Zurich Open Repository and Archive
University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2009

Histone deacetylase 7, a potential target for the antifibrotic treatment of systemic sclerosis

Hemmatazad, H ; Maciejewska Rodrigues, H ; Maurer, B ; Brentano, F ; Pileckyte, M ; Distler, J H W ; Gay, R E ; Michel, B A ; Gay, S ; Huber, L C ; Distler, O ; Jüngel, A

DOI: https://doi.org/10.1002/art.24494

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-18626
Journal Article
Accepted Version

Originally published at:
Hemmatazad, H; Maciejewska Rodrigues, H; Maurer, B; Brentano, F; Pileckyte, M; Distler, J H W; Gay, R E; Michel, B A; Gay, S; Huber, L C; Distler, O; Jüngel, A (2009). Histone deacetylase 7, a potential target for the antifibrotic treatment of systemic sclerosis. Arthritis and Rheumatism, 60(5):1519-1529.
DOI: https://doi.org/10.1002/art.24494

Histone deacetylase 7 – A potential target for the anti-fibrotic treatment of systemic sclerosis

Hossein Hemmatazad[1], Hanna Maciejewska[1], Britta Maurer[1], Fabia Brentano[1], Margarita Pileckyte[2], Jörg HW Distler [1/3], Renate E. Gay[1], Beat A. Michel[1], Steffen Gay[1], Lars C. Huber[1], Oliver Distler[1], Astrid Jüngel[1]

[1]Center of Experimental Rheumatology, University Hospital and Zurich Center of Integrative Human Physiology (ZIHP), Zurich, Switzerland

[2]Department of Rheumatology, Kaunas Medical University Hospital, Lithuania

[3]Department of Rheumatology, University of Erlangen-Nuremberg, Germany

To whom correspondence should be addressed. Center of Experimental Rheumatology, Zurich University Hospital, Gloriastr. 25, 8091 Zurich, Switzerland. E-mail: hossein.hemmatazad@usz.ch

Fellows` support list:

Hossein Hemmatazad: Schwyzer Stiftung

Hanna Maciejewska: EU Marie-Curie Grant/ZIHP

Britta Maurer: Olga-Mayenfisch Foundation

Astrid Jüngel: SNF – 320000-11684

All other co-authors are supported by their respective institutions

Abstract

Objective

Recently we have shown a significant reduction of cytokine-induced transcription of collagen type I and fibronectin in systemic sclerosis (SSc) skin fibroblasts upon treatment with Trichostatin A (TSA). Moreover, in a mouse model of fibrosis, TSA prevented the dermal accumulation of extracellular matrix. Here, we analyze silencing of HDAC7 as a possible mechanism of TSA to exert its anti-fibrotic function.

Materials and methods

Skin fibroblasts from patients with SSc were treated with TSA and/or TGF-β. Expression of HDACs (1-11), extracellular matrix proteins, connective tissue growth factor (CTGF) and intercellular adhesion molecule-1 (ICAM-1) were analyzed by Real-time PCR, Western blot and the Sircol Collagen assay. HDAC7 was silenced using siRNA.

Results

SSc fibroblasts did not show a specific pattern of expression of HDACs. TSA inhibited the expression of HDAC7 significantly whereas HDAC3 was up regulated. Silencing of HDAC7 decreased the constitutive and cytokine-induced production of collagen types I and III but not of fibronectin as TSA did. Most interestingly, TSA induced the expression of CTGF and ICAM-1, while silencing of HDAC7 had no effect on their expression.

Conclusions

Silencing of HDAC7 appears to be not only as effective as TSA, but also a more specific target for the treatment of SSc, because it does not up regulate the expression of profibrotic molecules like ICAM-1 and CTGF. This observation may lead to the development of more specific and less toxic targeted therapies against SSc.

Introduction

SSc is an autoimmune disease characterized by widespread vascular changes and progressive fibrosis of skin and internal organs. The etiology and pathogenesis of SSc are still unknown. Currently no effective treatment is available to inhibit the progression of SSc.

The term “epigenetics” refers to changes in gene activity, that are stable and inherited over rounds of cell division, but do not involve changes in the DNA sequence of the organism. Several epigenetic modifications have been described, such as DNA methylation and histone acetylation. In normal conditions, these modifications are balanced and reversible, but they may be altered in disease states. Epigenetic modifications have been shown to play a role in the pathogenesis of cancer as well as autoimmune and inflammatory disorders (1, 2). Recently, several publications reported epigenetic modifications in gene transcription in SSc (3-6). Therefore, the field of epigenetics may provide new therapeutic targets for treatment strategies.

The DNA in eukaryotic cells is tightly wrapped around octamers of histone proteins, restricting its accessibility to factors involved in DNA replication and transcription. Posttranslational modifications of histone proteins induce changes in the structure of chromatin and therefore modify gene expression. Hyperacetylated histones are generally found in transcriptionally active and hypoacetylated histones in transcriptionally silent regions. The acetylation state of chromatin proteins depends on the balance between the activities of HDACs and histone acetyltransferases (HATs). According to the structure and biologic activities, mammalian HDACs can be classified into four different classes: class I (HDAC1, 2, 3 and 8), class II (HDAC4, 5, 6, 7, 9 and 10), class III (Sirt1-7) and class IV (HDAC11) (7). HDACs have been

shown to be promising therapeutic targets for cancer therapy, as well as for inflammatory or fibrotic diseases (5, 8, 9).

