Systemic fibrotic diseases such as systemic sclerosis (SSc) and organ-specific fibrosis such as kidney fibrosis, idiopathic pulmonary fibrosis, and liver cirrhosis share common pathophysiological mechanisms (Bataller and Brenner, 2005; Varga and Abraham, 2007; Strieter and Mehrad, 2009). Tissue fibrosis results from an increased release of extracellular matrix from aberrantly activated fibroblasts (Varga and Abraham, 2007; Gabrielli et al., 2009). The accumulating extracellular matrix disrupts the physiological tissue structure, leading to organ dysfunction and contributing to the high morbidity and increased mortality of affected patients (Chung et al., 2007; Steen and Medsger, 2007). However, the mechanisms for pathological fibroblast activation are incompletely understood. Consequently, therapeutic approaches selectively targeting the molecular activation of fibroblasts are not yet available for clinical use.

Tissue fibrosis is preceded by microvascular injury in SSc, leading to a progressive loss of capillaries (Gabrielli et al., 2007). The vascular damage with exposure of subendothelial connective tissue results in ongoing activation of platelets. The levels of several platelet-derived molecules such as β-thrombomodulin, platelet factor 4, and platelet-derived growth factor are elevated in SSc patients (Kahaleh et al., 1982; Postlethwaite and Chiang, 2007). In addition, elevated levels of circulating platelet aggregates have also been described (Kahaleh et al., 1982). Moreover, platelets from SSc patients express markers of activation such as increased expression of the 65-kD receptor for type I collagen and enhanced activity of phosphatidylinositol-3
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5-HT did not enhance the stability of type I collagen or fibronectin-1 messenger RNA (mRNA; Fig. S1, A–C) but stimulated the release of collagen by increasing transcription in reporter assays (P = 0.05; Fig. S1 D).

The profibrotic effects of 5-HT are mediated by 5-HT2B

Consistent with a previous study, we observed that three different receptors for 5-HT are expressed in dermal fibroblasts: 5-HT1B, 5-HT2A, and 5-HT2B (Slominski et al., 2003). In contrast to 5-HT1B and 5-HT2A, the mRNA levels of 5-HT2B were up-regulated by 143 ± 17% in SSc fibroblasts compared with healthy controls (P < 0.05; unpublished data). To investigate which of these receptors mediates the profibrotic effects of 5-HT, dermal fibroblasts were incubated with selective serotonin receptor inhibitors. Inhibition of 5-HT1B by SB 224289 did not reduce the stimulatory effects of 5-HT on extracellular matrix production in SSc fibroblasts (Fig. 2, A–D). In contrast, the 5-HT2B inhibitor terguride decreased mRNA levels of col1a1, col1a2, and fibronectin-1 and also the release of collagen protein in a dose-dependent manner (Fig. S2, A–D). Most pronounced effects were observed at a concentration of 1.0 µM, with an almost complete blockade of 5-HT–induced extracellular matrix expression. To discriminate between 5-HT2A and 5-HT2B, the 5-HT2A inhibitor ketanserin and the 5-HT2B antagonist SB 204741 were used. No inhibitory effects were observed with ketanserin up to 1 µM, whereas SB 204741 at 1 µM completely prevented induction of extracellular matrix synthesis by 5-HT in SSc fibroblasts (Fig. 2, A–D). No differences in metabolic activity, as measured by conversion of microtiter tetrazolium (MTT), or in the number of

RESULTS

5-HT stimulates the production of extracellular matrix in SSc and healthy dermal fibroblasts

