Fussel-15, a New Player in Wound Healing, Is Deregulated in Keloid and Localized Scleroderma

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Dermal wound healing depends on highly complex interplay among various cytokines and cell types. Disruption of this process can result in impaired healing in the form of excessive scarring, as is the case in fibrotic diseases such as keloid and scleroderma. In the present study, we found Fussel-15, a new member of the Ski/Sno family of TGF- β /BMP signaling repressors, to be expressed in early wound healing and constantly overexpressed in keloid-derived and scleroderma-derived fibroblasts. Comparing the results of three-dimensional free-floating and attached-released *in vitro* wound healing assays, we observed that Fussel-15 is expressed during the migratory phase in the free-floating assay, indicating that Fussel-15 might play a role during fibroblast migration. Fussel-15-transfected fibroblasts showed greater migration ability in a scratch wound healing assay, compared with control-transfected cells. This migratory phenotype due to Fussel-15 was confirmed by increased peripheral F-actin localization and modifications in size, amount, and distribution of focal adhesion complexes, which were observed using F-actin and focal adhesion kinase (FAK) immunofluorescence staining, respectively. The present results suggest that expression of Fussel-15 during wound healing might promote fibroblast migration. Permanent expression of Fussel-15 in keloid and skin sclerosis fibroblasts could be involved in the pathogenesis of these conditions, but the molecular mechanism underlying this up-regulation remains to be determined. (*Am J Pathol* 2011, 178:2622–2631; DOI: 10.1016/j.ajpath.2011.02.009)

The process of wound healing occurs in the body to regenerate dermal and epidermal tissues. Upon injury, a set of complex biochemical events takes place in a closely orchestrated manner. These events overlap in time,^{1,2} but can be artificially categorized into separate

stages: the inflammatory, proliferative, re-epithelialization, and remodeling phases. In the inflammatory phase, macrophages and granulocytes invade the tissue and start to clean the wound. During the proliferative phase, the most important cell type is the fibroblast. Approximately 2 or 3 days after wounding, fibroblasts begin to migrate into the wound site, marking the onset of the proliferative phase, even before the inflammatory phase has ended.³ Fibroblasts are responsible for collagen production to form a new provisional extracellular matrix (ECM) (granulation) and for pulling together the wound edges (contraction).⁴ During re-epithelialization, keratinocytes migrate into the area of wounded skin, providing cover for the new tissue. The remodeling phase, in which the ECM is reorganized by fibroblasts, can take several weeks and even up to months or years.

Various physiological and mechanical factors may impair the healing response, resulting in aberrations such as chronic wounds or fibrotic lesions, which are characterized by increased ECM production by fibroblasts. Keloid and skin sclerosis are two fibroproliferative diseases caused by an exaggerated response to injury. The key alteration responsible for the pathogenesis of these conditions has not been identified, however, and no satisfactory treatment is thus far available. The fibroblasts involved in keloid and skin sclerosis formation are thought to differ phenotypically and functionally from those present in normal scars. In both diseases, excessive amounts of collagen and other ECM components are deposited in the skin, leading to fibrosis.

To understand the molecular causes leading to the development of keloid and skin sclerosis, it is essential to determine how normal wound healing is controlled. Regulation of scar metabolism related to collagen and wound matrix degradation shows promise for the development of alternative therapies to treat abnormal scars. The functional Smad-suppressing element on chromosome 15, Fussel-15, may represent a new role in the molecular network regulat-

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ing homeostasis in normal and diseased skin. Understanding the functional roles of Fussel-15 in the biological processes of both normal and abnormal wound healing can be expected to contribute to the development of new strategies to cure these pathological conditions.

Materials and Methods

Cell Lines and Cell Culture Conditions

Normal human dermal fibroblasts ($n = 3$) (Cambrex Charles City, Charles City, IA) and fibroblasts isolated from the skin of patients with keloid ($n = 4$) or localized scleroderma ($n = 5$) (gift from Rüdiger Hein, Technical University Munich, Germany) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Deisenhofen, Germany; St. Louis, MO) supplemented with penicillin (400 U/mL), streptomycin (50 μ g/mL), L-glutamine (300 μ g/mL), and 10% fetal calf serum (FCS; Sigma-Aldrich) and split in a ratio of 1:5 every 3 days.

For fibroblast activation, cells were incubated with 35 ng/mL of human recombinant platelet-derived growth factor PDGF-BB (Promokine; PromoCell, Heidelberg, Germany) in FCS-free medium for 4, 6, 8, and 24 hours before RNA was isolated. The activity of the cells was controlled by collagen α (1) real-time PCR analysis.

For regulatory analysis, fibroblasts were incubated with 100 ng/mL of recombinant TGF- β 1 (tebu-bio, Offenbach, Germany), TGF- β 2 (tebu-bio), or TGF- β 3 (R&D Systems, Wiesbaden-Nordenstadt, Germany; Minneapolis, MN) for 12 hours before RNA was isolated.

