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Human xylosyltransferase-I – A new marker for myofibroblast differentiation in skin fibrosis

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ABSTRACT

Skin fibrosis is a severe type of fibrotic disorder emerging in terms of hypertrophic scars or systemic sclerosis. Key event of fibrogenesis is the transition of fibroblasts to matrix-producing myofibroblasts. In the presence of fibrotic triggers, for instance secretion of profibrotic growth factors like transforming growth factor-β1 (TGF-β1) or mechanical strain, myofibroblasts persist. Current research focuses on discovering innovative myofibroblast biomarkers which are regulated in fibrotic development and accessible for antifibrotic inhibition.

Here, we consider the suitability of xylosyltransferase-I (XT-I) as a myofibroblast biomarker in skin fibrosis. XT-I catalyzes the initial step of glycosaminoglycan biosynthesis. Its increase in enzymatic activity is known to refer only to manifested diseases which are characterized by an abnormal rate of proteoglycan biosynthesis. In this study, treatment of normal human dermal fibroblasts (NHDF) with TGF-β1 was followed by increased relative *XYLT1* mRNA expression. Remarkably, this upregulation was strongly dependent on myofibroblast content, increasing during fibrogenesis. Moreover, XT activity increased time-dependently in response to progressive myofibroblast transformation. *XYLT1* expression was inhibited by TGF-β receptor I (ALK5) inhibitor SB431542. In contrast, *XYLT2* expression was only marginally affected by TGF-β1 as well as ALK5 inhibition.

Our results strengthen the significance of XT expression and activity in fibrotic remodeling. Therefore, we propose XT activity, in addition to α-SMA expression, as a new biomarker for myofibroblast differentiation and fibrotic development. Further studies are now needed to evaluate the option to control and inhibit fibrotic remodeling by interfering with XT expression.

1. Introduction

Fibrotic disorders are characterized by a dysbalance of extracellular matrix synthesis and degradation resulting in an excessive accumulation of matrix molecules. Skin fibrosis follows if molecular processes of dermal tissue regeneration proceed after wound closure. The key step of healing progression is the immigration and differentiation of resident fibroblasts to myofibroblasts [1–3]. In spite of their different origins, myofibroblasts are all characterized by specific properties such as high contractibility, *de novo* expression of alpha-smooth muscle actin (α-SMA) and the ability to produce extracellular matrix in response to mechanical strain or profibrotic molecules like transforming growth factor-β1 (TGF-β1) or ED-A fibronectin [2,4–6]. In nonpathological tissues, wound closure ceases after tissue restoration and myofibroblasts become apoptotic [7]. In the presence of fibrotic triggers, myofibroblasts persist and continue to produce scarring tissue. This dysfunctional matrix replaces the intact tissue, thus physiological function becomes lost and tissue stiffness increases, as it has been described for different types of abnormal skin scarring, such as keloids or hypertrophic scarring [8,9].

Today, a therapeutic strategy to treat dermal fibrotic disorders as well as general fibrogenesis does not exist. Since differentiation from fibroblasts to myofibroblasts is well known, this key cell is focused on the elucidation of therapeutic tools by searching for fibrotic predictors. Several antifibrotic targets have been discussed in recent publications. Blocking of stress fiber contraction by delivery of the NH_2-terminal sequence of α-SMA in the form of a fusion protein or stimulation of myofibroblast apoptosis are currently discussed [10,11]. The multifunctional cytokine and main fibrotic mediator TGF-β1 seems not to be a suitable therapeutic target [12,13]. So aiming of TGF-β1 downstream mediators is considered as an alternative.

Two of these downstream mediators are human xylosyltransferase-I and -II (XT). These isoenzymes catalyze the initial and

Abbreviations: ALK5, transforming growth factor-β receptor I; α-SMA/*ACTA2*, alpha-smooth muscle actin; Dpm, disintegrations per minute; GAG, glycosaminoglycan; NHDF, normal human dermal fibroblast; RT-PCR, quantitative real-time pcr; TGF-β1, transforming growth factor-β1; XT/*XYLT*, xylosyltransferase.