We first analyzed whether 5-HT stimulates the production of extracellular matrix. Therefore, fibroblasts from SSc patients and healthy subjects were stimulated with 5-HT at biologically relevant concentrations (Kasho et al., 1998; Wouters et al., 2007). 5-HT dose–dependently induced the expression of extracellular matrix proteins with maximal induction at a concentration of 1 µM (Fig. 1, A–D). Higher concentrations of 5-HT did not further increase the expression of extracellular matrix proteins but resulted in toxic effects (unpublished data). 5-HT increased the expression of col1a1 by 211 ± 14% (P < 0.05; Fig. 1 A). In addition to col1a1, 5-HT also induced the expression of col1a2 and fibronectin-1 in a dose–dependent manner (Fig. 1, B and C). Similarly, the release of collagen protein from SSc fibroblasts was increased by up to 198 ± 53% (P < 0.05; Fig. 1 D). 5-HT also induced the expression of collagen in dermal fibroblasts from healthy individuals without significant differences to fibroblasts from SSc patients (Fig. 1, A–D).
However, expression of 5-HT2B was strongly increased in fibrotic tissue compared with unaffected tissue from healthy controls (Fig. 3 A). Double staining for the fibroblast-specific marker prolyl-4-hydroxylase and 5-HT2B demonstrated that almost all fibroblasts stained positive for 5-HT2B in fibrotic tissue but not in controls (Fig. S2 E). Of note, intensive staining for 5-HT2B was observed in α-SMA (α-smooth muscle actin)–positive, SM22α (smooth muscle protein 22α)–negative myofibroblasts in skin of SSc patients (Fig. 3 B).

5-HT2B activates TGF-β/Smad signaling
TGF-β has been identified as a key player in the pathogenesis of fibrosis (Varga and Abraham, 2007). When we compared the effects of 5-HT and TGF-β in col 1a2 reporter assays, we observed different time kinetics. The stimulatory effects of 5-HT peaked after 24 h, whereas the maximal effects of TGF-β were observed after 6 h (Fig. S1 D). The delayed activation by 5-HT implicates that the profibrotic effects of 5-HT might be mediated indirectly by induction of a second messenger.

To investigate whether TGF-β itself might be this second mediator, we first analyzed the effects of 5-HT on the expression of TGF-β1 and of its target gene PAI-1 (plasminogen activator inhibitor 1). 5-HT dose-dependently increased the mRNA levels of TGF-β1 and PAI-1 in SSc fibroblasts (Fig. 4 A and B). Furthermore, 5-HT induced a time-dependent increase in nuclear levels of phospho-Smad3, the typical intracellular mediator of TGF-β signaling (Fig. 4 C). We next evaluated whether the profibrotic effects of 5-HT depend on TGF-β. Preincubation of

5-HT2B is overexpressed in skin of SSc patients
After demonstrating that 5-HT exerts its profibrotic effects via 5-HT2B, we were interested whether 5-HT2B might be up-regulated in human fibrotic diseases. 5-HT2B was detectable by immunohistochemistry in the fibrotic skin of SSc patients as well as in normal skin of healthy individuals (Fig. 3). However, expression of 5-HT2B was strongly increased in fibrotic tissue compared with unaffected tissue from healthy controls (Fig. 3 A). Double staining for the fibroblast-specific marker prolyl-4-hydroxylase β and 5-HT2B demonstrated that almost all fibroblasts stained positive for 5-HT2B in fibrotic tissue but not in controls (Fig. S2 E). Of note, intensive staining for 5-HT2B was observed in α-SMA (α-smooth muscle actin)–positive, SM22α (smooth muscle protein 22α)–negative myofibroblasts in skin of SSc patients (Fig. 3 B). Together with a previous report of an increased serum concentration of 5-HT in SSc (Biondi et al., 1988), these findings suggest that the 5-HT/5-HT2B axis is up-regulated in systemic fibrotic disease.

As off-target effects of the chemical inhibitors cannot be fully excluded, SSc fibroblasts were transfected with small interfering RNAs (siRNAs) against 5-HT2B, which efficiently reduced mRNA and protein levels of 5-HT2B (Fig. 2 E). Moreover, silencing of 5-HT2B in the presence of 5-HT significantly decreased mRNA expression for col1a1, col1a2, and fibronectin-1 compared with SSc fibroblasts transfected with nontargeting control siRNA (Fig. 2, F–H), suggesting that 5-HT2B plays a crucial role for the synthesis of extracellular matrix proteins in dermal fibroblasts.

To exclude the possibility that the decreased synthesis of extracellular matrix proteins is counter-regulated by induction of matrix-degrading enzymes or their inhibitors, the expression of MMPs (matrix metalloproteinases) and TIMPs (tissue inhibitors of MMPs) was analyzed in fibroblasts treated with 5-HT, terguride, and SB 204741 and in fibroblasts transfected with 5-HT2B siRNA. The expression of MMP-1, MMP-2, MMP-3, and MMP-13 and TIMP-1, TIMP-2, TIMP-3, and TIMP-4 was not affected upon stimulation with 5-HT or inhibition of 5-HT2B (unpublished data).

