that the majority of the TGFß1-treated cells expressed α-SMA that exhibited a cytoplasmic cable-like distribution and displayed typical MF morphology with cellular hypertrophy (Fig. 1B). In contrast, only a very small proportion of the fibroblasts not exposed to exogenous TGFβ1 exhibited such MF features. Importantly, TSA treatment fully prevented the induction of TGFβ1-mediated MF changes and reverted the small MF-like subpopulation present in the TGFβ1-non-treated condition to a fibroblastic phenotype. None of the TSA-treated fibroblasts displayed evident α-SMA expression. We thus concluded that

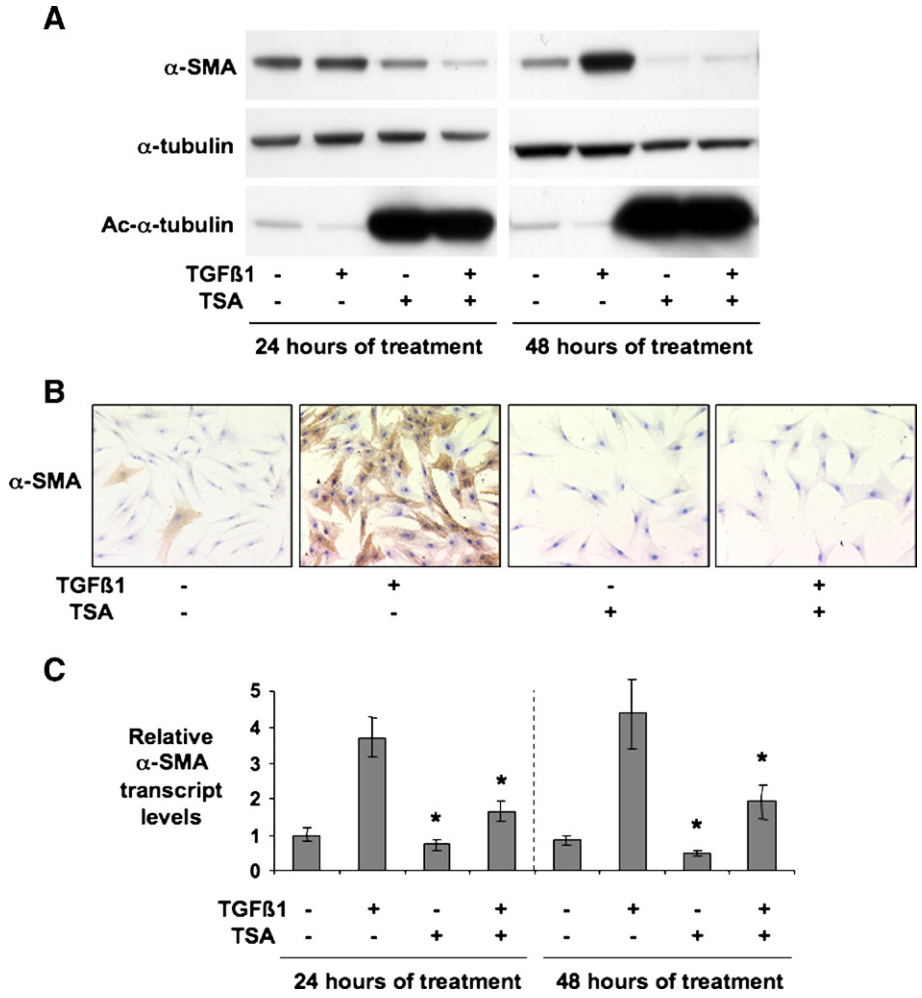

Fig. 1. Trichostatin A (TSA), a global class I and class II HDAC inhibitor, prevents TGFβ1-mediated myofibroblastic differentiation. After 24 or 48 h treatment of human primary fibroblasts with TGFβ1 (2.5 ng/mL), TSA (500 nM) or their respective vehicle (PBS and DMSO), the relative abundance of α-SMA transcript and protein was assessed using immunoblot (A), immunocytochemistry (B) and real time RT-PCR (C), as described in Materials and methods. Each experiment was repeated at least twice and consistent results were observed. Statistically significant reductions in relative α-SMA transcript levels ($p<0.05$) between TSA-treated and TSA-untreated conditions, either in absence or in presence of TGFβ1, are indicated as an asterisk.

TSA, an inhibitor of class I and class II HDACs, was able to prevent TGFβ1-mediated MF differentiation of human skin fibroblasts *in vitro*, as assessed morphologically as well as biochemically by analyzing cellular α-SMA abundance.