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rate-limiting step in glycosaminoglycan (GAG) biosynthesis by transferring an activated UDP-xylose molecule to selected serine residues in the proteoglycan core protein [14,15]. Within the last years, XTs have been comprehensively studied [16]. They are common biomarkers for quantification of the actual proteoglycan biosynthesis rate, so that diseases which are referred to a dysregulated proteoglycan metabolism may be monitored by analyzing serum XT activity [17,18]. The induction of XT explains the observation of higher GAG content in skin scar tissue than in normal skin tissue [19]. In addition, the increased proteoglycan synthesis in hypertrophic scars emphasizes a critical role of GAG-synthesizing enzymes like XT [19].

In summary, XT activity is associated with persistent fibrosis, but it has never been taken into consideration that XT activity or mRNA expression may be useful biomarkers for myofibroblast differentiation in the fibrotic onset, too. For the first time, we analyzed the fibrotic influence of TGF-β1 on *XYLT* mRNA expression in normal human dermal fibroblasts (NHDFs). We propose that XT activity and *XYLT1* mRNA expression are, in addition to α-SMA, a hitherto unconsidered but important marker for myofibroblast differentiation, which could serve as an antifibrotic target in the future.

2. Materials and methods

2.1. Materials

Recombinant TGF-β1 was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany) and ALK5 inhibitor SB431542 from Abcam (Cambridge, UK).

2.2. Cell culture

Normal human dermal fibroblasts (NHDF, 6th–9th passage) of four healthy controls were purchased from Genlantis (San Diego, USA), Cambrex (Walkersville, USA) and Coriell (New Jersey, USA). Cells were cultured routinely under humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen, San Diego, USA) supplemented with 10% fetal calve serum (PAA, Pasching, AT) and 1% antibiotic/antimycotic solution (100×; PAA, Pasching, AT) as specified by the manufacturer. Cells were seeded in appropriate cell densities of 40 or 500 cells/mm^2. After 24 h a serum withdrawal of 10–0.1% FCS was performed. The next day, cells were treated with TGF-β1 (5 ng/mL) or a combination of TGF-β1 and ALK5 inhibitor SB431542 (0.5 μM or 50 μM) for 48 h, 72 h or 120 h. Measurements were performed in triplicate. Cell lysates were resuspended in lysis buffer RA1 (Macherey–Nagel, Düren, Germany) and stored at −80 °C. Cell culture supernatants were stored at −20 °C.

2.3. Nucleic acid extraction and reverse transcription

Total RNA extraction from cell lysates was performed using the RNA Spin Blood Kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions, except removal of 50 μL of cell lysate after the first column-based cleaning step to determine DNA content. DNA extraction was executed using the DNA Spin Blood Kit (Macherey–Nagel, Düren, Germany). Nucleic acid concentrations were determined using the NanoDrop 2000 (Peqlab, Erlangen, Germany). 1 μg RNA was reverse transcribed to cDNA by using SuperScript II Reverse Transcriptase (Invitrogen, San Diego, USA).

2.4. Quantitative RT-PCR

The mRNA expression of the following target genes was analyzed by quantitative RT-PCR: hypoxanthine phosphoribosyltransferase 1 (*HPRT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β2-microglobulin (*B2M*), xylosyltransferase-I (*XYLT1*), xylosyltransferase-II (*XYLT2*), transforming growth factor-β1 (*TGF-β1*), alpha-smooth muscle actin (*ACTA2*), collagen type 1 alpha 1 (*COL1A1*) and decorin (*DCN*). Intron-spanning primer sequences are listed in Table 1. PCR products were amplified using the MasterCycler System (Eppendorf, Hamburg, Germany). For the RT-PCRs, 2.5 μL of cDNA (dilution 1:10) were mixed with 5 μL of a SYBR green Taq-DNA polymerase mixture (Invitrogen, San Diego, USA), 2 μL water and 0.25 μL of forward and reverse primer (each 25 pmol/μL). Mixtures were incubated at 50 °C for 2 min to enzymatically degrade uracil containing DNA, followed by the activation of DNA polymerase at 95 °C for 2 min and 45 cycles of denaturation (95 °C, 10 s), annealing (optimal annealing temperature, 15 s) and elongation (72 °C, 20 s). Finally, a melting curve analysis was performed. Relative transcription levels were verified in triplicate and calculated by the delta-delta Ct-method considering PCR efficiency [20]. The normalization factor calculation based on the geometric mean of the expression levels of *HPRT*, *GAPDH* and *B2M*.