5-HT2B is overexpressed in skin of SSc patients
After demonstrating that 5-HT exerts its profibrotic effects via 5-HT2B, we were interested whether 5-HT2B might be up-regulated in human fibrotic diseases. 5-HT2B was detectable by immunohistochemistry in the fibrotic skin of SSc patients as well as in normal skin of healthy individuals (Fig. 3).
The mRNA levels of col 1a1, col 1a2, and fibronectin-1 were only slightly reduced by neutralizing antibodies against TGF-β1 in unstimulated fibroblasts, suggesting that the observed effects of anti-TGF-β1 antibodies in 5-HT-stimulated fibroblasts were caused by blockade of the stimulatory effects of 5-HT rather than caused by effects on the basal synthesis (Fig. 4, D–F). Similar results were also obtained for healthy dermal fibroblasts (unpublished data).

SSc fibroblasts with neutralizing antibodies against TGF-β1 completely abrogated the profibrotic effects of 5-HT on mRNA expression of col 1a1, col 1a2, and fibronectin-1 (Fig. 4, D–F).

Figure 3. 5-HT2B is overexpressed in patients with SSc. (A) 5-HT2B was detectable by immunohistochemistry in fibroblasts, keratinocytes, and microvessels of SSc patients and controls. Two representative tissue sections and an isotype control are shown. (B) 5-HT2B expression in SM22α-negative and α-SMA-positive myofibroblasts. A representative skin section of an SSc patient is shown. SM22α as specific marker for vascular smooth muscle cells was stained with DAB, the myofibroblast marker α-SMA with Alexa Flour 594 (red), and 5-HT2B with Alexa Flour 488 (green; n = 8 each). The stainings were performed in three independent series. Bars: (A and B [top]) 50 µm; (B, bottom) 10 µm.

Figure 4. 5-HT exerts its profibrotic effects via activation of TGF-β/Smad-dependent pathways. (A and B) mRNA levels of TGF-β1 (A) and PAI-1 (B) in 5-HT-stimulated SSc fibroblasts (n = 9, three independent series). (C) Nuclear accumulation of p-Smad3 protein complexes after 12 and 24 h of stimulation with 1 µM 5-HT (n = 3). (D–F) mRNA expression of col 1a1 (D), col 1a2 (E), and fibronectin-1 (F) upon incubation with 500 ng/ml of neutralizing antibodies (Ab) against TGF-β1 in 5-HT-stimulated SSc fibroblasts (n = 5 each, two independent series). *, P < 0.05 compared with 5-HT-stimulated (D–F) or nonstimulated (A and B) controls. Error bars indicate SE.
The antifibrotic effects of the inhibition of 5-HT2 were further tested in a therapeutic approach in a modified bleomycin model. When the treatment with terguride was started after induction of fibrosis, terguride did not only prevent further progression, but induced regression of fibrosis (Fig. S3 B). Dermal thickening decreased by 78 ± 4% in mice treated with terguride for the last 3 wk compared with mice challenged with bleomycin for 6 wk (P = 0.003) and by 66 ± 4% compared with mice injected with bleomycin for 3 wk (P = 0.002; Fig. S3 C). The collagen content and the number of myofibroblasts were also efficiently reduced (Fig. S3, D and E).

To confirm the role of 5-HT2B in experimental fibrosis by a genetic approach, we evaluated the effects of bleomycin in mice lacking 5-HT2B. No histological changes or differences in dermal thickness were observed in untreated 5-HT2B−/− mice (Fig. 6 A). However, 5-HT2B−/− mice were almost completely protected from bleomycin-induced fibrosis. Bleomycin targeting of 5-HT2B exerts potent antifibrotic effects

