Bone Morphogenetic Protein-6 Inhibits Fibrogenesis in Scleroderma Offering Treatment Options for Fibrotic Skin Disease

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BMP6 is known to be crucial for regulating embryonic skin development. This study assessed the role of BMP6 in dermal fibrosis. We detected that BMP6 is significantly increased in skin-derived fibroblasts of patients with localized scleroderma. Moreover, it was shown that BMP6 significantly impacts proliferation, migration, cytoskeletal organization, and collagen expression, as well as activity of the major pro-fibrogenic transcription factor AP-1 in dermal fibroblasts. The importance of BMP6 in dermal fibrosis was further confirmed in an in vivo model of dermal fibrosis in which BMP6-deficient mice showed significantly enhanced fibrosis compared with wild-type mice. Conversely, application of recombinant BMP6 significantly ameliorated dermal fibrosis in this preclinical bleomycin-induced sclerosis model, and herewith provided proof of concept for the successful treatment of this fibrotic skin disease.


INTRODUCTION

BMPs are members of the transforming growth factor–β (TGF-β) superfamily. BMPs were initially identified as osteo-genic factors present in demineralized bone that are capable of inducing ectopic bone formation. Furthermore, BMPs are known to have several other functions, both during embryonic development and in adult tissue regeneration and homeostasis. Due to their importance as regulators of different biological functions throughout the body, deficiency in BMP production or functionality usually leads to marked defects or severe pathologies (Wang et al., 2014).

During skin development, BMP6 is expressed in a strictly coordinated pattern. The synthesis of BMP6 in the mouse epidermis starts at embryonal day 15.5 and accompanies the development of a multilayered structure of the skin. BMP6 expression persists during the perinatal period and declines to low levels in adult skin 6 days postpartum (Lyons et al., 1989; Wall et al., 1993). However, expression of BMP6 can be strongly induced during wound healing or pathological conditions, such as psoriasis or carcinogenesis (Blessing et al., 1996; Kaiser et al., 1998; Lian et al., 2013; Maegdefrau et al., 2012; Stieglitz et al., 2019). Additionally, BMP6 has emerged as a potential regulator of fibrosis in the kidney and the liver (Arndt et al., 2015; Dendooven et al., 2011). However, the role of BMP6 in skin fibrosis has not addressed so far.

BMP6 belongs to the same BMP subfamily as BMP5 and BMP7, and it has been shown that the role of especially BMP7 seems to vary in a tissue- and disease-specific manner (Kinoshita et al., 2007; Maric et al., 2003; Morrissey et al., 2002; Murray et al., 2008; Myllarniemi et al., 2008; Zeisberg et al., 2003, 2007a, 2007b).

Several fibrosing connective tissue disorders of the skin share the characteristic activation of fibroblasts that results in increased production and deposition of collagen (Canady et al., 2013). These diseases include localized and systemic scleroderma, eosinophilic fasciitis, keloids, hypertrophic scars, lichen sclerosus et atrophicus, and sclerotic graft-versus-host disease. The molecular mechanisms responsible for the fibrotic process are still only partly understood. In addition, therapeutic options are mostly unsatisfactory and not uniformly accepted because of the lack of large controlled studies. Topical and systemic immunosuppressants, such as corticosteroids, are commonly used as anti-fibrotic agents, but with considerable side effects and limited efficacy. Therefore, new effective and well-tolerated treatment modalities are urgently required.

The aim of this study was to investigate the relevance of BMP6 in skin fibrosis and to analyze its potential as a therapeutic agent. In the study, we focused on localized scleroderma (synonym: morphea) because of clinical as well as pathophysiologic considerations. Localized scleroderma is a potentially debilitating skin disease for which new effective therapeutic modalities are urgently needed. Furthermore, localized scleroderma is a useful model for sclerotic skin diseases because it shares many pathophysiological processes with other sclerosing skin diseases, particularly systemic sclerosis, such as changes in collagen metabolism and fibroblast activation.

RESULTS

BMP6 expression is increased in localized scleroderma

To obtain a first impression of the relevance of BMP6 in skin fibrosis, the mRNA expression levels of BMP6 and the