Monocytic cell lines (U937, THP-1) were maintained in DMEM with the supplements mentioned above, but with heat-inactivated (1 hour; 56°C) FCS (Sigma-Aldrich). Treatment with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL; Sigma-Aldrich) was performed for 24 hours to activate the cells; this entailed the attachment of the suspension monocytes.

IHC

Paraffin-embedded biopsy sections from normal dermis ($n = 4$), early wounds ($n = 3$), late wounds ($n = 2$), the skin of patients with keloids ($n = 8$), or localized sclerodermas ($n = 7$), and three-dimensional (3D) cultivated fibroblasts from control subjects and scleroderma and keloid patients were screened for Fussel-15 protein expression by immunohistochemistry (IHC). Tissue from normal skin was obtained from a malignant melanoma surgery (using the tumor-free safety margin). Early wound tissue was obtained from follow-up resections performed 1 to 2 days after primary surgery, and late wound tissue was provided by patients for whom a follow-up resection was performed 5 to 7 days after primary surgery. The use of all tissue samples included in this study, including keloid and scleroderma tissues, was approved by the ethics committee of the University of Regensburg (applications 03/151 and 09/11). Additionally, informed consent was given by all keloid and scleroderma patients.

The tissues were deparaffinized, rehydrated, and subsequently incubated with a primary rabbit anti-Fussel-15

antibody (BioGenes, Berlin, Germany; 1:20) overnight at 4°C. Incubation with the secondary antibody (biotin-labeled anti-rabbit/anti-mouse; Dako, Hamburg, Germany; Carpinteria, CA) was performed for 30 minutes at room temperature, followed by incubation with streptavidin-POD (Dako) for 30 minutes. Antibody binding was visualized using 3-amino 9-ethylcarbazole solution (AEC solution; Dako). Finally, the tissues were counterstained with hemalaun solution (Dako).

CD68 (clone KP1; Dako) macrophage-specific staining was performed with paraffin-embedded sections of normal dermis ($n = 4$) and dermis of early wounds ($n = 3$), as described previously.⁵ Evaluation of the staining was performed semiquantitatively by means of light microscopy (Carl Zeiss Vision, Halbergmoos, Germany).

RNA Isolation and Reverse Transcription

Total cellular RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany; Valencia, CA), and cDNA was generated by reverse transcription performed in a 20- μ L reaction volume containing 2 μ g of total cellular RNA, 4 μ L of 5 $\times$ first-strand buffer (Invitrogen, Groningen, The Netherlands; Carlsbad, CA), 2 μ L of 0.1 mol/L dithiothreitol, 1 μ L of dN6-primer (10 mmol/L), and 1 μ L of dNTPs (10 mmol/L) and diethylpyrocarbonate-treated water. The reaction mixture was incubated at 70°C for 5 minutes before 200 units of SuperScript II reverse transcriptase (Invitrogen) was added and RNA was transcribed for 1 hour at 37°C. The reverse transcriptase was inactivated at 70°C for 10 minutes, and the remaining RNA was digested with 1 μ L RNase A (10 mg/mL) at 37°C for 30 minutes.

Real-Time PCR Analyses

Quantitative real-time PCR for Fussel-15, collagen α (1), collagen α (2), and fibronectin was performed using a LightCycler system (Roche Applied Science, Mannheim, Germany; Indianapolis, IN) as described previously.⁶ The primers used are listed in Table 1.

The PCR reactions were evaluated by melting curve analysis, and PCR products were controlled on 2% agarose gels. β -Actin was amplified to ensure cDNA integrity and to normalize expression intensity. Each real-time PCR was performed in triplicate for each of three independent experiments.

Table 1. Real-Time PCR Primer Sequences

Target	Direction	Primer
Fussel-15	Forward	5'-CCACGAGCCAGATAAGGAAG-3'
	Reverse	5'-CCATTGTGTTCCAGGAGCAGT-3'
Collagen α (1)	Forward	5'-GGACCTCCGGCTCCTGCT-3'
	Reverse	5'-TCCAGGGATGCCATCTCG-3'
Collagen α (2)	Forward	5'-CCCAGCCGGAGATAGAGG-3'
	Reverse	5'-TCACCAGGCTCACCAGCAGG-3'
Fibronectin	Forward	5'-GAACCATCAAGCCAGATGTCAGAAGC-3'
	Reverse	5'-TGCCATGATACCAGCAAGGAATTGGG-3'
β -Actin	Forward	5'-CTACGTCGCCCTGGACTTCGAGC-3'
	Reverse	5'-GATGGAGCCGCCGATCCACACGG-3'