Since HDACs play a key role in the regulation of gene transcription by modifying core histone acetylation levels, we next asked whether TSA was able to influence α-SMA mRNA steady-state abundance in fibroblasts treated with or without TGFβ1. As shown in Fig. 1C, while TGF-β1 treatment induced a±3–4 fold increase in α-SMA mRNA steady-state levels (normalized to GAPDH mRNA levels), TSA treatment resulted in decreased levels of the transcript at both 24 and 48 h time points (2 fold decrease after 48 h of treatment; $p<0.05$). TGFβ1-mediated increase in α-SMA mRNA levels was significantly impaired when the cells were concomitantly exposed to TSA ($p<0.05$). Similar results were obtained when other reference genes, including cyclophilin A, β-actin, and 18S RNA, were used for normalizing α-SMA transcripts levels in the RT-PCR experiments (data not shown). Therefore, global inhibition of class I and class II HDAC activities was shown to repress α-SMA expression possibly at the transcriptional level. This repressive activity exerted by TSA was dose-dependent (Fig. 2A) and was not due to a cytotoxic effect. Indeed, when fibroblasts were relieved from TSA after 48 h of treatment, TGFß1 was still able to induce α-SMA transcript expression (Fig. 2A). Furthermore, TSA treatment did not significantly increase lactate dehydrogenase (LDH) activity levels in culture supernatants (Fig. 2B).

3.2. Influence of specific HDAC silencing on TGFβ1-mediated β-SMA induction

We next sought to determine which HDAC(s) might be involved in the process of TGFβ1-mediated MF differentiation. To this end, fibroblasts were transfected with siRNAs directed against most class I and class II HDACs (HDAC1 through HDAC8) for 16 h and then treated with TGFβ1 for 48 h. The capacity of each siRNA to silence its target HDAC was checked by real time RT-PCR analysis. Each siRNA transfection was found to reduce by at least 70% the amount of the corresponding HDAC mRNA, with the exception of HDAC8 (Fig. 3A). In addition, TGFβ1 treatment of the cells did not substantially affect the mRNA level of all the HDACs tested (less than 20% variation; data not shown).

Fold inductions of α-SMA transcript expression by TGFβ1 (±3–4 fold) were similar in fibroblasts transfected without

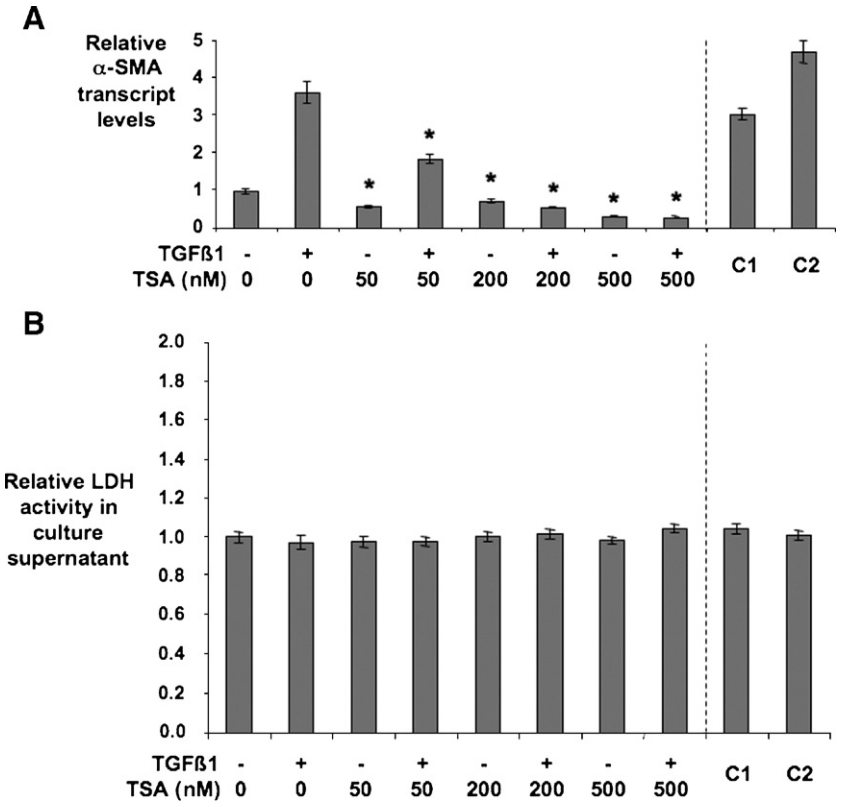

Fig. 2. TSA represses α-SMA transcript expression by human primary fibroblasts in a dose-dependent manner without triggering cellular toxicity. Human primary fibroblasts were treated with TGFβ1 (2.5 ng/mL) and/or TSA at various concentrations (0, 50, 200, and 500 nM) for 48 h. Fibroblasts were also treated either with TGFß1 (2.5 ng/mL) and DMSO (condition C1) or with TGFβ1 (2.5 ng/mL) and TSA (500 nM) (condition C2) for 48 h, and then further for 48 h in both conditions with TGFβ1 (2.5 ng/mL) and DMSO (no TSA). (A) The relative abundance of α-SMA transcripts was assessed in the different experimental conditions using real time RT-PCR, as described in Materials and methods. (B) Lactate dehydrogenase (LDH) activity in culture supernatants was determined using a fluorescent-based membrane integrity assay to evaluate potential cytotoxicity associated with TSA treatment of the cells. Each experiment was performed twice and consistent results were observed. Data were expressed as means±standard deviation. Statistically significant differences in α-SMA transcript levels and in LDH activity levels between TSA-treated and TSA-untreated conditions, either in absence or in presence of TGFβ1, are indicated as an asterisk ($p<0.05$).