Histone deacetylase inhibitors (HDACi) have emerged as a new class of agents for anticancer therapy. HDACi alter the balance of acetylation and affect many aspects of cellular function including cell growth, differentiation, cell death, cell-cell and cell-matrix interactions, and inflammatory responses (10). One of the first discovered HDACi with anti-tumor activity was TSA. However, the toxicity of TSA is still being controversially discussed (11). Even though individual HDAC isoforms have distinctive physiological functions, most known hydroxamate HDACi target members of class I and class II rather non-selectively. The production of inhibitors for specific HDAC isoforms are now in the focus of the pharmaceutical industry (12). Therefore, there is increasing interest to unravel the property and function of each isoforms and to explore their individual roles in the pathogenesis of certain diseases.

Recently, we have shown a significant reduction of cytokine-induced transcription of collagen type I and fibronectin in SSc skin fibroblasts upon treatment with TSA. In addition, we have demonstrated that the expression of total collagen protein in stimulated SSc skin fibroblasts was reduced by TSA. Moreover, TSA prevented the dermal accumulation of extracellular matrix in vivo in the mouse model of bleomycin-induced fibrosis (6). In the present study we analyzed the molecular mechanisms of TSA-mediated reduction of extracellular matrix in SSc. In order to define new target molecules, we measured the transcriptional level of individual HDACs (1-11) in SSc fibroblasts treated with TSA. We could show that TSA, as a non-specific HDACi, has different effects on individual HDACs. Most interestingly, TSA almost completely inhibited the transcription of HDAC7, whereas the transcripts for HDAC3 were up regulated. Specific gene knockdown of HDAC7 in SSc fibroblasts resulted in a reduced production of collagen types I and III both on the mRNA and protein level.

Therefore, therapies that specifically target HDAC7 could be safer than the non-selective HDACi TSA in the treatment of SSc, because silencing of HDAC7 does not increase the expression of pro-fibrotic molecules ICAM-1 and CTGF. Our results strongly support targeting HDAC7 to generate a more specific and less toxic anti-fibrotic agent for the treatment of SSc.

Materials and Methods

Patients and fibroblast cultures

Normal and SSc fibroblasts were obtained from the skin of patients with scleroderma and healthy controls. All patients fulfilled the criteria for SSc as suggested by LeRoy et al (13). All patients signed a consent form approved by the institutional review board of the Kaunas University, Lithuania. Primary cultures of human dermal fibroblasts were established by outgrowth and were cultured in Dulbecco`s modified Eagle`s medium (DMEM) containing 10% heat inactivated fetal calf serum (FCS), 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 2.5 µg/ml amphotericin B (all Gibco BRL, Basel, Switzerland). Fibroblasts of passages 3-8 in monolayer culture were used for experiments. Cells were treated with 10nM-2µM of TSA (Sigma, Buchs SG, Switzerland).

TaqMan RT-PCR

Total RNA was isolated from cultured cells using RNeasy-kit including DNAse treatment (Qiagen, Hombrechtikon, Switzerland) according to the instructions of the manufacturer. To generate cDNA, total RNA (300-500ng) was reverse transcribed using murine leukemia virus (MuLV) reverse transcriptase (RT) (2.5 U/µl), random hexamers (2.5 µM), dNTPs (2 mM each) and RNase inhibitor (1U/µl) (all Applied Biosystems, Rotkreuz, Switzerland). The reverse transcriptase reaction was performed in a total volume of 20 µl in a GeneAmp PCR cycler (Applied Biosystems, Rotkreuz, Switzerland) at 25°C for 10 min, followed by 30 min at 48°C and by 5 min at 95°C. Samples without enzyme in the reverse transcription reaction were used as negative controls (NRT) to exclude contamination with genomic DNA. Quantification of specific mRNA was performed by single reporter and Sybr Green Real-time PCR

using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) as described (14). Pre-developed primer-probes were used for HDAC1, 2, 8, PDGF-B (platelet-derived growth factor) and PDGFR-β (platelet-derived growth factor receptor) (Applied Biosystems). Sybr Green Real-time PCR was performed for human COL1A1, fibronectin (6), **ICAM-1**: fwd 5`-CCT ATG GCA ACG ACT CCT TC -3`, rev TGC GGT CAC ACT GAC TGA G -3; **COL3A1**: fwd 5`-GGC ATG CCA CAG GGA TTC T-3`, rev 5`-GCA GCC CCA TAA TTT GGT TTT-3`; **HDAC3:** fwd 5`-ATG CAA GGC TTC ACC AAG AG-3`, rev 5`-CAG TCA TCG CCT ACG TTG AA-3`; **HDAC4:** fwd 5`-TGT ACG ACG CCA AAG ATG AC-3`, rev 5`-CGG TTC AGA AGC TGT TTT CC-3`; **HDAC5:** 5`-CAG CAG GCG TTC TAC AAT GA-3`, rev 5`-CGATGC AGAGAG ATG TAG AGC A-3`; **HDAC6:** fwd 5`-GAA AGT CAC CTC GGC ATC AT-3`, rev 5`-TAG TCT GGC CTG GAG TGG AC-3`; **HDAC7:** fwd 5`-ATG GGG GAT CCT GAG TAC CT-3`, rev 5`-GAT GGG CAT CAC GAC TAT CC-3`; **HDAC9:** fwd 5`-CTG GAG CCC ATC TCA CCT T-3`, rev 5`-TCA TCA TCC TGA GGT CTG TCC-3`; **HDAC10:** fwd 5`-GCC GGA TAT CAC ATT GGT TC-3`, rev 5`-GAC GCT TCC TGT TGG ATG A-3`; **HDAC11:** fwd 5`-GGT CAG GAA GGG GTA CAG GT-3`, rev 5`-ATT GAG GGG GAA CTC CAG AT-3`. To confirm specific amplification by Sybr Green PCR, dissociation curve analysis was performed for each primer pair, and NRT controls and water controls were analyzed for all samples. Amounts of loaded cDNA were normalized using a predeveloped 18S assay (PE Applied Biosystems) as an endogenous control. Differential gene expression was calculated with the threshold cycle (Ct) and the comparative Ct method for relative quantification. Only samples with a difference of at least 4 cycles between the signals in cDNA samples and NRT (corresponding to a 16-fold difference in the expression) were considered for the calculations. All experiments were performed in duplicates.