Western Blotting

For total protein extraction, 1×10^6 trypsinized cells were incubated with 200 μ L of radioimmunoprecipitation assay buffer (Roche Applied Science) for 15 minutes at 4°C. Insoluble fragments were removed by centrifugation at $15,700 \times g$ for 10 minutes at 4°C, and the supernatant lysates were stored at -20°C. Because of the weak affinity of the Fussel-15 antibody, 80 μ g of radioimmunoprecipitation assay cell lysates were loaded onto a SDS polyacrylamide gel, separated by SDS-PAGE and then blotted onto a polyvinylidene difluoride membrane. After blocking for 1 hour with 3% bovine serum albumin/PBS, the membrane was incubated for 16 hours with primary antibodies against Fussel-15 (generated by BioGenes, Berlin, 1:200) or β -actin (Sigma-Aldrich, 1:2500). Then, the membrane was washed three times in PBS, incubated for 1 hour with an alkaline phosphatase-conjugated secondary antibody (Millipore-Chemicon, Temecula, CA; 1:5000), and washed again. Finally, immunoreactions were visualized by NBT/BCIP (Invitrogen-ZytoMed, San Francisco, CA) staining. All Western blot analyses were repeated at least three times.

Immunofluorescence Staining

For F-actin staining experiments, 1×10^6 normal human fibroblasts were transfected with 3 μ g of Fussel-15 expression vectors (Fussel-15-pCMV6-XL-5, generated by OriGene Technologies, Rockville, MD) or with 3 μ g of empty pCMV6-XL-5 vectors as a control and grown on collagen-coated (rat tail collagen, 3.85 mg/mL; BD Biosciences, San Jose, CA) 4-well chamber slides for 24 hours. Subsequently, the cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes, incubated for 5 minutes with 0.1% Triton-X-100 (Sigma-Aldrich) for permeabilization of the cell membrane, washed again, and covered with blocking solution (PBS/1% bovine serum albumin) for 1 hour. For F-actin/focal adhesion kinase (FAK) double-staining, the cells were incubated with rabbit anti-FAK (Millipore-Upstate Biotechnology, Lake Placid, NY; 1:30) for 1 hour after a washing with PBS. Subsequently, the cells were simultaneously incubated with the secondary antibody (FITC-conjugated Affinpure goat anti-rabbit IgG F antibody, 1:10; Jackson ImmunoResearch, West Grove, PA) and with 80 nmol/L rhodamine phalloidin (F-actin) (Cytoskeleton, 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 (Vectashield H-1500; Vector Laboratories, Burlingame, CA), and images were collected by fluorescence microscopy (Axio Imager Z1; Zeiss). F-actin single staining was accomplished according to the manufacturer's instructions (Cytoskeleton).

Free-Floating Collagen Contraction Assay

Free-floating collagen gels were assayed as described previously.⁷ The lattices were prepared in ultra-low attachment 6-well plates (Corning Life Sciences, Lowell, MA), combining 1.5 mL of a 1.5 $\times$ concentrated medium containing FCS with 0.35 mL of collagen solution (3.85

mg/mL; BD Biosciences) and 0.25 mL of cell suspension (1×10^6 cells/mL). This mixture polymerizes within 20 minutes at 37°C. The contraction of the collagen gel was analyzed by measuring the diameter of the collagen pellet over time, and images were collected for documentation after 2, 4, and 8 hours. For real-time PCR analysis, collagen cell pellets collected after 2, 4, and 8 hours were centrifuged, and RNA was isolated from the sedimented cells.

For knockdown experiments, fibroblasts were transiently transfected with 1.5 μ g of Fussel-15 small interfering RNA (siRNA; Qiagen) using a human dermal fibroblast Nucleofector kit (Amaxa; Lonza, Basel, Switzerland; Walkersville, MD). For Fussel-15 overexpression experiments, cells were transfected with 3 μ g of a Fussel-15 full-length expression plasmid (Fussel-15-pCMV6-XL-5, generated by OriGene Technologies) or with 3 μ g of an empty control vector.

Attached-Released Collagen Contraction Assay

Attached-released collagen gels were assayed as described previously.⁸ The lattices were prepared in high attachment 6-well plates (Corning Life Sciences). For each well, 0.4 mL of fibroblast suspension containing 2.5×10^5 cells were admixed with 0.4 mL of rat tail type I collagen solution (3.85 mg/mL; BD Biosciences) and placed into a plastic culture dish. After 1 hour of incubation at 37°C, 1.5 mL of 1.5 $\times$ concentrated medium containing FCS was trickled over the collagen lattice. Then, the attached lattices were incubated at 37°C for 2 hours and mechanically released from the culture dish by means of a sterile spatula. The culture dishes with the released lattices were returned to the incubator. Collagen lattices were photographed, measured, and used for RNA extraction immediately after detachment (<10 minutes) and after 2, 8, 24, and 48 hours of further incubation. All experiments were repeated at least three times, and real-time PCR analyses were performed in triplicate for three independent experiment.

Scratch Wound Healing Assay

The migratory behavior of the cells was examined by means of a scratch assay. For this, cells were seeded in high density into 6-well plates, coated with collagen (rat tail collagen; 3.85 mg/mL; BD Biosciences), and scratched with a pipette tip in a definite area. Migration into this area was documented and measured after 24 hours. The migration rate of control-transfected fibroblasts was measured after 24 hours using a Carl Zeiss microscope and set to 100%, compared with fibroblasts transfected with 3 μ g of a Fussel-15 expression plasmid (Fussel-15-pCMV6-XL-5). Each analysis was performed in duplicate and repeated three times.