2.5. Radiochemical xylosyltransferase activity assay

The determination of XT activity in cell culture supernatants is based on the incorporation of [^{14}C]D-xylose (Du Pont, Homburg, Germany) into silk fibroin receptor protein and was described previously [21]. Counts per minute were measured in duplicate for every sample and referred to total DNA concentration of the appropriate cell lysate.

2.6. Immunhistochemistry

To identify the dimension of myofibroblast differentiation by immunofluorescence staining of α-SMA, NHDFs were seeded at 40 or 500 cells/mm^2. Serum withdrawal and cell treatment was performed as described previously (Chapter 2.2). After 120 h cells were washed with phosphate-buffered saline (PBS), and fixed in a mixture of acetone and methanol (1:1) for 10 min. Cells were washed with PBS two times. Thereafter, blocking in 1% bovine serum albumin was performed for 1 h. After an additional washing step, cells were incubated with the primary monoclonal mouse anti-human smooth muscle actin antibody Clone 1A4 (1:50; Dako, Hamburg, Germany) for 2 h. Excess antibodies were removed by two washing steps, followed by the incubation with FITC conjugated goat-anti-mouse secondary antibody (Dianova, Hamburg, Germany) for 1 h. Finally, cells were washed and covered with PBS. Signals were visualized and photographed using microscope Eclipse TE2000-S (Nikon, Düsseldorf, Germany).

Table 1
Primer sequences for quantitative real-time PCR analysis.

Gene name	Protein name	Primer annotation	5′–3′-sequence
hACTA2	α-SMA	E8/1412U18	GACCGAATGCAGAAGGAG
		E9/1580L17	CGGTGGACAATGGAAGG
hB2M	B2M	E1/84U22	TGTGCTCGCGCTACTCTCTCTT
		E2/200L21	CGGATGGATGAAACCCAGACA
hDCN	DCN	E1/572U16	CCTTCCGCTGTCAATG
		E2/656L18	GCAGGTCTAGCAGAGTTG
hHPRT	HPRT	E3/311U18	GCTGACCTGCTGGATTAC
		E6/568L18	TGCGACCTTGACCATCTT
hGAPDH	GAPDH	E2-3/116U18	AGGTCGGAGTCAACGGAT
		E4/338L18	TCCTGGAAGATGGTGATG
hTGF-β1	TGF-β1	E3/1404U17	GCGATACCTCAGCAACC
		E6/1734L18	ACGCAGCAGTTCTTCTCC
hXYLT1	XT-I	E11/2489U18	ACTGCCGAATTCACACAC
		E11-12/2633L19	GTGCCTCCTCAGGTTTGAT
hXYLT2	XT-II	E10/2258U18	CCTTGTGCTGCCCTTGAC
		E11/2352L18	GCCCTGGAAACTCTGCTC

2.7. Statistics

Data are shown as mean ± SEM. Statistical analysis was performed using GraphPad Prism 5.0 (San Diego CA, USA). Experimental data were analyzed by Mann–Whitney-*U*-test. *p* Values less than 0.05 were considered statistically significant.

3. Results

3.1. XYLT1 and ACTA2 mRNA expression are both upregulated in myofibroblast differentiation of skin fibroblasts whereby XYLT2 is only marginally affected

To investigate XT as a putative myofibroblast biomarker, an appropriate cell culture model was established. Low- and high-density seeded fibroblasts were cultivated on a stiff matrix (cell culture dishes) and 24 h after seeding a serum withdrawal was performed. Cells were treated with TGF-β1 and the levels of mRNA expression were quantified by RT-PCR.