Collagen measurements

Total soluble collagen in cell culture supernatants was quantified using the SirCol collagen assay (Biocolor, Belfast, Northern Ireland). For these experiments, confluent cells were incubated for 24h with 40 µl DMEM/ 10% FCS per cm^2 of culture dish surface. 1 ml of the Sirius Red dye, an anionic dye that reacts specifically with [Gly-X-Y]n tripeptide in the triple helix sequence of mammalian collagens under assay conditions, was added to 100µl supernatant and incubated under gently rotation for 30 min at room temperature. After centrifugation for 10 min at 12000 g, the collagen-bound dye was re-dissolved with 1 ml of 0.5 M NaOH and the absorbance was measured at 540 nm in a MRX ELISA reader (Dynex technologies). All samples were measured in duplicates.

Western blot analysis

For Western blot analysis, SSc skin fibroblasts ($2x10^5$ cells per well) were incubated in the absence or presence of TSA for 48h. Whole cell lysates were prepared by lysing cells in 2x concentrated Lemmli buffer (100 m*M* Tris HCl [pH 6.8], 40% glycerol, 10% sodium dodecyl sulfate [SDS], 0.7*M* β-mercaptoethanol, and 0.0005% bromphenol blue). Proteins were separated on a SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked for 1h at room temperature in 5% non-fat dry milk with 0.05% Tween 20 in TBS (pH 7.4) and were probed overnight at 4°C with antibodies against HDAC7, collagen type I, collagen type III, ICAM-1, PDGF-B, PDGFR-β and CTGF (Santa Cruz, CA, USA) or α-tubulin (Sigma). The blots were washed and incubated for 1h at room temperature with HRP-conjugated secondary antibodies (Jackson Immunoresearch, Magden, switzerland) in 5% non-fat dry milk with 0.05% Tween 20 in TBS (pH 7.4). Bound

antibodies were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK). Evaluation of the expression of specific proteins was performed by the Alpha imager Software system (Alpha Innotech, San Leandro, CA) via pixel quantification of the electronic image.

Small interfering RNA (siRNA)

Fibroblasts from patients with SSc were transfected using Amaxa Basic Nucleofector Kit (Amaxa biosystems) as described (14). For silencing of HDAC7, we used HDAC7 siRNA (Santa Cruz / Qiagen) and scrambled siRNA representing an irrelevant coding siRNA (Applied Biosystems) as control siRNA which does not target any gene product. The protocol used has been optimized and recommended by the manufacturer. Briefly, proper amounts of cells (5×10^5 cells) were resuspended in 100µl of nucleofector solution, mixed with 5µl of siRNA (from a 10uM solution) and transfection was done using a nucleofector device (Program U23). After 24h incubation, fresh medium was added and the cells were incubated for another 24h before RNA extraction.

Statistics

All data are expressed as mean ± standard deviation. Statistical analysis was performed using the Mann–Whitney test (SPSS software). P-values <0.05 were considered significant.

Results

No disease specific pattern of mRNA expression of HDACs in SSc skin fibroblasts

SSc fibroblasts (n=5) and skin fibroblasts from healthy controls (n=4) were analyzed for the expression of all known HDACs (1-11) by TaqMan Real-time PCR. Comparing the delta Ct levels, there was no significant difference in the constitutive expression pattern of HDACs in fibroblasts from patients with SSc and healthy controls (Figure 1A). As we focus on HDAC7 in the present study, the expression of this particular HDAC was measured also on protein level. The expression of HDAC7 in SSc fibroblasts was not different from healthy controls (Figure 1B). In conclusion, fibroblasts from patients with SSc show no disease specific pattern of HDACs on the transcriptional level.