Colorimetric Proliferation Assay

Cell proliferation was determined using an XTT proliferation assay (Roche Applied Science). The XTT was prepared by mixing 5 mg of XTT solution (Roche Applied

Science) with 0.1 mL of electron coupling reagent, according to the manufacturer's instructions. Control-transfected (pCMV6-XL-5 vector; 3 μ g) and Fussel-15-transfected (Fussel-15-pCMV6-XL-5; 3 μ g) fibroblasts were seeded into a 96-well microtiter plate (1000 cells/well) and incubated for 1 to 5 days. After the incubation period, 50 μ L of the XTT solution was added to each well (final concentration 0.3 mg/mL), and plates were incubated for an additional 4 hours. The optical density of each well was measured with a 490-nm test wavelength and a 630-nm reference wavelength using a microplate reader with SoftMax software Pro 5 (Molecular Devices, Wokingham, UK; Sunnyvale, CA). Results were presented as the optical density after blank (cell-free medium) subtraction. Each sample was assayed in triplicate, and this whole experiment was performed twice.

Attachment Assay

For determination of the relative attachment of Fussel-15 and control-transfected cells to ECM proteins such as vitronectin, laminin, collagen type I, collagen type IV, and fibronectin [CytoMatrix (5) screen kit, ECM205; Millipore-Chemicon], 10,000 cells were seeded onto the coated substrates of a 96-well plate and incubated for 30 minutes, followed by determination of relative cell attachment using a microplate reader, according to the manufacturer's recommendations. Experiments were performed in triplicate and repeated at least twice.

G-Actin/F-Actin Assay

Differences in the amount of G-actin and F-actin were investigated in Fussel-15-transfected and control-transfected fibroblasts using a G-actin/F-actin *in vivo* assay kit (BK037; Cytoskeleton). Fibroblasts were homogenized in F-actin stabilization buffer, followed by centrifugation at $100,000 \times g$ to separate the insoluble F-actin (P) from the soluble G-actin (S) pool, according to the manufacturer's instructions. Supernatant (S) and pellet (P) samples were then proportionally loaded onto an SDS polyacrylamide gel and were separated by SDS-PAGE; actin was quantitated by Western blot analysis.

Statistical Analysis

Results are reported as means $\pm$ SD (range) or percent. Comparisons between two groups were performed using a Student's paired *t*-test. analysis of variance with Tukey's post hoc Analysis was used for more than two comparisons. A *P* value of <0.05 was considered statistically significant. All calculations were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

Results

Fussel-15 Is Expressed in Activated Fibroblasts during Wound Healing in Vitro and in Vivo

Previously, Fussel-15 expression was shown to be restricted to the nervous system. In this study, we show for

Figure 1. Fussel-15 expression in dermal fibroblasts *in vivo* and *in vitro*. **A:** IHC with Fussel-15 antibody. In early wound tissues, distinct expression of Fussel-15 was observed in spindle-shaped fibroblasts (arrows), but no expression was detected in fibroblasts of normal dermis and late wound sections. Representative samples are shown. **B:** IHC of normal and early wound tissues (representative samples). CD68-positive macrophages, round in shape (top right, arrows), and spindle-like Fussel-15-positive fibroblasts (bottom right, arrows) were found in wound tissues but in normal dermis staining for both was negative. **C:** Fussel-15 mRNA expression in activated fibroblasts. After cells had been treated with platelet-derived growth factor (PDGF) for 8 hours, Fussel-15 mRNA expression was induced in isolated fibroblasts. **D:** Real-time PCR analysis of Fussel-15 expression in activated fibroblasts versus expression in activated monocytes. In fibroblasts activated by means of PDGF treatment for 8 hours, Fussel-15 expression was induced, but monocytic cell lines (THP, U937) did not show any Fussel-15 expression, even after PMA activation. **P* < 0.05 (C and D).

the first time that Fussel-15 is also involved in wound healing and in pathophysiological processes such as keloid and skin sclerosis.

By analyzing formalin-fixed, paraffin-embedded (FFPE) samples of both normal and wounded skin by means of IHC, we observed staining of Fussel-15 in early wound tissue, whereas no Fussel-15 expression was detected in later wound healing stages, or in normal dermis (Figure 1A). We observed Fussel-15 staining in spindle-like cells, which were identified as dermal fibroblasts by a pathologist (Katharina Schardt). Given that there is no specific fibroblast marker available, we performed CD68 histological staining. CD68 is a specific marker for macrophages, which are cell types often confused with fibroblasts in histological sections. We clearly observed morphological differences among rounded CD68-positive macrophages in early wound tissue, compared with the spindle-shaped Fussel-15-positive fibroblasts (Figure 1B). Next, we analyzed the expression level of Fussel-15 in isolated fibroblasts. Of note, we observed an induction of Fussel-15 primarily after treating the cells for 8 hours with PDGF (Figure 1C), which is a common mediator of fibroblast activation.⁹ The cell-type specificity of Fussel-15 expression was further confirmed when we compared the Fussel-15 expression level of PDGF-activated fibroblasts with the expression level of PMA-activated monocytic cell lines (THP, U937). Neither normal cultivated nor PMA-activated monocytic cells expressed Fussel-15 (Figure 1D).