Abbreviations: BLM, bleomycin; wt, wild-type

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structurally related BMP5 and BMP7 were analyzed in human localized scleroderma-derived dermal fibroblasts and compared to those of normal fibroblasts from neonatal as well as adult donors. A significant induction was measured for BMP6 in scleroderma-derived fibroblasts (Figure 1a), while expression of BMP5 and BMP7 did not significantly differ (Supplementary Figure S1a, S1b online). The fibrogenic potential of scleroderma fibroblasts in comparison to normal fibroblasts was confirmed by induction of collagen αI (Supplementary Figure S1c) and alpha-smooth muscle actin (Supplementary Figure S1d), important markers of fibrosis in sclerotic skin (Kissin et al., 2006). Similarly, BMP6 mRNA expression was significantly higher in patient material derived from late scleroderma skin specimens in comparison to normal skin (Figure 1b). Additionally, the BMP6 amount in the cell culture supernatants from localized scleroderma fibroblasts was higher than in supernatants from neonatal or adult control fibroblasts (Figure 1c). Furthermore, immunohistology revealed BMP6 staining in all analyzed patients with early- and late localized scleroderma, whereas the control skin was BMP6 negative in all cases. Interestingly, the strongest BMP6 staining was observed in the fibrotic center of lesions from patients of late localized scleroderma (arrows), and the BMP6 staining in normal skin sections was negative in all cases. Stained cells were counted by a blinded investigator. Scale bar = 100 μm. ANOVA, analysis of variance; H&E, hematoxylin and eosin.

**BMP6 deficiency aggravates fibrosis in experimental skin sclerosis**

To get further insight into the impact of increased BMP6 expression in scleroderma, we applied a well-established
murine model of sclerotic skin induced by repeated local injection of bleomycin (BLM) (Yamamoto and Nishioka, 2002; Yamamoto et al., 1999, 2000). In this model, BMP6 increased in a time-dependent manner compared to the corresponding phosphate buffered saline control group (Figure 2a). Hereby, BMP6 induction correlated with the induction of collagen αI (1) (Supplementary Figure S2a online) and alpha-smooth muscle actin (Supplementary Figure S2b). To obtain more insight into the consequences of enhanced BMP6 expression during skin fibrosis, BMP6-deficient (BMP6−/−) mice (Solloway et al., 1998) and wild-type (wt) mice were compared in the BLM-induced skin sclerosis model. Without BLM treatment, histological analysis did not show any phenotypical differences between wt and BMP6−/− mice (Figure 2b).

Treatment with BLM caused excessive collagen deposition and enhanced maturation of collagen fibers in both groups of mice; however, BMP6−/− mice showed higher expression and deposition of collagen (Figure 2c–2f) and alpha-smooth muscle actin (Figure 2c, 2g) than wt mice. In contrast, the expression of the pro-inflammatory genes II-6 and TNF alpha (Supplementary Figure S3a, S3b online) and the immune cell infiltration as analyzed by CD3 staining (Supplementary Figure S3c) did not significantly differ between wt and BMP6−/− mice in the BLM-induced skin model. Still, there was a tendency for higher expression of inflammatory markers in the skin of BMP6−/− animals. However, in summary, our data suggest that elevated levels of BMP6 in skin sclerosis induce mainly anti-fibrogenic rather than anti-inflammatory effects.

**Figure 2. Elevated fibrosis in BMP6−/− mice in experimental localized scleroderma.** (a) BMP6 expression in the dermis of wt-mice in a BLM-induced sclerosis model during fibrosis progression after 15, 30, and 40 local BLM injections compared to PBS control. n = 6 animals per group. Significance was calculated by comparing PBS-treated versus BLM-treated dermal skin tissue of wt mice at day 15, 30, and 40 (*P < 0.05, Student t test). (b) Representative images of H&E-, Sirius Red/Fast Green, and alpha-SMA staining of skin sections from wt and BMP6−/− mice with normal skin (ctrl. skin) or (c) after 40 injections of BLM. n = 6 animals per group. Scale bar = 100 μm. (d, e) mRNA and protein quantification of collagen in the dermis of wt and BMP6−/− mice in normal skin (PBS-treated) or in a BLM-induced sclerosis model. Significance was calculated by comparing wt versus BMP6−/− in normal skin or in BLM-induced sclerosis (Student t test). n = 6 animals per group. (f) Quantification of the fibrotic region by quantifying the Sirius Red-positive area by image analysis. Data are presented as percentage of the total area (*P < 0.05, Student t test). n = 6 animals per group. (g) Determination of the fibrotic region by quantifying the Sirius Red-positive area by image analysis. Data are presented as percentage of the total area (*P < 0.05, Student t test). n = 6 animals per group. BLM, bleomycin; BMP6−/−, BMP6-deficient; H&E, hematoxylin and eosin; hpf, high-power field; PBS, phosphate buffered saline; SMA, smooth muscle actin; wt, wild-type.
BMP6 deficiency enhances a pro-fibrogenic phenotype of skin-derived fibroblasts

To further elucidate the anti-fibrotic effect of BMP6 on skin fibrosis, we analyzed functional differences between dermal fibroblasts isolated from wt and BMP6−/− mice using different in vitro assays. Real-time assays using the xCELLigence system revealed significantly higher proliferation of BMP6−/− compared with wt fibroblasts (Figure 3a, 3b). Similarly, Ki67 immunohistological analysis of formalin-fixed and paraffin-embedded fibroblasts showed a significantly higher mitotic activity of BMP6−/− fibroblasts (Supplementary Figure S4a, S4b online). Also in the 3-dimensional spheroid model BMP6−/− fibroblasts formed significantly larger spheroids than wt cells (Figure 3c, 3d).