Different effects of TSA on the expression of HDAC3 and HDAC7 in SSc fibroblasts

TSA is considered to be a non-specific HDAC inhibitor. Nevertheless, the direct effects of TSA on the expression of individual HDACs (1-11) in SSc fibroblasts have not been investigated so far. Therefore, we treated SSc fibroblasts with TSA (2μM) (6) for 24h and analyzed the mRNA expression of HDAC 1-11 by TaqMan Real-time PCR. HDAC7 mRNA was strongly reduced to 0.06 ± 0.01 fold ($p \leq 0.001$) after treatment with TSA. HDAC3 mRNA was increased by TSA to 5.13 ± 1.46 fold ($p \leq 0.002$) (Figure 1C). The transcription levels of all other HDACs were down regulated by TSA (**HDAC1**: 0.75 ± 0.46, **HDAC2**: 0.59 ± 0.19, **HDAC4**: 0.75 ± 0.14, **HDAC5**: 0.85 ± 0.23, **HDAC6**: 0.53 ± 0.15, **HDAC8**: 0.87 ± 0.06, **HDAC9**: 0.35 ± 0.06, **HDAC10**: 0.67 ± 0.20, **HDAC11**: 0.67 ± 0.10, n=3 for all). The results on mRNA level for HDAC3 and HDAC7 could be confirmed on the protein level by Western blot

(Figure 1D). Densitometric analysis of the Western blots showed that TSA reduced the expression of HDAC7 to 0.037 ± 0.05 fold (n=3, $p<0.05$) and increased the expression of HDAC3 to 2.19 ± 0.76 fold (n=3, $p<0.05$). In conclusion, we found the most pronounced effects of TSA on the expression of two individual HDACs, namely HDAC3 and HDAC7.

Expression of different types of collagen after TSA treatment

Patients with SSc have increased levels of collagen types I and III, with type I being the most abundant (15). In order to examine whether TSA regulates the expression of collagen, we performed a TaqMan Real-time PCR analysis for COL1A1 and COL3A1 in SSc fibroblasts (n=5). TSA down regulated the mRNA expression of COL1A1 and COL3A1 by 48 ± 6 and 67 ± 9% respectively (Figure 2A). Western blot was done to confirm the results on the protein level (Figure 2B). Densitometric analysis of the Western blots revealed a reduction of collagen types I and III by TSA to 0.23 ± 0.09 and 0.13 ± 0.02 fold respectively (n=3, $p<0.05$). To the same extent, expression of collagen types I and III was reduced by TSA after TGF-β stimulation (data not shown). Therefore, constitutive and induced expression of different types of collagen was down regulated after treatment with TSA.

Specific gene knockdown of HDAC7

As shown above, HDAC7 was considerably down regulated after treatment with TSA. To test whether the effects of TSA on SSc fibroblasts were mainly mediated through the down regulation of HDAC7, HDAC7 was knocked down using RNAi approach. As demonstrated in Figure 3A, gene expression of HDAC7 was suppressed by 76.20 ± 8.48% (n=10, $p=0.001$) in HDAC7 siRNA treated cells after 48h of transfection compared to RNAi control. HDAC7 was also down regulated on protein level after

72h of transfection (Figure 3B), demonstrating the successful knockdown of HDAC7 by siRNA.

Effect of specific gene knockdown of HDAC7 on the expression of HDAC3

Since we observed a significant down regulation of HDAC7 after TSA treatment, while the expression of HDAC3 was up regulated, we wanted to evaluate whether the expression of HDAC3 was affected by HDAC7 silencing. Therefore, we measured the expression level of HDAC3 after knockdown of HDAC7 in SSc fibroblasts (n=6). Using TaqMan Real-time PCR and Western blot, we observed no changes in the levels of HDAC3 expression, neither on the mRNA nor on the protein level (Figures 3C and D).

Expression of extracellular matrix proteins after HDAC7 silencing

To address the question whether the effects of TSA on extracellular matrix (ECM) components are mediated through HDAC7, we investigated the expression of ECM proteins after HDAC7 specific gene knockdown. Whereas the expression of fibronectin remained unchanged (data not shown), both constitutive and cytokine-induced gene expression of COL1A1 and COL3A1 were significantly reduced by silencing of HDAC7. The constitutive and TGF-β induced down regulation of mRNA levels for COL1A1 were 27.0 ± 2.4% (n=6, $p<0.05$) and 36.0 ± 14% (n=7, $p<0.05$) and for COL3A1 were 23.0 ± 4.0% (n=5, $p<0.05$) and 43.0 ± 9.0% (n=5, $p<0.05$) respectively (Figure 4A). Western blot analysis was done to confirm the results on protein level (Figures 4B and C). Additionally, using the Sircol Collagen assay, the production of total soluble collagen was found to be reduced by 26 ± 5.7%, as shown in Figure 4D (n=5, $p<0.05$). In conclusion, we found a significant down regulation in the expression of collagen types I and III after specific gene knockdown of HDAC7.

Effects of HDAC7 silencing on the expression of PDGF-B and PDGFR-β in SSc fibroblasts

PDGF plays an important role in the pathogenesis of SSc. Even though it is almost undetectable in healthy skin, studies have revealed the increased presence of PDGF and PDGF receptors in SSc skin biopsies (16). According to Mottet et al, HDAC7 silencing in endothelial cells up regulated the expression of PDGF-B and its receptor (PDGFR-β) (17). In order to examine whether gene knockdown of HDAC7 alters the expression of PDGF-B and PDGFR-β in SSc fibroblasts, we analyzed changes in the transcript expression levels of these genes in cells treated with HDAC7 siRNA (n=6 each). As it's shown in Figures 5A and B, there was no significant change in the levels of PDGF-B and PDGFR-β comparing HDAC7 siRNA with RNAi control cells. Western blot was done to confirm the results on protein level (Figures 5C and D). In conclusion, silencing of HDAC7 had no significant effect on the expression of PDGF-B and PDGFR-β in SSc fibroblasts.