To activate fibroblasts by imitating the *in vivo* events during wound healing, 3D collagen contraction assays were performed. Given that we had no data related to the function of Fussel-15 during wound healing, two different 3D collagen contraction models were used with different significances. The free-floating assay was originally developed by Bell et al,¹⁰ in which the development of tension is prevented in floating collagen lattices. This assay is therefore used specifically for measuring fibroblast migration and rearrangement of the collagen matrix. The attached-released assay is preferentially used to observe tension development.⁸ The rate of contraction immediately after release (<10 minutes) indicates the relative tension development, whereas cell movements and matrix organization can be observed during longer periods after lattice release.

In the free-floating assay, we observed a strong up-regulation of Fussel-15 mRNA expression 2 hours after contraction; a reduction of Fussel-15 mRNA was seen between 4 and 8 hours (Figure 2A). To evaluate Fussel-15 expression at the protein level, IHC was performed with the contracted collagen pellets; expression of Fussel-15 was observed 4 and 8 hours after contraction, but at later stages (24 hours) it was no longer detectable (Figure 2B).

In the attached-released assay, no Fussel-15 expression was observed <10 minutes after dislodgement of the collagen matrix from the cell culture dish, suggesting that Fussel-15 is not directly involved in tension development. Of note, we observed an up-regulation of Fussel-15 at 2 to 24 hours after release, with a maximum after 24 hours (Figure 2C). We speculate, therefore, that Fussel-15 is involved in fibroblast migration or rearrangement of the collagen structure.

Figure 2. Three-dimensional collagen contraction models showing the expression of Fussel-15 in fibroblasts *in vitro*. **A:** Real-time PCR analysis of Fussel-15 in 2-D versus 3-D cultured fibroblasts. In the 3-D free-floating assay, Fussel-15 mRNA expression was highly expressed after 2 hours of contraction, compared with 2-D cultured fibroblasts, which then decreased over time (between 4 and 8 hours). **B:** IHC with Fussel-15 antibodies. Protein expression of Fussel-15 was detected after 4 and 8 hours of contraction (arrows) in fibroblasts in 3-D free-floating collagen contraction pellets, whereas at later stages (24 hours) it was no longer detectable. **C:** Fussel-15 mRNA expression in fibroblasts in the 3-D attached-released model, compared with 2-D cultured fibroblasts. No induction of Fussel-15 expression was observed immediately (<10 minutes) after dislodgement of the collagen matrix from the cell culture dish. A consistent up-regulation of Fussel-15 was observed at 2 to 24 hours after release. * $P < 0.05$ and ** $P < 0.01$; ^{ns}, nonsignificant (**A** and **C**).

Changes in Fussel-15 Expression Result in Altered Collagen Contraction in the Free-Floating Contraction Assay

The functional role of Fussel-15 was analyzed by means of the free-floating collagen contraction assay. Transient transfection with a full-length Fussel-15 expression plasmid resulted in a 40-fold induction of Fussel-15 expression in fibroblasts at the mRNA level (Figure 3A) and in an increase of the amount of protein after 48 hours of transfection (Figure 3B).

Next, collagen contraction was analyzed using Fussel-15-transfected cells and cells transfected with a control plasmid (Figure 3C). Significantly enhanced lattice contraction was observed in Fussel-15-transfected cells 2

Figure 3. Effect of Fussel-15 overexpression on free-floating collagen contraction *in vitro*. **A:** Confirmation of efficient Fussel-15 transfection at the mRNA level. Transient transfection of fibroblasts with a Fussel-15 full-length expression plasmid was successful and resulted in a 40-fold induction of Fussel-15 mRNA expression. **B:** Confirmation of the overexpression of Fussel-15 at the protein level in fibroblasts by Western blot analysis. **C:** Free-floating collagen contraction of Fussel-15 and control-transfected fibroblasts. After 2 hours, collagen lattice contraction was significantly enhanced with Fussel-15-transfected cells, compared with cells transfected with a control plasmid. **D:** Confirmation of Fussel-15 siRNA transfection by means of RT-PCR. Transient transfection with two different Fussel-15 siRNAs resulted in 60% down-regulation of Fussel-15 mRNA expression using siRNA1 and in approximately 40% down-regulation using siRNA2. The siRNA1 was used for further experiments. **E:** Collagen contraction of Fussel-15 siRNA and control-transfected fibroblasts. Down-regulation of Fussel-15 expression by siRNA1 resulted in a significantly delayed collagen lattice contraction after 4 hours of contraction, compared with the collagen lattice with control-transfected cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (**A**, **C**, **D**, and **E**).

hours after contraction. In contrast, down-regulation of Fussel-15 expression by means of siRNA (Figure 3D) resulted in significantly delayed collagen contraction after 4 hours (Figure 3E).