We then assessed different inhibitors of 5-HT receptor for their antifibrotic activity in the mouse model of bleomycin-induced dermal fibrosis. Imatinib, a tyrosine kinase inhibitor with well established antifibrotic effects in this model, served as positive control (Wang et al., 2005; Distler et al., 2007a). No signs of toxicity were observed upon treatment with terguride, cyproheptadine, SB 204741, and imatinib. Injection of bleomycin potently stimulated the expression of 5-HT2B and induced dermal fibrosis (Fig. 5 A and Fig. S3 A). However, the 5-HT2 inhibitors terguride and cyproheptadine as well as the selective 5-HT2B inhibitor SB 204741 (Goldberg et al., 1979; Bonhaus et al., 1995; Glusa and Pertz, 2000; Jähnichen et al., 2005) efficiently prevented bleomycin-induced dermal thickening (Fig. 5, A and B). Collagen content and myofibroblast counts were also dose-dependently reduced by the inhibition of 5-HT2B (Fig. 5, C and D).

Figure 5. Inhibition of 5-HT signaling by the 5-HT2 inhibitors terguride, cyproheptadine, and SB 204741 prevents accumulation of extracellular matrix in experimental dermal fibrosis. (A) Representative trichrome-stained tissue sections are shown: control mice injected intracutaneously with NaCl (n = 10), bleomycin-challenged mice without antifibrotic treatment (n = 10), mice receiving bleomycin and terguride at a concentration of 0.2 mg/kg/bid (n = 8), mice treated with bleomycin and terguride at a concentration of 0.6 mg/kg/bid (n = 8), mice treated with bleomycin and cyproheptadine at a concentration of 10 mg/kg/d (n = 6), mice treated with bleomycin and SB 204741 at a concentration of 5 mg/kg/d (n = 6), and mice treated with bleomycin and imatinib at a concentration of 50 mg/kg/d (n = 6). Bars, 100 µm. (B) Dermal thickening upon challenge with bleomycin and treatment with terguride, cyproheptadine, SB 204741, or imatinib. (C) Collagen accumulation in lesional skin as analyzed with the SirCol collagen assay. (D) Myofibroblast counts after treatment of bleomycin-challenged mice with terguride, cyproheptadine, SB 204741, or imatinib. * P < 0.05 compared with bleomycin-treated mice without antifibrotic treatment. The experiment was performed in two independent series. Error bars indicate SE.
induced only minor dermal thickening, and the hydroxyproline content and the myofibroblast numbers did not increase in 5-HT2B−/− mice upon bleomycin challenge (Fig. 6, A–C).

To assess the role of 5-HT2B in a less inflammation-dependent model of fibrosis, the Tsk-1 (tight skin 1) model was used. The Tsk-1 model is characterized by increased hypodermal thickness compared with matched wild-type mice because of a tandem duplication in the fibrillin-1 gene (Green et al., 1976). Consistent with the findings in human SSc and in bleomycin-induced fibrosis, 5-HT2B was overexpressed in skin sections of Tsk-1 mice (Fig. 5A). Treatment with the 5-HT2B inhibitor SB 204741 normalized fibrotic changes observed in Tsk-1 mice (Fig. S4 B). Hypodermal thickening, collagen content, and the differentiation of resting fibroblasts into myofibroblasts in Tsk-1 mice were significantly reduced upon treatment with SB 204741 (Fig. S4, C–E).

Moreover, when 5-HT2B−/− mice were crossed with Tsk-1 mice, hypodermal thickening was significantly reduced in 5-HT2B+/−/Tsk-1 mice compared with 5-HT2B+/+/Tsk-1 mice (Fig. 7, A and B). Furthermore, the collagen content and the myofibroblast numbers in the skin of 5-HT2B−/−/Tsk-1 mice were significantly decreased compared with 5-HT2B+/+/Tsk-1 mice (Fig. 7, C and D).

**Inhibition of platelet activation ameliorates bleomycin-induced skin fibrosis and the Tsk-1 phenotype**

To confirm the link between increased platelet activation and increased 5-HT/5-HT2B signaling, we first measured the levels of 5-HT in fibrotic skin. The concentration of 5-HT in lesional skin increased to 5.8 ± 0.5 ng/mg in bleomycin-injected mice compared with 3.2 ± 0.2 ng/mg in control mice (P = 0.02). In the Tsk-1 model, the concentration of 5-HT was increased from 4.0 ± 0.4 ng/mg to 6.7 ± 1.0 ng/mg (P = 0.04). Thus, levels of 5-HT are up-regulated in different models of experimental fibrosis in accordance with the increased levels seen in human SSc (Stachów et al., 1979; Biondi et al., 1988).