TSA up regulates the expression of CTGF and ICAM-1 while the expression of these molecules remains unchanged in SSc fibroblasts silenced for HDAC7

CTGF is induced by TGF-β and modulates fibroblast cell growth but it also mediates many of the pro-fibrotic actions of TGF-β (18). ICAM-1, an inducible surface glycoprotein that promotes adhesion in immunological and inflammatory reactions, plays a role in a variety of inflammatory and neoplastic diseases. ICAM-1 also contributes significantly to the development of skin fibrosis, especially via ICAM-1 expression in skin fibroblasts (19). To investigate whether acetylation induced by TSA and specific gene knockdown of HDAC7 might play a role in the regulation of CTGF and ICAM-1 proteins, we analyzed the expression of CTGF and ICAM-1 after

TSA treatment as well as after silencing of HDAC7 by Western blot. As shown in Figure 6A, CTGF and ICAM-1 were up regulated after 48h treatment with TSA to 2.55 ± 0.19 fold (n=3, $p<0.05$) and to 21.34 ± 16.71 fold (n=3, $p\leq0.05$) respectively. Of interest, the expression of CTGF and ICAM-1 in HDAC7 siRNA treated cells remained unchanged compared to cells treated with siRNA control (Figure 6B). It can be concluded that silencing of HDAC7 in contrast to TSA does not induce the expression of the pro-fibrotic molecules CTGF and ICAM-1 in SSc fibroblasts and therefore might be a more specific anti-fibrotic therapeutic target than TSA.

Discussion

The results of our present study demonstrate that silencing of HDAC7, a member of class II HDACs, might be a more specific and effective anti-fibrotic therapeutic approach in SSc than the use of TSA. Silencing of HDAC7 significantly reduced the excessive production of extracellular matrix proteins, a characteristic feature of SSc fibroblasts, without increasing other known pro-fibrotic molecules such as ICAM-1 and CTGF.

Recently, we have shown that the non-selective HDAC inhibitor TSA blocks the cytokine-induced production of collagen type I and fibronectin in fibroblasts from patients with SSc. In addition, in the bleomycin-induced mouse model of skin fibrosis, TSA prevented the dermal accumulation of extracellular matrix in vivo (6). In the present study we show for the first time that TSA did not only block the enzymatic activity of HDAC, but additionally regulates the protein level of selective targets by influencing their transcription. Subsequently, almost all transcripts of HDACs were reduced by TSA. However, the most interesting finding was that although TSA almost completely inhibited the transcription of HDAC7, a member of class II HDACs; HDAC3, a member of class I HDAC was significantly up regulated after treatment by TSA. This dual function of regulating protein activity and protein expression by TSA in fibroblasts from patients with SSc has not been described yet.

Based on this observation we concluded that the down regulation of HDAC7 represents the anti-fibrotic mechanism of TSA and suggest here that silencing of HDAC7 may be a more specific and safer treatment for SSc than TSA.

TSA is one of the first discovered natural HDAC-inhibitors targeting the zinc-containing HDAC classes I, II and IV, but not the NAD+-dependent class III enzymes (11). Despite the ubiquitous distribution of HDACs in the cells, HDACi such as TSA selectively alter only a relative small proportion of the expressed genes (2-10%) (20).

Moreover, in one study with human lymphoid cell lines TSA altered 2% of the expressed genes only (8 genes out of approximately 340 examined) (21). It is remarkable that in all these studies, roughly similar numbers of genes were down regulated and up regulated. For example in a study with a T cell leukaemia cell line 22% of expressed genes were altered by HDACi with approximately similar numbers being up regulated and repressed (22).

TSA is still considered as the reference compound of hydroxamic-acid containing HDACi although the costly and highly inefficient production encouraged the search for alternative drugs (23). TSA is one of the most potent HDACi that exerts its effects at very low concentrations (nanomolar) but it is still controversially discussed because of its poor bioavailability in vivo due to an extensive biotransformation or instability (20, 24-26). Unfortunately, none of the numerous HDACi is specific for single isoforms of HDAC, but few drugs show preferences for groups or single HDACs. *FK228*, for example shows some preference for class I HDACs, and *tubacin* specifically targets HDAC 6 (27, 28). Therefore, there is a challenging task to develop a new generation of HDACi with improved specificity for certain HDAC isoforms and an increased efficacy compared to the pan-inhibitors such as TSA and suberoylanilide hydroxamic acid (SAHA) (29).

Recently, we gave evidence that TSA in vitro predominantly abrogates the cytokine-induced production of excessive extracellular matrix, and that it prevents fibrosis in a mouse model of bleomycin-induced fibrosis. Therefore, we favored the hypothesis that TSA might serve as early strategy for the treatment of fibrosis. However, in the present study, we demonstrate that TSA also up regulates the expression of CTGF and ICAM-1, both characteristic players in the pathogenesis of SSc. The production of extra-cellular matrix proteins is induced in fibroblasts by TGF-beta in the early stage and is subsequently maintained by CTGF (30, 31). Additionally, it has been

shown that the expression of CTGF correlates with the severity of fibrosis (32). Matsushita et al demonstrated that ICAM-1 deficiency attenuates the development of skin fibrosis in the tight-skin mouse model (19). Therefore, the up regulation of CTGF and ICAM-1 might counteract the value of TSA as an anti-fibrotic drug.