Figure 3. Induced proliferation and migration in BMP6−/− fibroblasts. (a) Cell proliferation using the Real-Time Cell Analyzer (xCELLigence; data are expressed as cell index). (b) Cell proliferation (wt set 100%) between 10 hours and 40 hours after starting the Real-Time Cell Analyzer system (*P < 0.05, Student t test). (c) Proliferation in a 3-dimensional spheroid model. Representative images are shown. Scale bar = 100 μm. (d) Spheroid area (μm²; average of 6 spheroids/group) (*P < 0.05, Student t test). (e) Wound healing assay. Representative images collected after the removal of the culture insert (0 hours) and 24 hours later. Scale bar = 500 μm. (f) Cell migration 24 hours after the removal of the culture insert (data represent “wound area” from at least four separate visual fields and three separate experiments.) (*P < 0.05, Student t test). (g) Cell migration out of 3-dimensional spheroids. Scale bar = 100 μm. (h) Area of the migrated fibroblasts (average from 6 spheroids) (*P < 0.05, Student t test). BMP6−/−, BMP6−/− deficient; wt, wild-type.
In addition to the enhanced fibroblast proliferation, a further hallmark of fibrotic processes is the elevated migratory ability. In a 2-dimensional wound-healing assay BMP6+/− fibroblasts facilitated significantly faster wound closure, indicating a higher migratory potential than control cells (Figure 3e, 3f). Similarly, BMP6+/− cells had a significantly higher potential to migrate into the collagen matrix than wt fibroblasts in a 3-dimensional spheroid assay (Figure 3g, 3h).

**Loss of BMP6 affects the contractility and the cytoskeletal organization in fibroblasts**

One critical pro-fibrotic property of fibroblasts is the ability to produce and organize extracellular matrix, particularly collagen. A free-floating collagen contraction assay using wt and BMP6+/− fibroblasts showed stronger collagen contraction through BMP6+/− cells than wt cells (Figure 4a, 4b). Moreover, collagen z1 (1) and alpha−smooth muscle actin mRNA expression was enhanced in BMP6+/− fibroblasts compared to wt cells (Figure 4c, 4d). These data further indicate that BMP6 promotes anti-fibrogenic properties in dermal fibroblasts.

Furthermore, BMP6 may be assumed to modify the cell structure or cell attachment. First, the attachment to different matrix components (laminin, collagen type IV, collagen type I, vitronectin, and fibronectin) was measured, but no differences were found between wt and BMP6+/− fibroblasts (Supplementary Figure S5 online). Next, the organization of the actin cytoskeleton was determined by F-actin immunofluorescence staining. In wt cells, F-actin was distributed throughout the cell, whereas in BMP6+/− cells, most of the stress fibers were located on the cell periphery (Figure 4e). Because each F-actin fiber is linked to focal contacts, it was determined by means of focal adhesion kinase staining whether loss of BMP6 influences the number of focal adhesion complexes (Figure 4e). The total number of adhesion complexes per cell was quantified, yielding significantly more adhesion points in wt cells than in BMP6+/− cells (Figure 4f). These results may lead to the assumption that loss of BMP6 has no effect on the attachment to the extracellular matrix, but modifies the cell structure by reducing the formation of F-actin fibers and the number of focal adhesion complexes.

**BMP6 deficiency causes enhanced AP-1 activity in dermal fibroblasts and fibrotic lesions**

Next, we aimed to analyze the signaling mechanism by which BMP6 affects dermal fibroblasts. Here, we focused on the transcription factor AP-1 that has major impact on cell growth, cell migration, proliferation, extracellular matrix degradation, and tissue reorganization (Angel et al., 1991, 2001; Karin et al., 1997). Inhibition of AP-1 signaling is known to abrogate the activation of fibroblasts, thus preventing experimental fibrosis (Avouac et al., 2012). In particular, c-Jun seems to be a major factor in these processes (Angel et al., 2001; Karin et al., 1997). Interestingly, the mRNA level of the AP-1 family member c-Jun in our study differed between wt and BMP6+/− fibroblasts. The c-Jun expression was higher in BMP6+/− fibroblasts than in wt cells (Figure 5a). Furthermore, luciferase assays revealed that loss of BMP6 in fibroblasts induced AP-1 activity (Figure 5b). Moreover, we performed immunohistochemical staining for phosphorylated (Ser73) c-Jun in the BLM-induced skin sclerosis model. Significantly more phospho-c-jun-positive fibroblast typical spindle-shaped cells appeared in BMP6+/− mice compared with wild-type mice (Figure 5c, 5d). Together, these data indicate BMP6 as negative regulator of AP-1 activity in dermal fibroblasts.