Because the vast majority of 5-HT in nonneuronal tissues is derived from platelets, we hypothesized that inhibition of platelet activation might reduce the tissue levels of 5-HT and prevent experimental fibrosis. Indeed, treatment with the P2Y12 receptor inhibitor clopidogrel reduced the 5-HT content in the fibrotic skin of bleomycin-challenged mice by 58 ± 21% (P = 0.03). In parallel, clopidogrel decreased dermal thickening by 61 ± 13% compared with control mice (P = 0.001; Fig. S5, A and B). Consistently, the collagen content and the myofibroblast counts were significantly reduced (Fig. S5, C and D).

Hypodermal thickening in Tsk-1 mice was also reduced by 51 ± 16% (P = 0.009) upon treatment with clopidogrel (Fig. S5, E and F). Additionally, the collagen content and the number of activated myofibroblasts in skin were significantly decreased (Fig. S5, G and H).

**Lack of 5-HT in platelets via knockout of TPH1 prevents experimental dermal fibrosis**

Platelet granules do not only contain 5-HT but also other growth factors. To demonstrate that 5-HT is the major profibrotic mediator released by platelets, we investigated the outcome of TPH1-deficient (TPH1−/−) mice in experimental fibrosis. TPH1−/− mice do not completely lack 5-HT, but total blood levels of 5-HT are reduced to 5% as compared with wild-type animals (Walther et al., 2003b). Consistent with an important role of platelet-derived 5-HT in fibrosis, the profibrotic effects of bleomycin were markedly reduced in TPH1−/− mice (Fig. 8 A). Dermal thickening was decreased by 61 ± 6% (P = 0.004), and the collagen content as well as myofibroblast counts were significantly reduced in TPH1−/− mice as compared with TPH1+/+ mice (Fig. 8, B–D).

TPH1−/− mice were interbred with Tsk-1 mice to yield Tsk-1 mice lacking 5-HT in platelets. Fibrosis was also efficiently ameliorated in TPH1−/−/Tsk-1 with reduced hypodermal thickening, decreased hydroxyproline content, and lower myofibroblast counts mice as compared with TPH1+/+/Tsk-1 (Fig. S6, A–D).

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and vascular endothelial growth factor, which are increased in the serum of SSc patients (Soma et al., 1993; Mercié et al., 1995; Distler et al., 2002). Platelets are also the largest storage pool for 5-HT, and elevated plasma levels of 5-HT have been observed in systemic fibrosis (Biondi et al., 1988). Thus, microangiopathy with subsequent platelet activation and increased release of 5-HT might further activate 5-HT/5-HT2B signaling in addition to the 5-HT2B overexpression in fibrotic diseases. Indeed, inhibition of platelet activation or selective inhibition of the synthesis of 5-HT prevents experimental fibrosis in different mouse models, thereby highlighting the role of platelet activation in fibrosis.

Our study could also explain the molecular mechanism of the clinical association between carcinoid tumors and fibrosis. Carcinoid tumors are neuroendocrine tumors producing large amounts of 5-HT. Tissue fibrosis is often found in patients with carcinoid tumors, both locally and systemically (Modlin et al., 2004). Local tissue fibrosis in the peritumoral tissue is found in most patients. The accumulation of extracellular matrix might be substantial and by far exceed the actual tumor volume (Moertel et al., 1961; Makridis et al., 1996). The most common systemic fibrotic manifestation is carcinoid heart disease with fibrotic changes of the cardiac...
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has been implicated in the development of fibrotic changes by selected ergots such as pergolide or bromocriptine, anorexins like fenfluramine, or drugs of abuse as for example MDMA (3,4-methylenedioxymethamphetamine; also known as ecstasy; Rothman et al., 2000; Setola et al., 2003; Hofmann et al., 2006; Roth, 2007). As we demonstrate herein that activation of 5-HT$_{2B}$ potently stimulates the production of extracellular matrix proteins, it might be concluded that activation of 5-HT$_{2B}$ by the metabolite methylergonovine might be sufficient to promote the development of fibrosis. The importance of 5-HT signaling for fibrotic diseases is supported by recent studies with 5-HT receptor antagonists in experimental pulmonary or liver fibrosis (Ruddell et al., 2006; Fabre et al., 2008). Thus, our findings might have widespread implications, namely that inhibition of the 5-HT$_{2B}$ pathway might be a promising strategy for fibrotic disorders.