We found, as depicted elsewhere, that *ACTA2* mRNA expression was upregulated by profibrotic factor TGF-β1 [5]. The enhancement gave approximately equivalent results in low- and high-density cultivated cells (6.5- or 7.2-fold; Fig. 1A). It has to be emphasized, that the high upregulation factor in high-density cultured cells is caused by a lowered basic expression level in response to low-density cultured cells. Nevertheless, *ACTA2* mRNA expression level of high-density cells is always below the expression of low-density cultured cells. To verify a TGF-β1 dependent signaling, the ALK5 inhibitor SB431542 was added and concentration-dependently minimized *ACTA2* gene expression to basic mRNA expression level of the negative control (Fig. 1A).

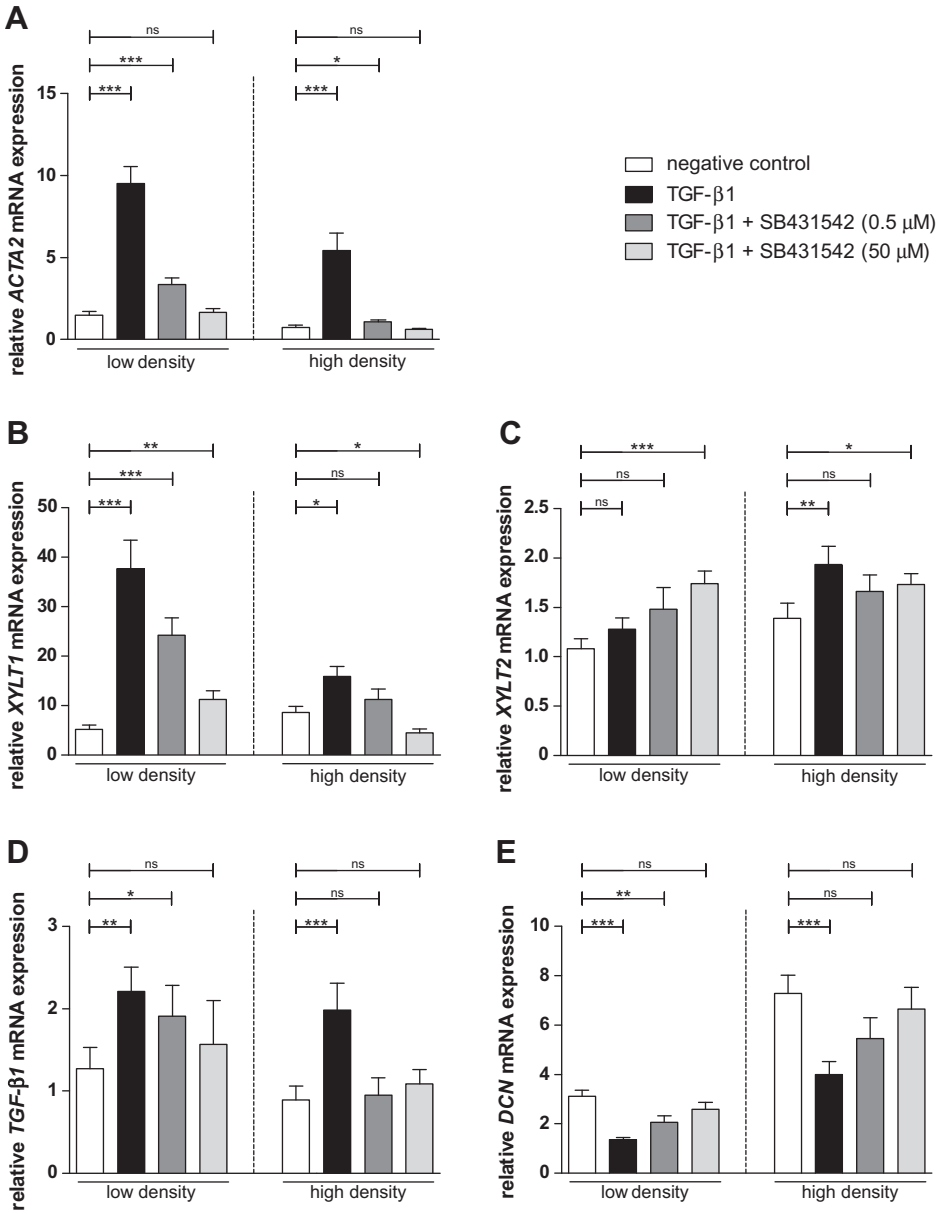

Fig. 1. Relative mRNA expression of myofibroblast-related genes in response to TGF-β1 treatment and ALK5 inhibition. Four cell lines of NHDF were seeded in low or high density (40 or 500 cells/mm^2), incubated for 24 h in serum-depleted medium and treated with TGF-β1 (5 ng/mL) or TGF-β1 + ALK5 inhibitor SB431542 (0.5 or 50 μM) for 48 h. RNA was isolated from cell lysates and reverse transcribed. Relative mRNA expression levels of *ACTA2* (A), *XYLT1* (B), *XYLT2* (C), *TGF-β1* (D) and *DCN* (E) were analyzed by quantitative RT-PCR. Data were normalized to a normalization factor, determined by calculating the geometric mean of *HPRT*, *GAPDH* and *B2M* mRNA expression levels, and expressed as a ratio to one cell line. Values are means ± SEM. ns: not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (Mann–Whitney *U*-test).

The relative mRNA expression of *XYLT1* was principally regulated in accordance with *ACTA2* mRNA expression after TGF-β1 treatment of low- and high-density cultures (upregulation 7.3- or 1.9-fold; Fig. 1B). ALK5 inhibitor SB431542 reduced *XYLT1* gene expression as well. In this regard, it should be pointed out, that the ratio of gene expression enhancement in low to high cell density culture was much greater for *XYLT1* than *ACTA2*, whereby myofibroblast-rich culture systems expressed more mRNA in both cases. As described before, basic *ACTA2* mRNA expression of low-density cultured cells extends the basal level of high-density cultured cells, whereby *XYLT1* basic gene expression level was higher in low-density culture conditions. *XYLT2* mRNA expression level was neither upregulated by TGF-β1 nor inhibited by supplementation of ALK5 inhibitor SB431542 to a large extent. Interestingly, data show a slight *XYLT2* mRNA expression upregulation (1.6-fold) after inhibition (Fig. 1C).

3.2. Regulation of TGF-β1 and DCN mRNA expression in onset of skin fibrosis

To assure myofibroblast differentiation, *TGF-β1* and *DCN* mRNA expression were analyzed (Fig. 1D–E). In matters of cell density, the two mRNA expression patterns only differed slightly. Myofibroblast differentiation was revealed to be related to a higher or lower basic expression level of *TGF-β1* (1.4-fold) or *DCN* (0.4-fold) respectively, as evidenced by comparing negative control expression levels. In general, *TGF-β1* gene expression was upregulated in response to cell treatment with TGF-β1, while ALK5 inhibition was followed by a diminished mRNA expression. *DCN* was expressed reversely to *TGF-β1*.

3.3. Time-dependent increase of XT activity and α-SMA protein expression after TGF-β1 induction correlates with the grade of fibroblast-to-myofibroblast transition

It was further investigated whether XT activity increases with higher myofibroblast content of cell culture, as it is known for increased α-SMA protein expression. To directly compare changes in α-SMA and XT protein expression level, low- and high-density cultivated fibroblasts were treated with TGF-β1 or a combination of TGF-β1 and ALK5 inhibitor SB431542 for up to 120 h. The expression of α-SMA protein was estimated by immunofluorescence assay, which could only be performed after 120 h because of low signals at earlier points in time. α-SMA protein expression level was strengthened by supplementation with TGF-β1 and reduced to the basic level in a concentration dependent manner after inhibitor treatment. The observed effect could be shown to be much more pronounced in low-density (Fig. 2A–D) than in high-density cultured cells (Fig. 2E–H).