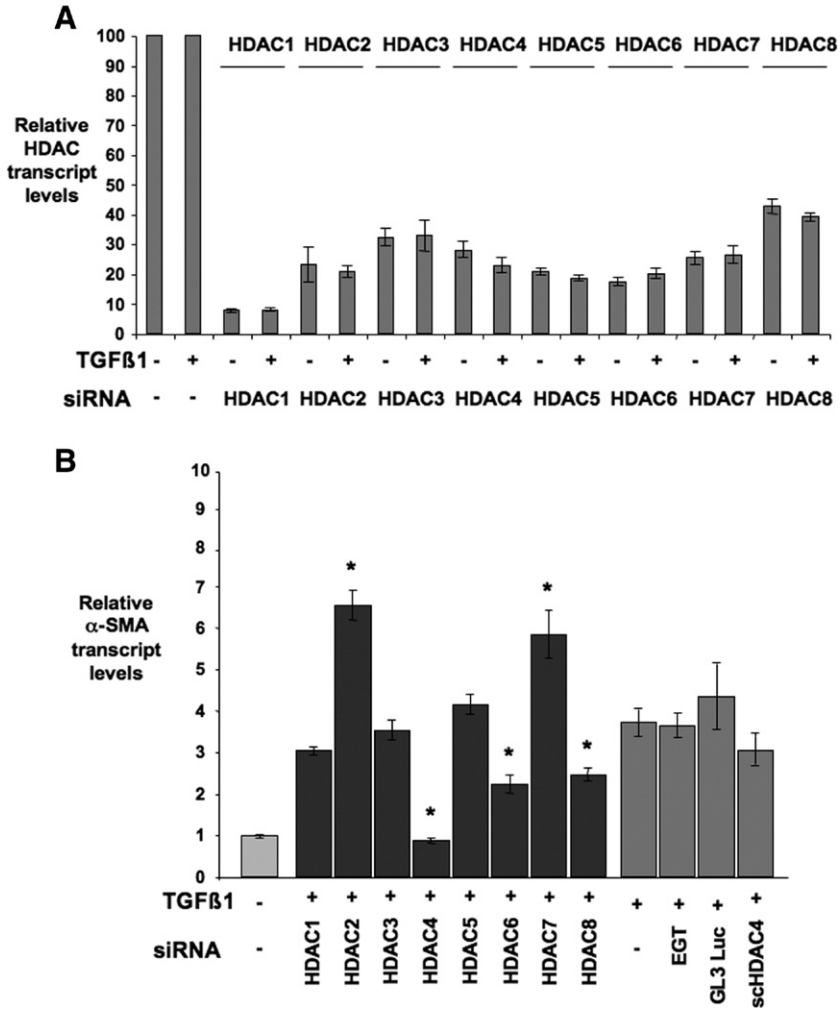

Fig. 3. Screening of the influence of specific HDAC silencing on TGFβ1-mediated myofibroblastic differentiation. Primary human fibroblasts were transfected with siRNAs directed against each of the 8 first members of the HDAC family (HDAC1 through HDAC8) and then treated with or without TGFβ1 (2.5 ng/mL in PBS) for 48 h. Mock transfections with transfection reagent alone served as negative control. Controls also included three different RNA duplexes: a scrambled version of HDAC4 siRNA (scHDAC4 siRNA), a siRNA directed against GL3 luciferase (GL3 Luc), and Eurogentec (EGT) negative control siRNA. The relative transcript levels of each HDAC (A) as well as those of α-SMA (B) (normalized to GAPDH transcript levels) were assessed by real time RT-PCR, as described in Materials and methods. Each experiment was repeated at least twice and consistent results were observed. Data were expressed as mean ratios ± standard deviation. Statistically significant differences in relative α-SMA transcript levels between control cells (mock-, EGT siRNA-, GL3 Luc siRNA-, and scHDAC4 siRNA-transfected cells) and cells transfected with each specific HDAC siRNA are indicated as an asterisk ($p<0.05$).