MATERIALS AND METHODS

Patients, mice, and fibroblast cultures. Fibroblast cultures were obtained from skin biopsies of SSC patients and healthy volunteers as described previously (Akhmetshina et al., 2008). All patients fulfilled the criteria for SSC as suggested by LeRoy and Medsger (2001). Patient characteristics are summarized in Table S1. All patients and controls signed a...
consent form approved by the local institutional review boards. All human protocols were approved by the ethical board of the University of Erlangen-Nuremberg.

TPH1−/− mice and 5-HTβ−/− mice have been described previously (Nebigil et al., 2000; Walthner et al., 2003b). All mouse experiments were approved by the Government of Mittelfranken.

ELISA. 4-mg skin biopsies of the injected area were analyzed by competitive ELISA for 5-HT using a serotonin ELISA kit (GenWay).

Stimulation with 5-HT and 5-HT receptor inhibition. For stimulation experiments, a special FCS depleted of 5-HT was used (HyClone). Dermal fibroblasts were stimulated with 5-HT (Sigma-Aldrich) in concentrations from 0.01 to 1.0 µM.

Cells were incubated with the nonselective 5-HT2 inhibitor terguride (0.01–1.0 µM) or the selective inhibitors SB 224289 (5-HT2 subtype), ketanserin (5-HT2A), and SB 204741 (5-HT2B) at concentrations of 0.1 and 1.0 µM (Kasho et al., 1998; Lawre et al., 2005). The Ki values of all chemical inhibitors according to the National Institute of Mental Health Ki Database are given in Table S2. 5-HT was added 1 h after the inhibitors at a concentration of 1.0 µM.

Transfection with siRNAs against 5-HTβ. Ssc fibroblasts were transfected with 1.5 µg siRNA duplexes against 5-HTβ using the human dermal fibroblast Nucleofector kit (Lonza; Jungel et al., 2007). Two different siRNA duplexes with the following sequences were used: siRNA duplex 1 sense, 5′-CCGCAUCCAUCAUGCAUCU-3′; and antisense, 5′-ACUGGUAGGUAGUGGG-3′; and siRNA duplex 2 sense, 5′-GUGGGUGUAAUCAUCAAC-3′; and antisense, 5′-UUAGAAUUAUACACACGACCC-3′. Fibroblasts transfected with nontargeting control siRNAs (Invitrogen) were used as controls. Medium was changed after 6 h to remove the Nucleofector solution. Cells were harvested after 48 h.

Selective inhibition of TGF-β signaling. Fibroblasts were stimulated with 1 µM 5-HT in the presence of neutralizing mouse anti-human TGF-β1 or IgG control antibodies (R&D Systems) at a concentration of 500 ng/ml as described previously (Dusler et al., 2007b).

mRNA stability assay. The mRNA stability assay was performed to investigate whether 5-HT stabilizes collagen or fibronectin mRNA as described previously (Dusler et al., 2004). Ssc fibroblasts were stimulated with 1 µM 5-HT. Actinomycin D was added at a concentration of 10 µg/ml 6 h later. Nonstimulated fibroblasts with and without actinomycin D served as controls. Fibroblasts were harvested 4, 8, 12, 18, and 24 h after the addition of actinomycin D and analyzed with real-time PCR.

 Luciferase reporter assay. The -553 COL1A2/LUC construct was provided by M. Trojanowska (Boston University School of Medicine, Boston, MA; Czuwara-Ladykowska et al., 2001). HEK293T cells were transfected in serum-free medium with 0.5 µg of reporter construct mixed with polyethylenimine. A common lacZ reporter vector was used as control. Cells were transfected with 5-HT or TGF-β directly after transfection.