48 h, 72 h and 120 h after cell treatment changes in XT enzymatic activity were revealed (Fig. 3). We found that a greater induction of enzymatic XT activity by TGF-β1 was detected in myofibroblast-rich cell density cultures (4.8-fold) than in, myofibroblast-poor, high cell density cultures (3.6-fold) after 48 h (Fig. 3A). Inhibition of XT activity by addition of ALK5 inhibitor occured concentration-dependently. Determination of XT activity was also performed after 72 h and 120 h (Fig. 3B and C). Because of the enzyme accumulation in cell culture supernatants, measured XT activities increased proportionally with incubation time. By comparing basic and upregulated XT activity after addition of profibrotic growth factor TGF-β1, data show an enlarging ratio of XT activity upregulation in low to high cell density cultivated cells over the course of time (Table 2). Besides, the elongation of incubation time resulted in a change of basal activity in untreated negative controls. So the primary observed rate of a higher XT activity in low-density cultured cells in contrast to high-density cultured cells was completely inverted after 120 h (Fig. 3C).

4. Discussion

Fibrosis of the skin is reported to emerge in terms of systemic sclerosis or hypertrophic scars and gives rise to physical, esthetic, psychological and social consequences [22,23]. The activation of fibroblasts to myofibroblasts is modulated by pro-fibrotic cytokines (like TGF-β1 or connective tissue growth factor) or mechanical strain and requires ED-A fibronectin. TGF-β1 is the key player of fibrotic onset. Embryonic wounds which heal scar-free are defined by lowered levels of TGF-β1 in contrast to adult wounds [24]. Oral mucosal wounds display less scar formation than skin wounds, too. This observation likely depends not only on myofibroblast content but also on matrix composition [25]. Hence, there is a great research interest in developing antifibrotic strategies, but none is applicable yet.

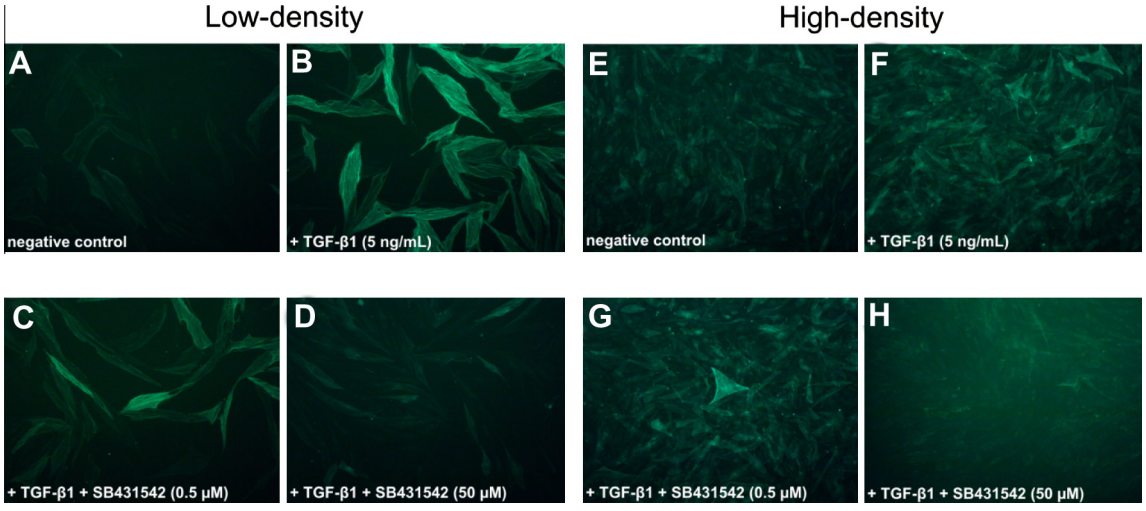

Fig. 2. Immunofluorescence staining of α-SMA after myofibroblast differentiation and its inhibition. NHDF were seeded in low-density (40 cells/mm^2; A–D) or high-density (500 cells/mm^2; E–H) and, after a serum withdrawal for 24 h, incubation with TGF-β1 (5 ng/mL) or a combination of TGF-β1 and ALK5 inhibitor SB431542 (0.5 or 50 μM) was performed for 120 h. Cells were fixed, incubated with specific antibodies and analyzed for α-SMA-positive cells by microscopy.