siRNA (mock) or transfected with siRNAs directed against HDAC1, HDAC3, HDAC5 or with various control siRNAs (EGT negative control siRNA, GL3 luciferase siRNA, or a scrambled HDAC4 siRNA) ($p>0.05$). A weaker TGFβ1-mediated α-SMA induction (±2 fold) was observed in HDAC6- and HDAC8-silenced cells ($p<0.05$) while a stronger α-SMA transcript induction (±6–7 fold) was found in HDAC2- and HDAC7-silenced, TGFβ1-treated fibroblasts ($p<0.05$) (Fig. 3B). Among the HDACs investigated, it appeared that HDAC4 silencing most efficiently abrogated TGFβ1-induced α-SMA overexpression. Thus, while global inhibition of class I and class II HDACs by TSA lead to a complete prevention of TGFβ1-mediated α-SMA induction, the specific silencing of a number of these HDACs yielded various effects on α-SMA abundance (stronger, weaker, or similar induction). Since HDAC4 silencing resulted in the most profound blocking of α-SMA induction – similar to that obtained when fibroblasts were treated with TSA – we further attempted to characterize the impact of HDAC4 in TGFβ1-mediated MF differentiation.

3.3. HDAC4 is necessary for TGFβ1-mediated MF differentiation

We first evaluated the toxicity of HDAC4 RNA interference using 3 different siRNAs directed against human HDAC4 mRNA. Measurements of LDH activity levels in culture supernatants following transfection of the cells with these siRNAs showed no cytotoxic effect of the siRNAs as compared with mock-, scrambled HDAC4 siRNA, and EGT negative control siRNA transfections (data not shown). In addition, no induction of 2′, 5′-oligoadenylate synthase-1 (OAS-1) transcript expression was observed following siRNA transfections (data

not shown), indicating that the small double-stranded RNAs did not trigger any interferon response by the transfected cells.

Among the 3 HDAC4 siRNAs tested, HDAC4 siRNA#2 and HDAC4 siRNA#3 most efficiently down-regulated HDAC4 transcripts in fibroblasts while the silencing effect of HDAC4 siRNA#1 was more modest ($p<0.05$) (Fig. 4A). As shown in Fig. 4B, TGFβ1-mediated α-SMA transcript induction was prevented in fibroblasts transfected with the most efficient HDAC4 siRNAs (#2 and #3) ($p<0.05$) while it was only modestly repressed in fibroblasts transfected with the less efficient HDAC4 siRNA#1 ($p<0.05$). In addition, HDAC4 silencing did not significantly decrease the transcript levels of HDAC1 through HDAC8 (Fig. 5A) ($p>0.05$). Similar results were observed when 2 reference genes, GADPH and β-actin, were used for normalizing α-SMA transcripts levels in the RT-PCR experiments (data not shown).

At the protein level, immunoblot analysis showed that silencing of HDAC4 using HDAC4 siRNAs #2 and #3 prevented the induction of α-SMA expression mediated by TGFβ1 (Fig. 5B). This repressive effect was weaker in HDAC4 siRNA#1-transfected cells. Immunocytodetection of α-SMA confirmed the immunoblot data and further showed that HDAC4 silencing using HDAC 4 siRNAs #2 and #3 fully prevented the induction of MF morphologic changes mediated by TGFβ1 (Fig. 6). Therefore, these results indicated that HDAC4 is required for efficient TGFβ1-mediated MF differentiation.