 Quantitative real-time PCR. Gene expression was quantified by TaqMan or by SYBR Green real-time PCR (ABI Prims 7300 sequence detection system; Applied Biosystems) as described previously (Akhmetshina et al., 2008). Total RNA was isolated with the NucleoSpin RNA II extraction system (Macherey-Nagel) according to the instructions of the manufacturer. RT into cDNA was performed as described previously (Akhmetshina et al., 2008) using random hexamers. Samples without enzyme in the RT reaction (non-RT controls) were used as negative controls. Unspecific signals caused by primer dimers were excluded by dissociation curve analysis and samples without cDNA (no template controls). A predeveloped 18S assay (Applied Biosystems) was used to normalize for the amounts of loaded cDNA. Differences were calculated with the comparative Ct method for relative quantification.

 Collagen measurements. Total soluble collagen in cell culture supernatant was quantified using the SirCol collagen assay (BioColor; Akhmetshina et al., 2008). To detect also highly cross-linked collagen, the collagen content in skin samples was analyzed by determination of the hydroxyproline content (Woessner, 1961).

 Western blot. Nuclear and cytoplasmic extracts were prepared as previously described (Andrews and Fallar, 1991; Khelifi et al., 2005). Polyvinylidene fluoride membranes were incubated with polyclonal rabbit anti–5-HTβ (GeneTex) or monoclonal rabbit anti–phospho-Smad3 antibodies (Cell Signaling Technology). Equal loading of proteins was confirmed by visualization of the nuclear protein lamin A/C (Cell Signaling Technology) and the cytoplasmic protein β-actin (Sigma-Aldrich). Quantification was performed with ImageJ software (version 1.41; National Institutes of Health).

 Immunohistochemistry for 5-HTβ, α-SMA, and SM22-α. Skin sections from Ssc patients and controls were stained with polyclonal rabbit anti–5-HTβ antibodies. A subset of 5-HTβ-stained sections was double stained with the fibroblast marker prolyl-4-hydroxylase β (Acris Antibodies). To analyze the expression of 5-HTβ in myofibroblasts, we performed triple staining for 5-HTβ, the myofibroblast marker α-SMA (clone 1A4; Sigma-Aldrich), and SM22-α (Abcam) as specific marker for vascular smooth muscle cells to distinguish myofibroblasts from vessels. Antibodies labeled with horse-radish peroxidase (Dako), alkaline phosphatase (Jackson Immunoresearch Laboratories, Inc.), and Alexa Fluor 488 or Alexa Fluor 594 (both Invitrogen) were used as secondary antibodies. The expression of SM22-α and 5-HTβ in single- and double-stained sections was visualized with DAB (diaminobenzidine) peroxidase substrate solution (Sigma-Aldrich), and the expression of prolyl-4-hydroxylase β with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Vector Laboratories). Isotype antibodies in the same concentration were used for controls. Myofibroblasts in mouse sections were identified by staining for α-SMA as described previously (Dusler et al., 2007a).

 MTT assay. The metabolic activity of dermal fibroblasts incubated with inhibitors of 5-HTβ was measured using the MTT (3, [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) method (Dusler et al., 2005). In brief, Ssc and healthy dermal fibroblasts were incubated with terguride or SB 204741 in concentrations of 0.1 and 1.0 µM in 96-well plates for 20 h. After removing 100 µl of the medium, MTT was added at a final concentration of 1 mg/ml, and the cells were incubated at 37°C for 4 h. After dilution with 300 µl of 0.04 N HCl in isopropanol, the cells were analyzed using the ELISA reader (Spectra MAX 190 microplate spectrophotometer; Molecular Devices) at a test wavelength of 570 nm with a control wavelength of 630 nm. Untreated fibroblasts were used as negative controls, and all other results were normalized to untreated cells. Fibroblasts incubated with 50% DMSO served as positive controls.

 Quantification of apoptotic and necrotic cells. Dermal fibroblasts were incubated with terguride or SB 204741 for 7 d. The number of apoptotic and necrotic cells was analyzed as described previously (Dusler et al., 2007a).