**BMP6 application reduced formation of skin sclerosis in mice**

To further study the anti-fibrogenic potential of BMP6 on skin sclerosis in vivo, we applied recombinant BMP6 in the experimental model of sclerotic skin. Control mice were treated with solvent (phosphate buffered saline) only. BMP6 application was started on day 14 after the first BLM treatment. Histological analysis revealed that BMP6 markedly reduced the development of sclerotic responses compared to control (BLM) mice (Figure 6a, 6b). Furthermore, collagen z1 (1) mRNA expression and the total collagen amount in skin areas with BLM induced fibrosis was reduced in BMP6 treated compared with untreated (BLM) mice (Figure 6c, 6d).

In summary, BMP6 application exhibited strong anti-fibrogenic effects in a preclinical model of skin sclerosis.

**DISCUSSION**

The aim of this study was to enhance the understanding of the role of BMP6 in skin fibrosis using human and murine in vitro and in vivo model systems. Notably, previous studies of other BMP subfamily members, especially BMP7, showed that their effects on fibrosis vary in a tissue- and cell-specific manner (Cervantes-Garcia et al., 2017; Higgins et al., 2017; Jin et al., 2018; Murray et al., 2008).

In the case of localized scleroderma, our investigations indicated BMP6 to be the most affected BMP molecule within this BMP family. BMP6 expression was elevated in localized scleroderma-derived fibroblasts and in tissue samples obtained from patients with localized scleroderma. Interestingly, the expression of BMP6 appeared to correlate with fibrosis progression and not with inflammation, and the induced BMP6 expression could be seen as a protective mechanism against the development of fibrosis. The first indication for this hypothesis was obtained from the BLM-induced sclerosis model in which the expression of BMP6 increased steadily with increasing BLM treatments, whereas inflammation markers like IL-6, TNF-α, and CD3 do not significantly alter between wt and BMP6+/− mice. This hypothesis was further supported by the histological evaluation of human whole skin preparations of early (inflammatory phase) and late (fibrotic phase) localized scleroderma. Here, the strongest BMP6 staining was observed in the fibrotic center of lesions from late-phase patients.

The anti-fibrotic properties of BMP6 in the skin were clearly shown by means of a BLM-induced sclerosis model. Furthermore, functional assays with wt and BMP6+/− skin derived fibroblasts provided an insight into the molecular mechanisms underlying the fibro-protective effect of BMP6. Loss of BMP6 induced pro-fibrogenic hallmarks, such as elevated migration, proliferation, and collagen contraction. The strong production and modulation of extracellular matrix components provided further evidence on the anti-fibrotic potential of BMP6 in skin fibroblasts. Further investigations
Figure 4. The effect of loss of BMP6 on contractility and cytoskeletal organization in fibroblasts. (a) Free-floating collagen contraction assays of wt and BMP6−/− fibroblasts. Representative images show the contraction potential after 24h. (b) Collagen contraction of wt cells compared to that of BMP6−/− cells (n = 3 strains per genotype) from three independent experiments (*P < 0.05, Student t test). (c, d) mRNA expression of collagen αI (1) and alpha-SMA from wt and BMP6−/− cells 24 hours after the start of collagen contraction (n = 3 strains per genotype) (Student t test). (e) Staining for F-actin immunofluorescence staining (first row), FAK (second row), and merged images of F-actin/FAK double staining (third row) of wt and BMP6−/− fibroblasts. Scale bar = 20 μm. (f) Adhesion complexes from wt (set to 100%) and BMP6−/− fibroblasts (n = 10 cells per group) were counted and averaged (*P < 0.05, Student t test). BMP6−/−, BMP6-deficient; FAK, focal adhesion kinase; SMA, smooth muscle actin; wt, wild-type.
showed that elevated AP-1 activity seems to be responsible for the pro-fibrotic hallmarks in BMP6\textsuperscript{−/−} cells. The upregulation of the AP-1 member c-Jun was already detected in mouse models of dermal fibrosis induced by BLM or by adenoviral overexpression of a constitutive active TGF-\(\beta\) receptor type I by Avouac et al. (2012). These authors concluded that AP-1 is upregulated in scleroderma in a TGF-\(\beta\) dependent manner and that the selective AP-1 inhibitor T-5224 may efficiently prevent the development of experimental dermal fibrosis (Avouac et al., 2012). It is known that the activation of SMAD-2/3 via TGF-\(\beta\) or activins regulates the expression of several profibrotic genes, including different collagens (Verrecchia et al., 2001a, 2001b), plasminogen activator inhibitor-1 (Dennler et al., 1998; Hua et al., 1998), various proteoglycans (Dadlani et al., 2008; Schonherr et al., 1991), integrins (Margadant et al., 2010), connective tissue growth factor (Chen et al., 2002), and matrix metalloproteinases (Yuan et al., 2001). BMPs are capable of suppressing TGF-\(\beta\) mediated fibrotic gene expression via activation of SMAD-1/5/8 (Wang et al., 2003). Based on this context, our results lead to the assumption that BMP6 is a natural endogenous counter player of TGF-\(\beta\) during the development of skin fibrosis and that the development of fibrosis may be reduced by the exogenous administration of BMP6. Still, further investigations are necessary to fully elucidated the molecular mechanisms how BMP6 exhibits its anti-fibrogenic effects.