Of interest, in the present study we could demonstrate that TSA significantly blocked the transcription of HDAC7. Our results are supported by the recent publication from Dokmanovic et al who reported that the hydroxamatic acid-based HDACi *Vorinostat* (SAHA), which is similar to TSA, selectively down regulated HDAC7 in several cancer cell lines and to a lesser extent also in normal foreskin fibroblasts with little or no effect on the expression of other class II HDACs. *Vorinostat* has been approved for clinical treatment, and the authors suggest that the reduced expression of HDAC7 might serve as a biomarker for a response to treatment (33).

HDAC7 belongs to the class II HDACs that show a tissue or cell-specific expression and shuttle between the nucleus and the cytoplasm upon certain cellular signals. Nucleocytoplasmatic shuttling has been observed for all class II HDACs and reflects a putatively important regulatory mechanism. However, it needs to be stressed that HDAC7 is expressed in heart and lung tissues, placenta, pancreas, and skeletal muscle as well as in CD4/CD8 double positive thymocytes (34-37). Null mouse embryos for HDAC7 display defects in the development of blood vessels and their integrity (38). Moreover, HDAC7 protein has been implicated into several biological processes, including regulation of gene expression either as co-activator or co-repressor (39, 40), and plays a role in T cell differentiation by inducing Nur77 (35). The enzymatic activity of HDAC7 maps to the carboxyl-terminal domain and seems to be dependent on the interaction with the class I HDAC, HDAC3. The binding of these two HDACs might be mediated by the transcriptional co-repressors SMRT and N-CoR that simultaneously bind class II HDACs and HDAC3 (41). Accordingly, it has

been recently reported that silencing of HDAC7 has also profound effects on endothelial cells. Mottet et al gave evidence of an altered migration of endothelial cells (HUVECs) upon silencing of HDAC7 (17). They could show that this disturbance was at least in part due to an up regulation of PDGF-B and PDGFR-β. However, in our study, silencing of HDAC7 in SSc dermal fibroblasts did not affect the expression of PDGF-B and PDGFR-β.

The impact of HDAC7 on the vascularisation was underlined by another study performed by Chang et al. This study showed that HDAC7 plays a key role in the maintenance of vascular integrity by repressing matrix metalloproteinase 10 in HUVEC cells (38). Whether the influence of silencing of HDAC7 on angiogenesis, shown in HUVEC cells, also occurs in the skin of patients with SSc, is not yet clear.

In our study we could show that TSA inhibited the expression of HDAC7 and up regulated the expression of HDAC3 in fibroblasts from patients with SSc. However, silencing of HDAC7 by siRNA did not affect the level of expression of HDAC3 neither on the mRNA nor on the protein level. Therefore, we conclude that the anti-fibrotic effects of TSA are mediated by HDAC7 independently of HDAC3. In the present study we demonstrated that specific gene knockdown of HDAC7 similarly to TSA reduces significantly both, the cytokine (TGF-β)-induced as well as the constitutive production of the extracellular matrix proteins collagen types I and III. As compared to TSA, silencing of HDAC7 does not affect the expression of fibronectin. However, the most pronounced advantage of silencing HDAC7 is the specific anti-fibrotic effect. In contrast to TSA, silencing of HDAC7 did not influence the expression levels of the pro-fibrotic molecules CTGF and ICAM-1 in SSc fibroblasts. Therefore, silencing of HDAC7 might be a new and promising approach for the anti-fibrotic treatment of SSc.

Figure Legends

Figure 1: Expression of HDACs in SSc vs. normal skin fibroblasts and effects of TSA on the expression levels of HDAC3 and HDAC7 in SSc fibroblasts

(A) mRNA expression levels of HDAC1 to -11 were analyzed by Real-time PCR in SSc fibroblasts (n=5) and fibroblasts from healthy controls (n=4). Results are expressed as mean ± SD of x-fold change. Levels of expression in healthy fibroblasts are considered as 1. **(B)** Total cellular extracts from normal and SSc fibroblasts were analyzed by Western blot with anti-HDAC7 antibodies. The same samples were analyzed with antibodies against α-tubulin for normalization. **(C)** mRNA levels of HDAC3 (n=6) and HDAC7 (n=7) in untreated and TSA treated (2μM for 24h) SSc fibroblasts, analyzed by TaqMan Real-time PCR. Values are mean ± SD. Levels of expression in untreated cells (control) is considered as 1 (white bars), and the effect of TSA (black bars) is shown as x-fold change relative to control. **(D)** Protein levels of HDAC3 and HDAC7 after TSA treatment, determined by Western blot analysis. Untreated cells were used as controls, and the expression level of α-tubulin was used for normalization. Results shown are from 3 independent experiments.

Figure 2: Effects of TSA on the expression of different types of collagen

(A) Real-time PCR quantification of COL1A1 and COL3A1 after treatment with TSA, as compared to control (untreated cells). The expression level of 18S was used for normalization and results show the mean ± SD of 5 individual samples. **(B)** Western blot was used to assess the expression of collagen types I and III on the protein level after incubation with TSA for 48h. The same cell extracts were analyzed with antibodies against α-tubulin for normalization.