 Bleomycin-induced dermal fibrosis. Skin fibrosis was induced in 6-wk-old, pathogen-free, female DBA/2 mice (Charles River) by local injections of bleomycin for 21 d. Seven groups received subcutaneous injections of 100 µl bleomycin dissolved in 0.9% NaCl at a concentration of 0.5 mg/ml in defined areas of the upper back every other day to induce dermal fibrosis. Six groups of bleomycin-challenged mice were treated with the P2Y12 receptor inhibitor clopidogrel (25 mg/kg/d) by oral gavage, terguride (0.2 mg/kg/bid and 0.6 mg/kg/bid) by intraperitoneal injections, ciprofloxacin (10 mg/kg/d) by oral gavage, SB 204741 (5 mg/kg/d) subcutaneously, or imatinib (50 mg/kg/d) by intraperitoneal injections, which served as positive control. One group with subcutaneous injections of 100 µl of 0.9% NaCl served as control group. After 21 d, animals were sacrificed by cervical dislocation.

 The mouse model of bleomycin-induced dermal fibrosis was also used to evaluate a potential protection from fibrosis in mice lacking TPH1 or 5-HTβ. Four groups of mice were analyzed. Two groups consisted of TPH1−/− deficient.
FVB mice (n = 5) or 129/PAS mice lacking 5-HT2B (n = 8), whereas the other two groups consisted of wild-type FVB (n = 7) and 129/PAS mice (n = 8). Dermal fibrosis was induced in one group each by repeated injections of bleomycin, whereas the other two groups received sham treatment with subcutaneous injections of NaCl and served as controls.

Treatment of established fibrosis. For the treatment of established fibrosis, a modified model of bleomycin-induced dermal fibrosis was used. Six groups of mice were analyzed. Two groups of mice were challenged with bleomycin for 3 wk and for 6 wk, respectively. The third group received bleomycin injections for 3 wk followed by NaCl injections for the next 3 wk to control for spontaneous regression of fibrosis. To assess the effects of tergure for treatment of established fibrosis, mice were challenged with bleomycin for 6 wk and treated in parallel with tergure (0.6 mg/kg/d) for the last 3 wk. Two groups of mice receiving intracutaneous injections of 100 µl of 0.9% NaCl for 3 wk and 6 wk, respectively, were used as controls.

Inhibition of platelet aggregation and 5-HT\textsubscript{2} signaling in Tsk-1 mice. B6.Cg-Fbn\textsuperscript{13659+/+}\textsuperscript{+}/Pld\textsuperscript{1720}\textsuperscript{+}/J (Tsk-1) mice and age-matched pa/pa (wild type) controls were purchased from the Jackson Laboratory (Charles River). Four groups of mice were analyzed. Three groups of Tsk-1 mice were treated with clopidogrel (25 mg/kg/d) by oral gavage, SB 24741 (5 mg/kg/d) subcutaneously, or the solvent DMSO subcutaneously. The fourth group consisted of pa/pa (control) mice, which also received subcutaneous injections of DMSO. The treatment was started at an age of 3 wk, and mice were sacrificed after 6 wk of treatment.

In addition, Tsk-1 mice were crossed with 5-HT\textsubscript{2}\textsuperscript{−/−} or TPH1\textsuperscript{−/−} mice to yield Tsk-1 mice deficient for 5-HT\textsubscript{2} or TPH1. The F2 generations consisting of 5-HT\textsubscript{2}\textsuperscript{−/−}/−/−/Tsk-1 (TPH1\textsuperscript{−/−}/Tsk-1), 5-HT\textsubscript{2}\textsuperscript{−/−}/−/−/Tsk-1 (TPH1\textsuperscript{−/−}/Tsk-1), 5-HT\textsubscript{2}\textsuperscript{+}/−/−/Tsk-1 (TPH1\textsuperscript{+}/−/−/Tsk-1), and 5-HT\textsubscript{2}\textsuperscript{+}/+−/−/Tsk-1 (TPH1\textsuperscript{+}/+−/−/Tsk-1) were analyzed.

Histological analysis. Paraffin-embedded histological sections were stained with hematoxylin and eosin for the determination of dermal thickness as described previously (Distler et al., 2007a). For direct visualization of collagen with hematoxylin and eosin for the determination of dermal thickness as described previously (Distler et al., 2007a).

Support Award of Medicine of the Ernst Jung Foundation, and a research grant from ErgoNex Pharma GmbH, Switzerland. L. Marteau was supported by grants from the Centre National de la Recherche Scientifique, Institut National de la Sante et de la Recherche Medicale, and Fondation de France. The authors have no conflicting financial interests.

Submitted: 9 August 2010
Accepted: 23 March 2011

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