Our results indicate that AP-1 may be one trigger of changing migration, proliferation, collagen contraction, and matrix organization when comparing wt and BMP6\textsuperscript{−/−}.
fibroblasts. Several studies have already described correlations between AP-1 and the above-mentioned cellular mechanisms. Enhanced mRNA and protein levels of the AP-1 family member c-Jun has been found in keloids and hypertrophic scars that are characterized by hyperproliferation of dermal fibroblasts and accelerated production of components of the extracellular matrix (Teofili et al., 1999). In addition to the regulation of cellular proliferation (Holt et al., 1986), a governing role has emerged for AP-1 in the regulation of cell shape and motility in fibroblasts. Fos-transformed fibroblasts exhibit gross cytoskeletal reorganization, resulting in fewer actin stress fibers and focal contacts and altered motility (Hennigan et al., 1994; Lamb et al., 1997; Miller et al., 1984). In the present study, loss of BMP6 changed both, AP-1 activity and cytoskeletal organization. Stress fibers were reduced in BMP6/−/− fibroblasts, and the number of focal adhesion points was diminished. These observations corresponded with the obvious increase in the migration ability of BMP6/−/− fibroblasts with strong AP-1 activity.

In summary, the present study showed for the first time the anti-fibrotic potential of BMP6 in a preclinical skin sclerosis model, suggesting that BMP6 could be an interesting target in the treatment of fibrotic skin lesions. Further clinical studies are required to prove the efficacy of BMP6 for treating or preventing fibrotic skin diseases, such as localized scleroderma, systemic sclerosis, hypertrophic scars, or keloids.

**MATERIALS AND METHODS**

**Cells and cell culture**

Normal neonatal human dermal fibroblasts (n = 7), normal adult human dermal fibroblasts (n = 6), and fibroblasts isolated from the skin of patients with localized scleroderma (n = 4) were cultured as described (Arndt et al., 2011). Detailed information on gender, race,
and location on organ, age of the patient, and source/company of the cells was summarized in Supplementary Figure S6 online.

Primary murine fibroblasts were freshly isolated from three 4-week-old 129Sv/Ev wt and BMP6−/− mice as described in detail in Supplementary Materials and Methods online.

Mice and murine models of sclerotic skin
129Sv/Ev wt and BMP6−/− mice on a 129Sv/Ev inbred background were obtained from the Robertson Laboratory (Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA) (Solloway et al., 1998). All animals were 8 weeks old at the start of the study and maintained under specific pathogen-free and controlled conditions. Female wt and BMP6−/− mice (n = 6 per group) were subjected to a well-established BLM-induced sclerosis model (Yamamoto 2002, 2006; Yamamoto and Nishioki, 2005; Yamamoto et al., 1999) based on the Yamamoto et al. (1999) study on BALB/C mice with small modifications adapted for the 129Sv/Ev mouse strain: Subcutaneous BLM injection (10 µg/ml) was performed for up to 40 days; control animals received subcutaneous injections of phosphate buffered saline; after 15, 30, and 40 days, the animals were killed, and the BLM-treated skin area was removed and divided into three parts: one part was immediately snap-frozen and stored at −80°C, one part was fixed for histological preparations, and one part was provided for immediate RNA analysis.

For therapeutic intervention, 15 ng of recombinant BMP6 (R&D Systems, Wiesbaden-Nordenstadt, Germany; 100 ng/ml) was simultaneously injected subcutaneously with BLM for 26 days starting on day 14 after the first BLM treatment.

Immunohistochemical and immunofluorescence analysis
Immunohistochemistry of human and murine tissues and immunofluorescence analysis of cells is described in detail in Supplementary Materials and Methods.

Analysis of collagen content
Collagen analyses in mouse tissues were performed with the QuickZyme Total Collagen assay according to the manufacturer’s instructions (QuickZyme Biosciences, Leiden, The Netherlands).