Figure 3: Transfection efficacy of HDAC7 siRNA and expression of HDAC3 after specific gene knockdown of HDAC7

(A) Levels of mRNA for HDAC7 after specific gene knockdown using the siRNA, assessed by TaqMan Real-time PCR. Results are the mean ± SD from 10 different experiments. **(B)** Western blot shows the expression of HDAC7 protein after inhibition with siRNA. The same extracts were incubated with antibodies against α-tubulin as control. **(C)** Expression of HDAC3 in cells treated with HDAC7 siRNA was analyzed by TaqMan Real-time PCR. Results are the mean ± SD fold change in levels of mRNA relative to the control group (considered as 1). Data are representative of 6 individual samples from different patients. **(D)** Effect of HDAC7 gene knockdown on the levels of HDAC3 protein in SSc fibroblasts (n=3), measured by Western blot. To control for variability of loading and transfer, membranes were reprobed with human anti-α-tubulin.

Figure 4: Expression of different types of collagen in HDAC7 siRNA treated SSc fibroblasts

(A) Constitutive and TGF-β induced levels of mRNA for COL1A1 and COL3A1 in HDAC7 siRNA fibroblasts (black bars) compared to RNAi control (white bars), estimated by quantitative TaqMan Real-time PCR. Values are the mean ± SD change in percent from 6 individual samples. **(B)** Total cellular extracts from HDAC7 siRNA and RNAi control fibroblasts were analyzed by Western blot with anti-collagen type I and anti-collagen type III antibodies. The same extracts were analyzed with antibodies against α-tubulin as control. **(C)** Western blot analysis for collagen types I and III in whole cell lysates of TGF-β stimulated SSc fibroblasts transfected with HDAC7 siRNA and RNAi control. Expression of α-tubulin was used for normalization. **(D)** Total soluble collagen production in supernatants of HDAC7/siRNA fibroblasts as

compared to siRNA control, measured by the Sircol collagen assay. The amount of collagen in cells transfected with RNAi control considered as 100% (white bar) and the regulation by gene knockdown of HDAC7 (black bar) is shown relative to the control. The data represent the mean ± SD from 5 independent experiments.

Figure 5: Expression of PDGF-B and PDGFR-β in HDAC7 siRNA treated fibroblasts

(A, B) mRNA expression of PDGF-B and PDGFR-β in cells transfected with HDAC7 siRNA (black bar) compared to RNAi control (white bar) was analyzed by Real-time PCR. Results are shown as the mean ± SD (n=6 each). **(C, D)** Western blots showing PDGF-B (n=2) and PDGFR-β (n=3) in whole cell lysates of SSc fibroblasts transfected with HDAC7 siRNA and RNAi control. The same extracts were analyzed with antibodies against α-tubulin as control.

Figure 6: Expression of CTGF and ICAM-1 in TSA treated cells and HDAC7 siRNA treated fibroblasts

(A) Western blot analysis of CTGF and ICAM-1 in cells obtained from patients with SSc (n=2) treated with TSA after 48h. Levels of α-tubulin served as a loading control. **(B)** SSc fibroblasts (n=2) silenced for HDAC7 (HDAC7 siRNA) and controls (control siRNA) were analyzed for the expression of CTGF and ICAM-1 by Western blot. Expression of α-tubulin was used for normalization.

References

1. Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC. Clinical development of histone deacetylase inhibitors as anticancer agents. Annu Rev Pharmacol Toxicol 2005;45:495-528.
2. Chung YL, Lee MY, Wang AJ, Yao LF. A therapeutic strategy uses histone deacetylase inhibitors to modulate the expression of genes involved in the pathogenesis of rheumatoid arthritis. Mol Ther 2003;8(5):707-17.
3. Wang Y, Fan PS, Kahaleh B. Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. Arthritis Rheum 2006;54(7):2271-9.
4. Ghosh AK, Varga J. The transcriptional coactivator and acetyltransferase p300 in fibroblast biology and fibrosis. J Cell Physiol 2007.
5. Ghosh AK, Mori Y, Dowling E, Varga J. Trichostatin A blocks TGF-beta-induced collagen gene expression in skin fibroblasts: involvement of Sp1. Biochem Biophys Res Commun 2007;354(2):420-6.
6. Huber LC, Distler JH, Moritz F, Hemmatazad H, Hauser T, Michel BA, et al. Trichostatin A prevents the accumulation of extracellular matrix in a mouse model of bleomycin-induced skin fibrosis. Arthritis Rheum 2007;56(8):2755-64.
7. Walkinshaw DR, Yang XJ. Histone deacetylase inhibitors as novel anticancer therapeutics. Curr Oncol. 2008;15(5):237-43.
8. Huang L. Targeting histone deacetylases for the treatment of cancer and inflammatory diseases. J Cell Physiol 2006;209(3):611-6.
9. Ellmers LJ, Scott NJ, Piuhola J, Maeda N, Smithies O, Frampton CM, et al. Npr1-regulated gene pathways contributing to cardiac hypertrophy and fibrosis. J Mol Endocrinol 2007;38(1-2):245-57.
10. Dokmanovic M, Marks PA. Prospects: histone deacetylase inhibitors. J Cell Biochem 2005;96(2):293-304.
11. Yoshida M, Kijima M, Akita M, Beppu T. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem 1990;265(28):17174-9.
12. Khan N, Jeffers M, Kumar S, Hackett C, Boldog F, Khramtsov N, et al. Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. Biochem J 2008;409(2):581-9.
13. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr., et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988;15(2):202-5.
14. Jungel A, Distler O, Schulze-Horsel U, Huber LC, Ha HR, Simmen B, et al. Microparticles stimulate the synthesis of prostaglandin E(2) via induction of cyclooxygenase 2 and microsomal prostaglandin E synthase 1. Arthritis Rheum 2007;56(11):3564-74.
15. Charles C, Clements P, Furst DE. Systemic sclerosis: hypothesis-driven treatment strategies. Lancet 2006;367(9523):1683-91.
16. Trojanowska M. Role of PDGF in fibrotic diseases and systemic sclerosis. Rheumatology (Oxford) 2008;47 Suppl 5:v2-4.
17. Mottet D, Bellahcene A, Pirotte S, Waltregny D, Deroanne C, Lamour V, et al. Histone deacetylase 7 silencing alters endothelial cell migration, a key step in angiogenesis. Circ Res 2007;101(12):1237-46.
18. Ihn H. Autocrine TGF-beta signaling in the pathogenesis of systemic sclerosis. J Dermatol Sci 2008;49(2):103-13.