The determination of a myofibroblast biomarker would implicate the opportunity to diagnose a fibrotic disorder and to estimate its outcome and prognosis. In previous studies it could be demonstrated that serum XT activity is a reliable biomarker for revealing manifested liver fibrosis or systemic sclerosis [17,18]. XT, catalyzing the initial step in GAG biosynthesis, is reported to be regulated by mechanical strain and TGF-β1. This has been demonstrated by studying increased GAG content and *XYLT1* mRNA expression as well as XT activity of fibroblasts in cardiac fibrosis [26]. Here, we describe a corresponding upregulation of XT activity and *XYLT1* mRNA expression after treatment of dermal fibroblasts with TGF-β1. Alongside to the increased serum activity in systemic sclerosis, this regulation in skin fibroblasts verifies XT-I as an initiator of dermal fibrosis. XT-II is involved in fibrosis neither in this nor in other studies [26]. Interestingly, a slight *XYLT2* mRNA expression upregulation was determined after ALK5 inhibition. This effect possibly underlies a compensatory regulation. *XYLT1* inhibition by an ALK5 inhibitor confirms regulation by SMAD signaling pathway, whereby – due to the pleiotropic effects of TGF-β1 – targeting of ALK5 as an antifibrotic strategy should be carefully balanced [27]. In contrast, siRNA-mediated *XYLT* knockdown is correlated to a diminished relative GAG synthesis and therefore impairs tissue remodeling [28].

We suggest not only that XT-I is upregulated in the context of a manifested fibrosis, but also that initial upregulation is dependent on tissue myofibroblast content. The more myofibroblasts abound, the stronger the upregulation of XT activity is. Variable myofibroblast content was implemented by variation of cell-density as described earlier [29]. Until now, this dependency could only been demonstrated for α-SMA, which is the only accepted myofibroblast biomarker so far. Therefore, several studies were performed to verify the key components of transition (TGF-β1 or ED-A fibronectin) as targets of antifibrotic strategies. However, it could be demonstrated that none of these targets is perfectly qualified [10,13]. The development of an effective antifibrotic strategy should aim not only at the initial fibrotic step of myofibroblast transformation but also at the biological process of a manifested fibrotic disorder. Targeting XT satisfies both criteria.

In comparison with α-SMA, XT exhibits different advantages in application as a myofibroblast biomarker. The rise of the *XYLT1* mRNA expression level after fibrotic induction obviously exceeds the *ACTA2*-correlated one, so that the effect is more intensive. Besides, increased XT activity after TGF-β1 treatment was already detectable after 48 h, while elevated α-SMA protein expression was firstly detectable after 120 h. At this particular time, XT activity was still heightened and the ratio of activity induction among high- and low-density cultured fibroblasts had increased even more. It is therefore possible to monitor fibroblast differentiation at any time of fibrotic development by an easy-to-perform assay

Table 2
Time-dependent amplification factor of XT activity in NHDF low- and high-density culture supernatants after TGF-β1 treatment.

	48 h	72 h	120 h
Low-density (40 cells/mm^2)	4.8×	5.3×	6.0×
High-density (500 cells/mm^2)	3.6×	3.1×	2.9×