Modification of Fussel-15 Expression Affects Fibroblast Migration but Has No Influence on Matrix Synthesis, Cell Proliferation, or Cell Attachment in Vitro

To investigate the functional role of Fussel-15 during wound healing, we performed different *in vitro* assays using Fussel-15-overexpressing and control-transfected fibroblasts.

First, we analyzed whether Fussel-15 is involved in matrix synthesis. We observed no significant changes in collagen

$\alpha(1)$, collagen $\alpha(2)$, or fibronectin expression in Fussel-15-transfected fibroblasts, compared with control-transfected cells (data not shown). To analyze whether Fussel-15 plays a role during fibroblast proliferation or migration, functional XTT proliferation and scratch migration assays were performed. Proliferation was not influenced by Fussel-15 overexpression (data not shown), but the migration rate of Fussel-15-transfected fibroblasts was significantly higher than that of controls (Figure 4A). The greater migration ability of Fussel-15-transfected cells into the wound bed of a scratched wound within 24 hours is shown in Figure 4B. These results suggest that Fussel-15 might be involved in fibroblast migration, whereas adhesion to different matrix proteins seems to be unaffected by Fussel-15 (Figure 4C).

Fibroblast movement during wound healing is a complex phenomenon driven primarily by the actin network.¹¹ To analyze whether Fussel-15 is involved in actin organization processes, we performed Western blot quantification of globular (G-actin) and filamentous actin (F-actin) in cellular fractions of Fussel-15-transfected and control-transfected cells. The amount of G-actin was not changed by Fussel-15, but we observed an increase in the F-actin level in Fussel-15-transfected cells, compared with control cells (Figure 4D). These results led us to speculate that Fussel-15 might be involved in the formation of F-actin.

Overexpression of Fussel-15 in Fibroblasts Changes the Formation of F-Actin Patterns and the Number, Size, and Distribution of Focal Adhesion Points

Based on observations suggesting that Fussel-15 might be involved in F-actin formation, we analyzed the F-actin pattern after Fussel-15 transfection by means of F-actin immunofluorescence staining. According to the method of Verderame et al,¹² we classified the F-actin distribution of cultured fibroblasts into four categories: category 1, >90% of the cell area is filled with cables; category 2, with at least two thick cables running under the nucleus, while the rest of the cell area is filled with fine cables; category 3, no thick cables, but some fine cables are present; and category 4, only a few cables are visible in the central area of the cell, with thick cables running at the periphery. In comparing control cells with Fussel-15-transfected cells, the latter were classified into category 4, whereas control-transfected cells were assigned to category 1 (Figure 5A).

Given that each F-actin fiber is linked to focal contacts, we next determined whether Fussel-15 influences the number, size, and distribution of focal adhesion complexes using F-actin/focal adhesion kinase (FAK) co-staining (Figure 5A). First, we quantified the total number of adhesion complexes per cell in 10 Fussel-15-transfected and 10 control-transfected cells and observed an approximately 10% reduction of adhesion complexes after Fussel-15 transfection (Figure 5B). Second, the number of peripheral adhesion complexes was determined. Here, we counted 20% more adhesion complexes at the

Figure 4. Influence of Fussel-15 overexpression on fibroblast behavior. **A:** The migration rate was measured 24 hours after scratching. The migration ability of Fussel-15-transfected cells within 24 hours was calculated and is displayed as the percentage relative to controls (set at 100%). The results are measurements of the scratch area from at least 12 separate visual fields from three separate experiments. **B:** Scratch assay showing that Fussel-15 promotes fibroblast migration; representative images are shown, immediately after scratch and at 24 hours. **C:** Attachment assay showing that Fussel-15 has no influence on adhesion to different matrix proteins. Attachment of Fussel-15 and control-transfected fibroblasts to vitronectin, laminin, collagen type I and type IV, and fibronectin was analyzed, but no significant difference was observed in attachment to any of the matrix proteins examined. **D:** Fussel-15 changes the proportion of F-actin. Actin levels were quantified by Western blot analysis. The ratio of globular actin (G-actin; supernatant cell fraction) was not altered by Fussel-15, but the amount of filamentous actin (F-actin; pellet cell fraction) was increased after Fussel-15 transfection. Total protein lysates of Fussel-15 and control-transfected cells were applied to confirm equal loading. ** $P < 0.01$ (A).

cell periphery after Fussel-15 transfection, compared with control-transfected cells (Figure 5C). Third, the distribution of adhesion complexes per cell was examined. Of note, Fussel-15-transfected cells exhibited more attachment points at the cell margin (areas I and III) than in the central area (area II) (Figure 5D). Finally, we analyzed the size of adhesion complexes. We detected a greater amount of medium and tall complexes after Fussel-15 transfection, whereas control-transfected cells presented a greater amount of small attachment points (Figure 5E).