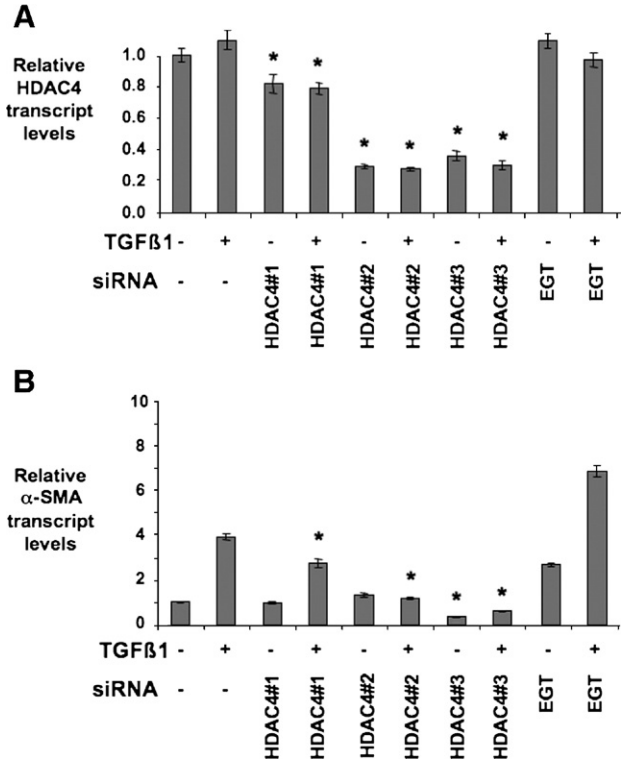

Fig. 4. HDAC4 is necessary for TGFβ1-mediated myofibroblastic differentiation. Primary human fibroblasts were transfected with 3 different siRNAs directed against HDAC4 and then treated with or without TGFβ1 (2.5 ng/mL in PBS) for 48 h. Mock transfections with transfection reagent alone and transfections with Eurogentec negative control siRNA served as negative controls. The relative levels of HDAC4 (A) and α-SMA (B) transcripts (normalized to GAPDH transcript levels) were assessed by real time RT-PCR, as described in Materials and methods. Each experiment was repeated at least twice and consistent results were observed. Statistically significant reductions in relative HDAC4 and α-SMA transcript levels between mock-transfected cells and cells transfected with each of the other siRNAs, either in absence or in presence of TGFβ1, are indicated as an asterisk ($p<0.05$).

3.4. HDAC4 silencing induces the expression of TGIF and TGIF2, two endogenous inhibitors of the TGFβ1 signaling pathway

Since histone deacetylases are usually associated with repression of gene expression, we next sought to investigate whether HDAC4 silencing would result in the induction of known endogenous inhibitors of the TGFβ pathway, including Smad7 [33–37], 5′-TG-3′-interacting factor (TGIF), and 5′-TG-3′-interacting factor 2 (TGIF2). As shown in Fig. 7, TGFβ1 slightly upregulated the transcripts of these inhibitors. No significant increase in Smad7 mRNA levels was observed in HDAC4-silenced TGFβ1-treated fibroblasts ($p>0.05$). In contrast, HDAC4 silencing in fibroblasts resulted in increased levels of TGIF and TGIF2 transcripts, whether cells were exposed to TGFβ1 or not ($p<0.05$).

4. Discussion

With the exception of a subset of myofibroblasts residing in specific normal human tissues [31], most myofibroblasts are thought to arise locally from resident fibroblasts as a consequence of tissue injury and acquire some of the contractile proteins normally expressed by smooth muscle cells such as α-SMA. These hypertrophic stromal cells typically contain numerous smooth muscle actin stress fibers that contribute to the generation of isometric mechanical force required for wound contraction and closure [13,38]. Chronic myofibroblast activation has been associated with a number of pathological conditions associated with tissue remodeling, including hypertrophic scarring, interstitial fibrosis, and stromal response to certain neoplasias. TGFβ1 is commonly considered as a master molecule for the general MF activation program. Other cytokines and growth factors, such as platelet-derived growth factor and tumor necrosis factor-α, do also bear profibrotic activity, but do not induce α-SMA in fibroblasts [3].

In this study, we have shown that inhibition of class I and class II HDAC activities by TSA abrogates TGFβ1-mediated *de novo* α-SMA transcript and protein expression and morphological changes characteristic of MF differentiation in human skin fibroblasts. These findings are consistent with those of a previous study by Rombouts et al., who have reported that TSA suppresses the inductive effect of TGFβ1 on α-SMA mRNA abundance in rat skin fibroblasts [24]. TSA treatment has also been shown to interfere with other biological processes mediated by TGFß1, such as the epithelial-to-mesenchymal transition of human renal proximal tubular epithelial cells [39] and the repression of the interferon α-induced MHC II transactivator (CIITA) gene in mouse microglial cells[40]. Thus, the results of our current study not only suggest that global HDAC inhibition may be a useful approach to prevent TGFß1-