19. Matsushita Y, Hasegawa M, Matsushita T, Fujimoto M, Horikawa M, Fujita T, et al. Intercellular adhesion molecule-1 deficiency attenuates the development of skin fibrosis in tight-skin mice. J Immunol 2007;179(1):698-707.
20. Vanhaecke T, Papeleu P, Elaut G, Rogiers V. Trichostatin A-like hydroxamate histone deacetylase inhibitors as therapeutic agents: toxicological point of view. Curr Med Chem 2004;11(12):1629-43.
21. Van Lint C, Emiliani S, Verdin E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Expr 1996;5(4-5):245-53.
22. Peart MJ, Smyth GK, van Laar RK, Bowtell DD, Richon VM, Marks PA, et al. Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. Proc Natl Acad Sci U S A 2005;102(10):3697-702.
23. Papeleu P, Vanhaecke T, Elaut G, Vinken M, Henkens T, Snykers S, et al. Differential effects of histone deacetylase inhibitors in tumor and normal cells-what is the toxicological relevance? Crit Rev Toxicol 2005;35(4):363-78.
24. Elaut G, Torok G, Vinken M, Laus G, Papeleu P, Tourwe D, et al. Major phase I biotransformation pathways of Trichostatin a in rat hepatocytes and in rat and human liver microsomes. Drug Metab Dispos 2002;30(12):1320-8.
25. Charrier C, Bertrand P, Gesson JP, Roche J. Synthesis of rigid trichostatin A analogs as HDAC inhibitors. Bioorg Med Chem Lett 2006;16(20):5339-44.
26. Vigushin DM, Ali S, Pace PE, Mirsaidi N, Ito K, Adcock I, et al. Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo. Clin Cancer Res 2001;7(4):971-6.
27. Furumai R, Matsuyama A, Kobashi N, Lee KH, Nishiyama M, Nakajima H, et al. FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. Cancer Res 2002;62(17):4916-21.
28. Haggarty SJ, Koeller KM, Wong JC, Grozinger CM, Schreiber SL. Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci U S A 2003;100(8):4389-94.
29. Yang XJ, Gregoire S. Class II histone deacetylases: from sequence to function, regulation, and clinical implication. Mol Cell Biol 2005;25(8):2873-84.
30. Xiao R, Liu FY, Luo JY, Yang XJ, Wen HQ, Su YW, et al. Effect of small interfering RNA on the expression of connective tissue growth factor and type I and III collagen in skin fibroblasts of patients with systemic sclerosis. Br J Dermatol 2006;155(6):1145-53.
31. Denton CP, Abraham DJ. Transforming growth factor-beta and connective tissue growth factor: key cytokines in scleroderma pathogenesis. Curr Opin Rheumatol 2001;13(6):505-11.
32. Leask A. Transcriptional profiling of the scleroderma fibroblast reveals a potential role for connective tissue growth factor (CTGF) in pathological fibrosis. Keio J Med. 2004;53(2):74-7.
33. Dokmanovic M, Perez G, Xu W, Ngo L, Clarke C, Parmigiani RB, et al. Histone deacetylase inhibitors selectively suppress expression of HDAC7. Mol Cancer Ther 2007;6(9):2525-34.
34. Kasler HG, Verdin E. Histone Deacetylase 7 Functions as a Key Regulator of Genes Involved in Both Positive and Negative Selection of Thymocytes. Mol Cell Biol 2007.
35. Dequiedt F, Kasler H, Fischle W, Kiermer V, Weinstein M, Herndier BG, et al. HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. Immunity 2003;18(5):687-98.
36. Dressel U, Bailey PJ, Wang SC, Downes M, Evans RM, Muscat GE. A dynamic role for HDAC7 in MEF2-mediated muscle differentiation. J Biol Chem 2001;276(20):17007-13.
37. Dokmanovic M, Clarke C, Marks PA. Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res 2007;5(10):981-9.

38. Chang S, Young BD, Li S, Qi X, Richardson JA, Olson EN. Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. Cell 2006;126(2):321-34.
39. Kato H, Tamamizu-Kato S, Shibasaki F. Histone deacetylase 7 associates with hypoxia-inducible factor 1alpha and increases transcriptional activity. J Biol Chem 2004;279(40):41966-74.
40. Kao HY, Downes M, Ordentlich P, Evans RM. Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. Genes Dev 2000;14(1):55-66.
41. Fischle W, Dequiedt F, Fillion M, Hendzel MJ, Voelter W, Verdin E. Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. J Biol Chem 2001;276(38):35826-35.