In summary, we suggest that Fussel-15 influences the formation of F-actin and the formation of adhesion complexes due to F-actin modifications.

Fussel-15 Is Permanently Expressed in Keloids and in Skin Sclerosis

After observing the temporally strictly controlled expression pattern of Fussel-15 in normal wound healing and the functional changes resulting from deregulation of Fussel-15, we decided to examine human keloid and scleroderma tissues with respect to Fussel-15 expression. IHC revealed strong Fussel-15 protein expression in disease-derived dermal fibroblasts, compared with very low expression in fibroblasts from control dermis (Figure 6A). These results could be confirmed *in vitro* at the mRNA level, with significantly increased expression of Fussel-15 detected in keloid-derived and scleroderma-derived fibroblasts after 8 hours of collagen contraction (Figure 6B). IHC staining of sections from formalin-fixed, paraffin-embedded collagen contraction pellets con-

firmed the presence of strong Fussel-15 expression in keloid and skin sclerosis fibroblasts, compared with normal fibroblasts, at 8 hours after contraction (Figure 6C). Of note, keloid and skin sclerosis fibroblasts basically did not produce more Fussel-15 than the maximal level observed in normal 3D activated fibroblasts after 2 hours of contraction (data not shown). We therefore speculate that a thus far unknown regulation mechanism prevents the down-regulation of Fussel-15 in keloids and in skin sclerosis, leading to sustained elevated expression.

TGF- β s Are Not Able to Regulate the Expression of Fussel-15

To better understand why Fussel-15 is permanently expressed in keloids and skin sclerosis, we conducted a preliminary investigation of the regulation mechanisms underlying Fussel-15 expression. TGF- β family members are considered to be central mediators of both wound healing and pathological organ fibrosis. In mammals, this family comprises three types: TGF- β 1, - β 2, and - β 3. The function of TGF- β s during skin morphogenesis and wound healing has been well characterized; we were therefore interested in whether these growth factors are able to regulate the expression of Fussel-15. We incubated fibroblasts with recombinant TGF- β 1, TGF- β 2, or TGF- β 3 (100 ng/mL) for 12 hours, after which RNA was isolated. Notably, none of these TGF- β molecules appeared to exert any influence on the expression of Fussel-15 (data not shown).

Figure 5. Overexpression of Fussel-15 changes the localization of F-actin and the number, distribution, and size of attachment complexes. **A:** F-actin immunofluorescence staining (upper row) of Fussel-15-transfected cells shows a reduction of F-actin fibers in the central area of the cell, whereas peripheral actin fibers seem to be strengthened, compared with control-transfected cells. F-actin/focal adhesion kinase (FAK) double-staining (lower row) was performed to analyze changes in attachment complexes due to Fussel-15. Note change in original magnification between rows. **B–E:** Number, distribution, and size of adhesion complexes was determined. **B:** Fussel-15 reduces the total number of adhesion complexes per cell by approximately 10%. Three visual fields of 10 different Fussel-15 and control-transfected cells were counted under $\times 40$ optical magnification. **C:** Fussel-15 enhances the number of peripheral adhesion complexes by 20%. Adhesion complexes on the cell periphery of Fussel-15-transfected fibroblasts were counted and compared with control-transfected cells. The calculation was performed analyzing three visual fields of 10 different Fussel-15 and control-transfected cells under $\times 40$ optical magnification. **D:** The distribution of adhesion complexes was influenced by Fussel-15. For quantification of changes in the distribution of adhesion complexes, cells were divided into three areas (areas I, II, and III, as indicated in the cell schematic). Three visual fields of each area from 10 different Fussel-15 and control-transfected cells were analyzed under $\times 40$ optical magnification. **E:** Size of adhesion complexes was influenced by Fussel-15 transfection. The adhesion points were divided into three different sizes: small, $\leq 1 \mu\text{m}^2$; medium, $1\text{--}2 \mu\text{m}^2$; and tall, $> 2 \mu\text{m}^2$. Three visual fields from 10 different Fussel-15 and control-transfected cells were analyzed using $\times 40$ optical magnification. * $P < 0.05$ and *** $P < 0.001$; ns, nonsignificant (**B–E**). Blue circles, small adhesion complexes; orange circles, F-actin; yellow circles, tall adhesion points; black lines, peripheral actin fibers (**B–E**).

Further extensive studies will be required to reveal the mediator of the constitutive expression of Fussel-15 in keloid and skin sclerosis.