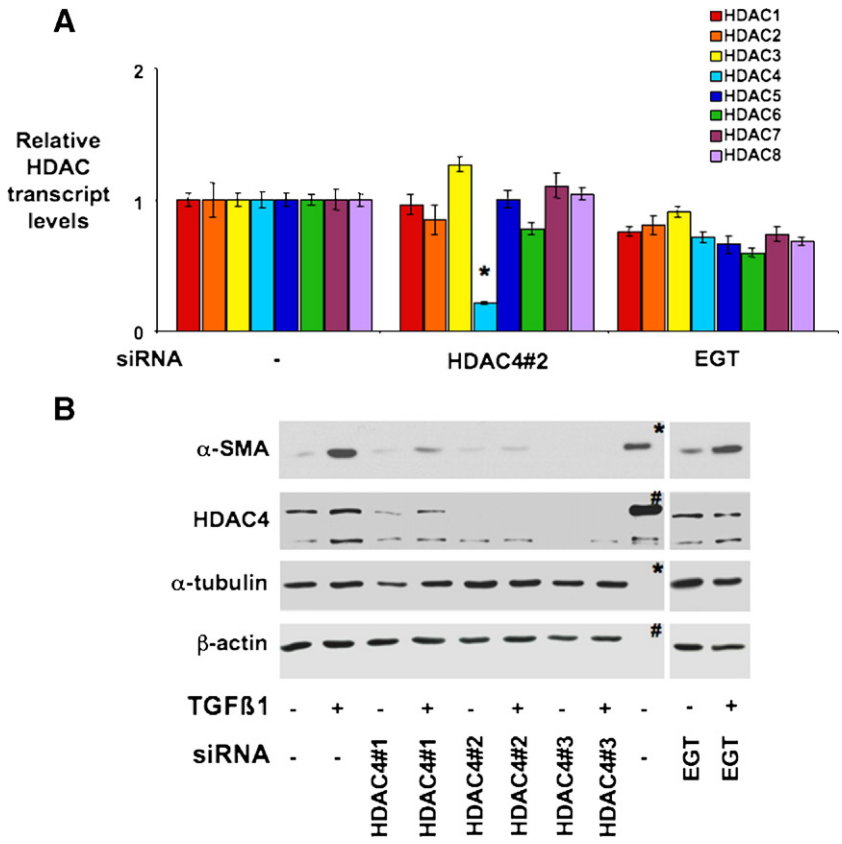

Fig. 5. (A) HDAC4 silencing does not significantly modify the transcript levels of HDAC1 through HDAC8 in primary human fibroblasts. Primary human fibroblasts were transfected with a siRNA directed against HDAC4 (HDAC4 siRNA#2). Mock transfections with transfection reagent alone and transfections with Eurogentec negative control siRNA served as negative controls. The relative transcript levels of HDAC1 through HDAC8 (normalized to GAPDH transcript levels) were assessed by real time RT-PCR, as described in Materials and methods. Data were expressed as mean ratios ± standard deviation. Statistically significant reductions in relative HDAC transcript levels between cells transfected with HDAC4#2 siRNA and both mock- and EGT siRNA-transfected cells are indicated as an asterisk ($p<0.05$). (B) TGFβ1 fails to induce α-SMA protein expression in the absence of HDAC4 in primary human fibroblasts. Primary human fibroblasts were transfected with 3 different siRNAs directed against HDAC4 and then treated with or without TGFß1 (2.5 ng/mL in PBS) for 48 h. Mock transfections with transfection reagent alone and transfections with Eurogentec (EGT) negative control siRNA served as negative controls. The relative levels of α-SMA, HDAC4, α-tubulin, and β-actin were assessed by immunoblot, as described in Materials and methods. Lanes marked by an * (α-SMA and α-tubulin) were loaded with total protein extracts from primary human smooth muscle cells. Lanes marked by a # (HDAC4 and β-actin) were loaded with total protein extracts from Saos2 cells transfected with a flag-tagged human HDAC4 expression vector. Experiments were repeated at least twice and consistent results were observed.

induced MF differentiation but also add weight to the growing evidence indicating that TGFß1 mediates its biological functions at least in part by epigenetic mechanisms of gene regulation involving histone deacetylase activities.

The negative impact of TSA on TGFβ1-mediated MF differentiation obviously represents the net effect of the cumulative inhibitory activity of the drug on all sensitive HDACs present within the fibroblasts. In order to investigate the involvement of individual HDACs in this process, we have evaluated the impact of specific HDAC gene silencing by RNA interference on α-SMA gene expression by fibroblasts treated with TGFβ1. Silencing of each of the eight first members of the histone deacetylase family resulted in diverse effects on α-SMA transcript abundance. On one hand, forced downregulation of HDAC4, HDAC6, and HDAC8 repressed TGFβ1-mediated α-SMA transcript levels; the most profound repression was observed with HDAC4 silencing, which resulted in an almost complete failure of TGFβ1-treated fibroblasts to overexpress α-SMA transcript and protein. Thus, at least 3 different HDACs may operate to drive or promote TGFβ1-mediated myofibroblastic differentiation. On the other hand, while knockdown of HDAC1, HDAC3, and HDAC5 had no effect on α-SMA transcript expression, silencing of HDAC2 and HDAC7 substantially potentiated the induction of α-SMA transcript in response to TGFβ1 exposure. The differential effects of each specific HDAC silencing on α-SMA expression are not unexpected and are in keeping with the results of previous reports on TGFß1-responsive genes. Indeed, prior studies have shown opposed effects of different HDAC inhibitors on the expression of one single gene (e.g. TSA/sodium butyrate versus valproic acid on Timp-1 expression [41]) as well as divergent impacts of one single HDAC inhibitor on the expression of different genes (e.g. TSA on type I collagen versus PAI-1 [26]). Sirtuins have not been considered in this study. However, these class III HDAC's may also prove in the future to be involved in the MF differentiation program. Indeed, it has been very recently shown that SIRT1, through its capacity to promote the deacetylation of Smad7 (and subsequently its ubiquitin/proteasome-mediated degradation), attenuates TGFβ1-induced apoptosis of murine mesangial cells [20].