ELISA
Cell supernatants from normal adult (n = 6), normal neonatal (n = 7), and localized scleroderma (n = 4) fibroblasts were collected 48 hours after cultivation and were analyzed for BMP6 amount using the human DuoSet ELISA from R&D Systems (Minneapolis, MN) according to the manufacturer’s instructions with small modifications in the assay procedure. Instead of 100 µl of sample, 300 µl of cell supernatant was added, and incubation was done overnight by 4°C instead of 2 hours as stated in the protocol. Finally, the BMP6 amount was normalized to total protein amount using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA). Each sample was assayed in duplicates, and the entire experiments were performed three times.

RNA isolation and quantitative real-time PCR analysis
For RNA isolation from murine skin, an approximately 0.4-cm³ piece of skin from the central fibrotic area was used. The RNA extraction was carried out from the previously separated dermis. Dermis was separated from epidermis by dispase digest (Life Technologies GmbH, Darmstadt, Germany) overnight at 4°C. Total dermal RNA was subsequently isolated using the RNeasy kit (Marchery-Nagel, Düren, Germany) according to the instructions for tissue isolation and cDNA was generated using the AMV reverse transcriptase kit (Promega, Mannheim, Germany) using 2 µg of total RNA for transcription. Isolation of total cellular RNA of cultured cells and reverse transcription were carried out as described (Arndt et al., 2010). Quantitative real-time reverse transcription–PCR was performed with specific sets of primers and conditions (Supplementary Figure S7 online) applying LightCycler technology (Roche Diagnostics, Mannheim, Germany) as described (Maegdefrau et al., 2009). Each reverse transcriptase–PCR was performed in duplicate and results were normalized to β-actin.

Transfection and luciferase assay
Overall, 2 × 10⁴ fibroblasts of wt and BMP6−/− mice (passage 2) were seeded into each well of a six-well plate and transfected with 0.5 µg of AP-1 reporter construct using Lipofectamine Plus (Life Technologies GmbH). The AP1-Luc reporter vector, containing seven repeats of the AP-1 Enhancer Elements (TGACTAA), was purchased from the PathDetect in Vivo Signal Transduction Pathway cis-Reporting Systems (Agilent Technologies, Santa Clara, CA). Twenty-four hours after transfection, the cells were lysed with Passive Lysis Buffer 1x (Promega, Mannheim, Germany), and luciferase activity was determined. To normalize transfection efficiency, 0.2 µg of a pRL-TK plasmid (Promega, Mannheim, Germany) was co-transfected in each sample reaction, and Renilla luciferase activity was measured with a luminometric assay (Dual-Luciferase Reporter Assay; Promega). Basal activity resulting from the pGL2 basic vector was set to 1.

Functional in vitro assays
Proliferation, attachment, migratory activity, and contractility of cells were analyzed in different functional assays as described in previous studies (Arndt et al., 2011, 2013a, 2013b) and in detail in Supplementary Materials and Methods.

Statistical analysis
Data were expressed as mean values ± standard deviation (SD). Data were analyzed with GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Two groups were compared with the Student unpaired t test, and more than two groups were compared by one-way analysis of variance with Tukey’s honestly significant difference post hoc test analysis. Differences were considered statistically significant at P < 0.05.

Study approval
All animal studies were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the appropriate permission from the Animal Rights Commission of the State of Bavaria, and approved by the Committee on Ethics of Animal Experiments of the University of Regensburg, Germany (permit numbers 54-2531.2-13/08).

Routine paraffin-embedded skin sections obtained from affected areas of patients with localized scleroderma were used anonymized. The diagnosis of localized scleroderma had been previously confirmed histologically by a dermatopathologist. Normal skin samples were obtained from remnants of full-thickness skin grafts. Patient consent for experiments was not required because German laws consider human tissue left over from surgery as discarded material.

DATA AVAILABILITY STATEMENT
The data sets generated during and/or analyzed during the current study are available from the corresponding author on request.

CONFLICT OF INTEREST
The authors state no conflict of interest.


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AUTHOR CONTRIBUTIONS

Conceptualization: SA, AKB; Methodology: SA, CH, AKB; Investigation: SA; Resources: SK, AKB; Writing - Original Draft Preparation: SA, CH; Writing - Review and Editing: SK, AKB. Funding Acquisition: SA, AKB, KH; Supervision: AKB

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.02.020.

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large versican-like chondroitin sulfate proteoglycan by arterial smooth

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BMP6 in Dermal Fibrosis

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SUPPLEMENTARY MATERIALS AND METHODS

Isolation and culture of primary murine fibroblasts
Hairless skin pieces were cut in 1-cm² squares and washed with Betaisodona solution (Mundipharma GmbH, Limburg, Germany) followed by one washing cycle with phosphate buffered saline (PBS). The skin pieces were then transferred to Dispase II solution (Gibco, Darmstadt, Germany) and incubated overnight at 4°C. Afterwards, the dermis and epidermis were separated with a pair of tweezers. The dermis was further incubated in collagenase solution (Serva, Amstetten, Austria) at 37°C for 6 hours and filtered through a 100-μm Falcon Cell Strainer (Corning GmbH, Kaiserslautern, Germany) at 1,200 rpm for 5 minutes. The cell pellet was resuspended and transferred to a T25 cell culture dish and cultured as described (Canady et al., 2013a). Isolation was performed from three 4-week-old 129Sv/Ev wt and BMP6−/− mice.