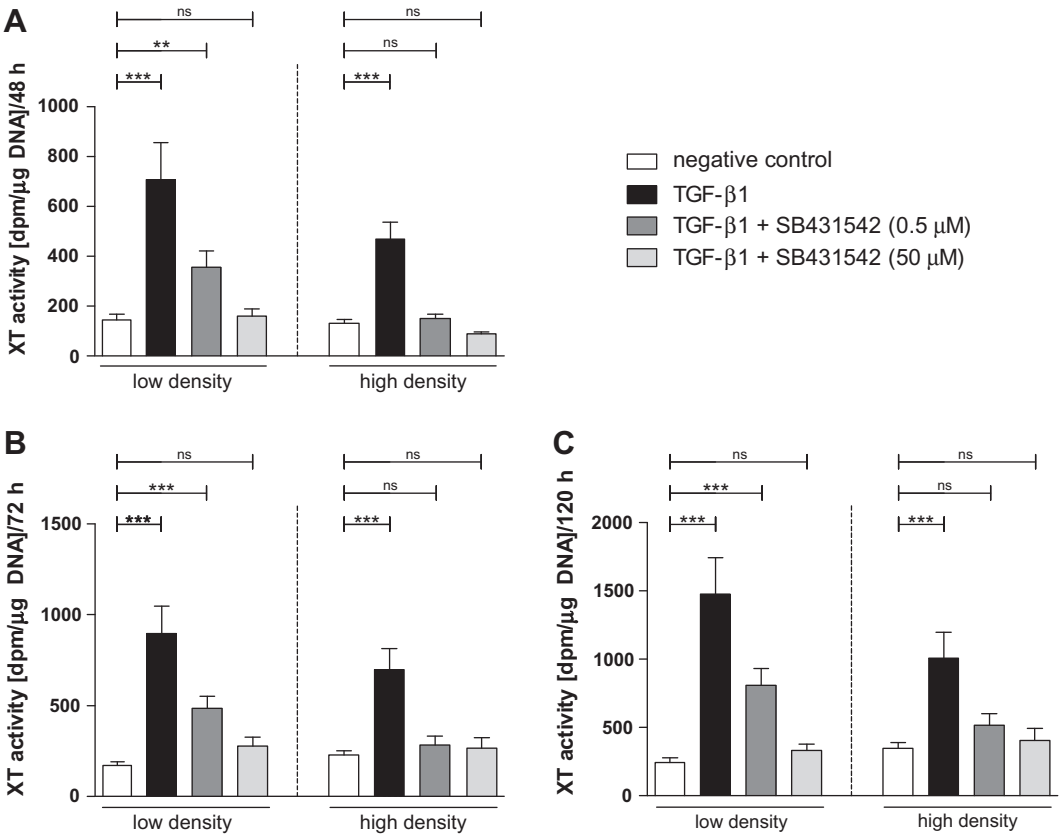

Fig. 3. Determination of XT activity in cell culture supernatants of NHDF transformed to myofibroblasts in variable extent. Four low- or high-density cultured NHDF cell lines (40 or 500 cells/mm^2) were cultivated in serum-depleted medium for 24 h and after that treated with TGF-β1 (5 ng/mL) or TGF-β1 + ALK5 inhibitor SB431542 (0.5 or 50 µM). After 48 h (A), 72 h (B) and 120 h (C) cells were harvested and total lysate DNA was extracted. XT activity was measured in cell culture supernatants by radioactive enzyme assay. XT activity is expressed as dpm/µg DNA. Values represent means ± SEM. ns: not significant; $^{**}p < 0.05$; $^{***}p < 0.001$ (Mann–Whitney *U*-test).

which allows determination of *in vivo* XT activity in any body fluid or *in vitro* XT activity in cell culture supernatants of nearly all common cell lines. The widespread XT expression in various human tissues has already been investigated in a broad study [30].

Above all, we could show a higher *TGF-β1* basic mRNA expression level and, correspondingly, a lower *DCN* basic mRNA expression level in myofibroblast-rich populations. DCN affects collagen fibril formation and also regulates activity of TGF-β1 by its binding and inhibiting [31]. The lowered *DCN* mRNA expression level correlates with the increased autocrine synthesis of TGF-β1 in myofibroblasts [32]. Notably, xylosylated heparin sulfate proteoglycans participate in regulation of TGF-β availability [33]. Furthermore, an artificial downregulation of *XYLT1* by SMAD-2 or SMAD-3 specific shRNA is connected to a diminished proteoglycan biosynthesis and lowering in CS-GAG content in astrocytes [34]. The following reduction in the molecule count as well as the shrinking stiffness of matrix would achieve a reduced storing rate of TGF-β1 and therefore a disruption of matrix accumulation.

In summary, the data reported here reflect the reliable suitability of XT-I as a biomarker not only for manifested dermal fibrosis but also for skin myofibroblast differentiation. As we could show, XT-I and α-SMA seem to contribute equally to the onset of skin fibrosis. Because of their regulation by the same signal transduction pathways, it has to be considered whether specific inhibition of both TGF-β1 downstream mediators could be the method of choice in antagonizing skin fibrosis in the future.

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