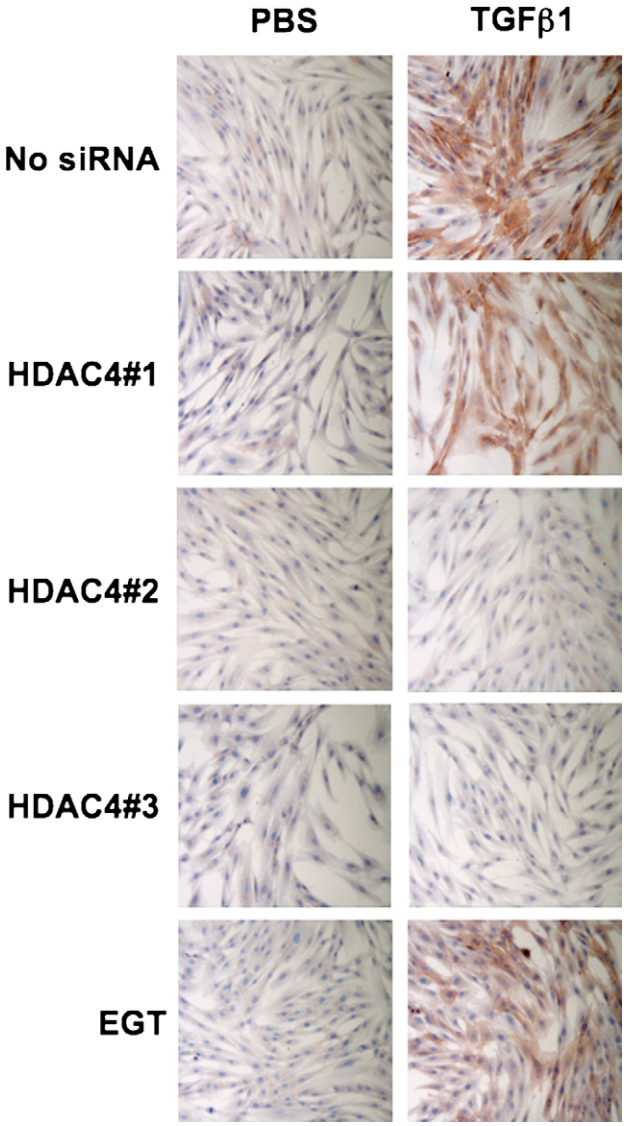

Fig. 6. HDAC4 silencing prevents TGFβ1-mediated induction of myofibroblastic morphologic changes in primary human fibroblasts. Primary human fibroblasts were transfected with 3 different siRNAs directed against HDAC4 and then treated with TGFβ1 or vehicle (PBS). Mock transfections with transfection reagent alone and transfections with Eurogentec (EGT) negative control siRNA served as negative controls. Expression of α-SMA was assessed by immunocytostaining, as described in Materials and methods. Experiments were repeated at least twice and consistent results were observed.

Our data have clearly indicated that HDAC4 is essential for TGFß1-mediated MF transdifferentiation, as assessed phenotypically. Remarkably, under our experimental conditions, HDAC4 silencing was almost as efficient as TSA to prevent the induction of α-SMA gene expression by TGFβ1. In fact, it may not be intriguing that HDAC4 is necessary for MF differentiation. This HDAC indeed represents a major contributor to several other differentiation programs, including those related to osteoblasts [42], chondrocytes [43], and muscle cells [44–48]. Whether common pathways are affected during these differentiation processes remains to be elucidated.

Since HDAC activities are generally associated with transcriptional repression and the stimulation of α-SMA expression by TGFβ1 is repressed by HDAC4 silencing, we have reasoned that HDAC4 silencing may exert its negative regulation on α-SMA expression by inducing endogenous repressing activities of the TGFß pathway. In other words, HDAC4 may be necessary to silence the expression of TGFβ pathway repressors to enable α-SMA expression. In addition, it has been previously observed that the inhibitory effect of TSA on TGFβ1-mediated α-SMA transcription is cycloheximide-sensitive, i.e. requiring *de novo* protein synthesis of a putative TGFβ pathway repressor [24]. Other potential mechanisms by which HDAC4 silencing may negatively affect the expression of α-SMA encompass