Immunohistochemical and immunofluorescence analysis
Immunohistochemistry was conducted on full-skin preparations from control donors (n = 4); scleroderma patients with early localized sclerosis (inflammatory phase) (n = 7) and late sclerosis (fibrotic phase) (n = 11). All sections were reviewed by a dermatopathologist. Two-micrometer human paraffin-embedded skin sections were screened for BMP6 expression, as described elsewhere (Arndt et al., 2010) using the antibody anti-BMP6 (AF507) (1:50; R&D Systems, Wiesbaden-Nordenstadt, Germany). Two-micrometer paraffin-embedded skin sections of wt and BMP6−/− mice were stained with anti-CD3 antibody (C7930) (1:2,000; Sigma-Aldrich, Taufkirchen, Germany), anti-alpha-smooth muscle actin antibody (EPR5368) (1:1,000; Abcam, Cambridge, UK), and anti-phospho-c-Jun antibody (Ser73) (D47G9) XP (1:50; Cell Signaling, Frankfurt am Main, Germany) according to the following protocol: sections were deparaffinized, rehydrated, and placed in Tris/EDTA buffer (pH 8) in a steamer for 30 minutes. After cooling down to room temperature, the tissue slides were washed in PBS and incubated for 1 hour with the primary antibody at room temperature, washed again with PBS, and incubated with Histofine solution (Nichirei Biosciences, Tokyo, Japan) for 30 minutes. Tissue slides were washed again with PBS and incubated with AEC substrate chromogen (ready to use; Dako-Agilent, Santa Clara, CA) for 15 minutes by room temperature in the dark. Staining reaction was stopped with bicarbonate solution; Sigma-Aldrich, Munich, Germany), and with 80 nmol/L rhodamine phalloidin (F-actin) (Cyto-skeleton, Denver, CO) in PBS containing 10% goat serum for 1 hour. After washing with PBS, the cells were mounted using hard set mounting medium with DAPI (Vector Laboratories, Burlingame, CA), and images were collected by fluorescence microscopy (Axio Imager Z1; Carl Zeiss Vision GmbH, Halbergmoos, Germany). Total adhesion complexes/cell were counted from 10 wt and 10 BMP6−/− fibroblasts and averaged.

Migration assay
The migratory behavior of wt and BMP6−/− fibroblasts was assessed by means of a wound healing assay as described previously (Arndt et al., 2013a). The migration rate into the “wound area” was documented and measured with a Carl Zeiss (Carl Zeiss Vision GmbH) immediately (0 hours) and after 24 hours. The migration rate of wt fibroblasts was set to 100% and compared to that of BMP6−/− cells. Proliferation was inhibited by treating the cells with 1.6 μg/ml mitomycin D (Sigma-Aldrich, Munich, Germany) before seeding into the culture-inserts (ibidi GmbH, Martinsried, Germany) according to the manufacturer’s instruction.

Ki67 staining to quantify proliferation
Proliferation analysis on 1 × 10⁶ formalin-fixed and embedded fibroblasts (passage 2) of wt and BMP6−/− mice was done with the Shandon Cytoblock Cell Block Preparation System (Thermo Electron Corporation, Pittsburgh, PA) and the subsequent Ki67 IHC analysis with Ki67 (SP6) antibody (1:500; Abcam, Cambridge, UK). Ki67-positive fibroblasts were counted from six fields of views, averaged, and were normalized to total cell counts.

Spheroid proliferation assay
“Hanging drop” spheroids were generated with fibroblasts of wt and BMP6−/− mice according to a modified protocol published by Ruedel et al. (2013). Cells were trypsinized, adjusted to 50,000 cells/ml in Dulbecco’s modified Eagle medium, and mixed with 20% methocel (6 g methyl cellulose; Sigma-Aldrich, Munich, Germany, 250 ml Dulbecco’s modified Eagle medium). Twenty-five microliters of the cell suspension were dropped onto the cover of a 9-mm petri dish filled with PBS. The cover dish was inverted and incubated under a humidified atmosphere of 8% CO₂ at 37°C for 72 hours. The spheroid area was determined in μm². The spheroids were harvested and collected in a Falcon tube filled with Dulbecco’s modified Eagle medium for subsequent experiments.

Spheroid migration assay
Harvested spheroids were embedded into a collagen matrix (1 part x10 minimum essential medium, 1 part 7.5% sodium bicarbonate solution; Sigma-Aldrich, Munich, Germany), and rat tail collagen type I (BD Biosciences, Heidelberg, Germany) at a final concentration of 2.5 mg/ml and covered
with 2 ml of Dulbecco’s modified Eagle medium and 1.6 μg/ml of mitomycin D (Sigma-Aldrich, Munich, Germany) per well. Migration of fibroblasts out of the spheroid was photographed, and the migrated area was determined 2 days after the start of the assay.

**Real-time cell analysis**

Wt and BMP6−/− fibroblasts were seeded in duplicates onto a 16-well E-plate at a density of 3,000 cells/well. Changes in cell proliferation were measured with the Real-Time Cell Analyzer of the xCELLigence System (Roche, Mannheim, Germany) for approximately 57 hours as described previously (Arndt et al., 2013b) and expressed as unit-less parameter termed cell index. Experiments were repeated three times.

**Attachment assay**

The relative attachment of wt and BMP6−/− fibroblasts to extracellular matrix proteins such as laminin, collagen type I, collagen type IV, vitronectin, and fibronectin was determined using the CytoMatrix (5) screen kit (ECM205; Millipore-Chemicon) as described previously (Arndt et al., 2011).

**Free-floating collagen contraction assay**

Free-floating collagen gels were assayed as described (Arndt et al., 2011) using cells of wt and BMP6−/− fibroblasts (passage 2). Contraction of the collagen gel was analyzed by measuring the diameter of the collagen gel over time, and images were collected for documentation after 24 hours. Experiments were independently conducted three times, and results were averaged.

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**Supplementary Figure S1. Gene expression in cell culture of normal and scleroderma-derived fibroblasts.**

(a) BMP5, (b) BMP7, (c) Col I, and (d) alpha-SMA expression in control fibroblasts (ctrl. fibr., neonatal n = 7, adult n = 6), and fibroblasts derived from scleroderma patients (scl. fibr., n = 4) (*P < 0.05, analysis of variance with Tukey’s post hoc test). Col I, collagen α1; SMA, smooth muscle actin.
Supplementary Figure S2. Expression of pro-fibrogenic gene expression in an experimental model of skin sclerosis. (a) Expression of Col I and (b) alpha-SMA in the dermis during fibrosis development (after \( \times 15 \) and \( \times 40 \) treatment with BLM). Corresponding PBS control was set to 1. \( n = 6 \) animals per group. Significance was calculated by comparing PBS-treated (\( \times 15 \)) versus BLM-treated (\( \times 15 \)) and PBS-treated (\( \times 40 \)) versus BLM-treated (\( \times 40 \)) (*\( P < 0.05 \), Student t test). BLM, bleomycin; Col I, collagen \( \alpha I \); PBS, phosphate buffered saline; SMA, smooth muscle actin.

Supplementary Figure S3. Expression of inflammatory markers in an experimental model of skin sclerosis. (a, b) Interleukin 6 and TNF-alpha mRNA expression in wt and BMP6 \( ^{-/-} \) dermal tissue of BLM-induced sclerosis (\( n = 6 \) per group) (Student t test). (c) Representative images of immunohistochemical staining for CD3 of control skin and skin tissue from BLM-induced sclerosis mice. \( n = 6 \) mice per group. Scale bar = 500 \( \mu \)m. BLM, bleomycin; BMP6 \( ^{-/-} \), BMP6-deficient; hpf, high-power field; wt, wild-type.
Supplementary Figure S4. Proliferation is induced in BMP6−/− fibroblasts. (a) Ki67 proliferation analysis was performed on formalin-fixed and embedded fibroblasts from wt and BMP6−/− mice and (b) Ki67-positive fibroblasts were counted in 6 hpf, averaged, and normalized to total cell counts (*P < 0.05, Student t test). Scale bar = 100 μm. BMP6−/−, BMP6-deficient; hpf, high-power field; wt, wild-type.

Supplementary Figure S5. Loss of BMP6 does not modify cell attachment to different matrix proteins. Attachment to laminin, collagen type IV, collagen type I, vitronectin, and fibronectin was analyzed using wt and BMP6−/− fibroblasts (n = 3 per genotype). Significance was calculated by comparing wt versus BMP6−/− cells (Student t test). BMP6−/−, BMP6-deficient; wt, wild-type.
<table>
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<tr>
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**Supplementary Figure S6. Information about the cells used.** Abbreviations: HDFa, human dermal fibroblast adult; HDFneo, human dermal fibroblasts neonatal; HDFsclero, human dermal fibroblasts from localized scleroderma patients; y.o., years old.
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*Supplementary Figure S57. Primers and conditions.* Ann, annealing temperature; mt, melting temperature.