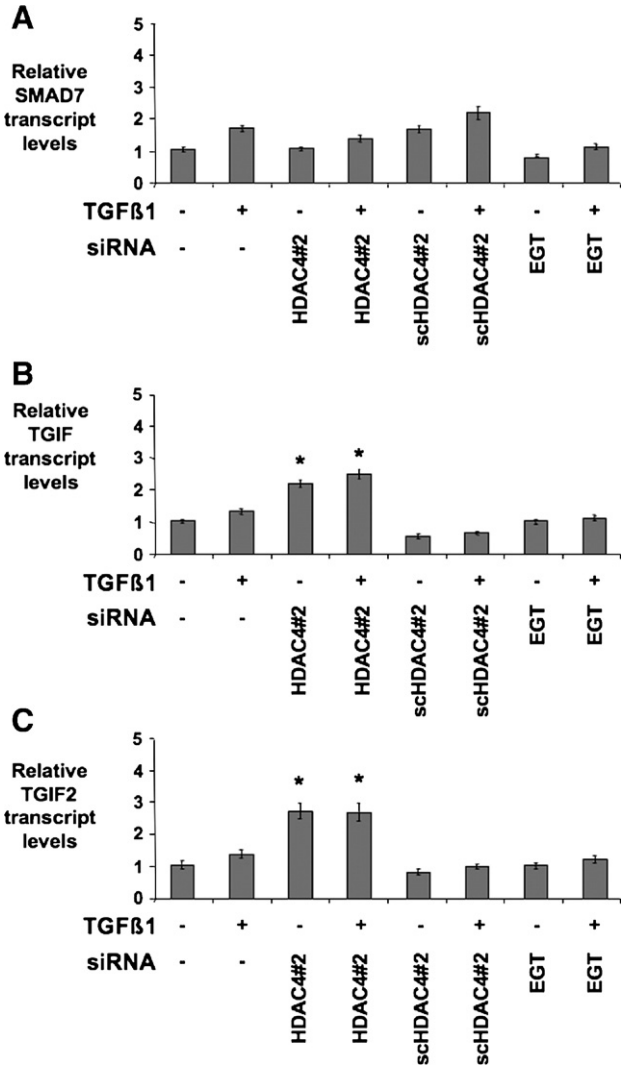

Fig. 7. HDAC4 silencing induces the expression of TGIF and TGIF2, two endogenous inhibitors of the TGFβ1 signaling pathway, in primary human fibroblasts. Primary human fibroblasts were transfected with a siRNA directed against HDAC4 (HDAC4 siRNA#2), with transfection reagent alone, with Eurogentec negative control siRNA, or with a scrambled HDAC4 siRNA (scHDAC4#2 siRNA), and then treated with TGFβ1 or vehicle (PBS). The relative levels of Smad7, TGIF, and TGIF2 transcripts were assessed by real time RT-PCR analysis, as described in Materials and methods. Data were expressed as mean ratios ± standard deviation. Statistically significant increases in relative Smad7, TGIF, and TGIF2 transcript levels between cells transfected with HDAC4#2 siRNA and mock-, scrambled HDAC4#2 siRNA, and EGT siRNA-transfected cells are indicated as an asterisk ($p<0.05$). Experiments were repeated at least twice and consistent results were observed.

direct hyperacetylation – and subsequent stabilization and/or increased activity – of transcriptional corepressors of the α-SMA gene and HDAC4 deacetylase activity-independent effects. A number of TGFß pathway repressors have been characterized and include the well known inhibitory Smad7 protein [33,37] as well as TGIF and TGIF2. The latter are DNA-binding homeoproteins, which share two major blocks of similarity encompassing the homeodomain and a conserved carboxyl terminal repression domain and act as TGFβ specific Smad transcriptional corepressors through direct interactions with Smad2, Smad3, the Sin3 corepressor complex and HDAC1 [49–51]. TGIF may also be a component of a ubiquitin ligase complex that mediates the degradation of Smad2 in response to TGFß signaling [52]. Previous studies have shown that (i) Smad7 inhibits α-SMA expression in different cell types [53,54], (ii) ectopic expression of TGIF markedly suppresses Smad-mediated activation of TGFß1-responsive promoter activity and completely blocks TGFß1-induced α-SMA expression in cultured rat and mesangial cells [55] and (iii) TSA upregulates the expression of both TGIF and Smad7 transcripts in TGFß1-treated rat fibroblasts [24]. Therefore, we have been interested to find out whether HDAC4 silencing would result in an upregulation of these TGFβ pathway repressor molecules. We have found that Smad7, TGIF, and TGIF2 transcript abundance was increased in response to TGFß1 treatment, which is in agreement with previous studies suggesting that these proteins may act as negative feedback effectors to desensitize TGFβ1 action [24,56,57]. However, when fibroblasts were transfected with HDAC4 siRNAs, TGIF and TGIF2, but not Smad7 transcript levels were significantly upregulated, suggesting that (i) HDAC4 is involved in the regulation of the two homeodomain proteins and (ii) HDAC4 silencing mediates its inhibitory effects on TGFß1-mediated phenotypic changes through TGIF and TGIF2 induction. In addition, to our knowledge, these are the first data to indicate that TGIF and TGIF2 expression may be regulated by a HDAC. It will take another study to dissect the precise mechanisms whereby HDAC4 regulates TGIF and TGIF2 expression.

Overall, the data presented in the current study point at a necessary role for HDAC4 in the phenotypic transition of fibroblasts to myofibroblasts mediated by TGFß1, thereby unveiling HDAC4 as a potential target for treating MF differentiation-related pathological conditions.

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