Discussion

Recently, our research group discovered Fussel-15, a new member of the c-Ski/SnoN transcriptional co-repressor family involved in the inhibition of the bone morphogenetic (BMP) pathway.⁶ In contrast to the broad expression pattern observed for c-Ski and Sno,^{13,14} Fussel-15 was detected exclusively in the brain and in the dorsal spinal cord.⁶

Through screening a variety of different tissues for Fussel-15 expression *inter alia*, a variety of different tissues, including tumorous and fibrotic tissues, we detected strong expression of Fussel-15 in the dermis during the physiological process of dermal wound healing. Because of these findings, we concluded that Fussel-15 may play a role not only in the nervous system, but also in the regulation of the healing processes in the skin.

Dermal wound healing is a complex process that occurs in several overlapping stages. Its successful progression depends on the complex signal integration of various factors. The interplay of these factors is highly coordinated and susceptible to interference. Understanding the molecular mechanisms underlying normal wound healing is absolutely necessary for finding new therapeutic options to treat dermal lesions such as keloids and skin sclerosis.

In the present study, we found that Fussel-15 was expressed in fibroblasts during wound healing in a temporally restricted manner. We could show this regulation process to be faulty in keloids and sclerodermas, so we hypothesized that the observed ongoing expression of Fussel-15 in keloid-derived and scleroderma-derived fibroblasts leads to their excessive activity.

To confirm this hypothesis, our primary aim was to investigate the molecular function of Fussel-15 during normal wound healing. Functional wound healing scratch assays showed that Fussel-15 influences the migratory behavior of fibroblasts (Figure 4, A and B). Increased fibroblast migration is important during the proliferative phase of wound healing, when fibroblasts enter the wound site from the surrounding dermis to promote matrix production and contraction of the leading edges. During this wound healing phase, not only fibroblast migration but also fibroblast proliferation plays an important role. We observed no influence on cell proliferation of Fussel-15 overexpression. Both functions, fibroblast migration and proliferation, must be well orchestrated to achieve effective wound healing. Thus, combinatorial signaling pathways are essential to determine fibroblast proliferation or migration, but the mechanisms essential for the coordination of these processes have yet to be fully elucidated. For this reason, we believe that our results showing that Fussel-15 induces enhanced fibroblast migration, but not proliferation, are important and are of interest for investigation in future studies.

Figure 6. Fussel-15 expression in keloid-derived and localized scleroderma-derived fibroblasts. **A:** IHC with Fussel-15 antibody in normal and pathological dermal sections. Histological analysis of human keloid ($n = 4$) and scleroderma ($n = 4$) dermal tissues reveals strong Fussel-15 protein expression in both keloid and scleroderma fibroblasts (arrows), compared with fibroblasts of control dermis ($n = 4$). **B:** Real-time PCR analysis of Fussel-15 expression in normal and disease-derived fibroblasts. Significantly increased expression of Fussel-15 mRNA was detected after 8 hours of free-floating collagen contraction in keloid-derived ($n = 4$) and scleroderma-derived ($n = 5$) fibroblasts, compared with control fibroblasts ($n = 3$); $*P < 0.05$. **C:** IHC staining of normal and disease-derived 3D cultured fibroblasts with Fussel-15 antibody. Expression of Fussel-15 protein in collagen contraction pellets (paraffin-embedded after 8 hours of contraction) was considerably increased in pellets with scleroderma-derived and keloid-derived fibroblasts (arrows), compared with control fibroblasts.

A further important aspect of wound healing is the contraction of the ECM during this process. This step is necessary to bring the wound edges together to seal the injury. First, we suggested that Fussel-15 might play a role in tension because Fussel-15-overexpressing fibroblasts promote the contraction of 3D free-floating lattices (Figure 3C). However, after careful analysis of the results of both the free-floating and attached-released assay analyses, we concluded that Fussel-15 is not involved in tension development, which was analyzed primarily by means of the attached-released model. We observed no induction of endogenous Fussel-15 expression immediately (<10 minutes) after release of the collagen matrix from the cell culture dish (Figure 2C). Additionally, we observed no difference in the rate of contraction after release between control-transfected and Fussel-15-transfected cells (data not shown). In the free-floating assay, tension development is prevented because cells are not able to attach to the cell culture dish. In this setting, the processes of cell migration and collagen reorganization can be observed. Of note, we observed strong endogenous induction of Fussel-15 using this assay, providing a further hint that Fussel-15 could play a role during cell migration.

Cell migration, a complex phenomenon driven primarily by the actin network, can be divided into three general components: protrusion of the leading edge of the cell, adhesion of the leading edge and de-adhesion of the cell body and rear, and cytoskeletal contraction to pull the cell forward.¹¹ In the present study, we observed that Fussel-15 promotes cell migration in a scratch wound healing assay (Figure 4, A and B), but matrix protein adhesion was not influenced by Fussel-15 (Figure 4C). We speculate that the cell adhesion process pulling the cell forward is independent of Fussel-15. Of note, we

observed that Fussel-15 increases the amount of F-actin (Figure 4D) and is involved in the distribution of F-actin and focal adhesion complexes within fibroblasts (Figure 5). We suggest that Fussel-15 can bind to the F-actin network (unpublished observation) and promotes the organization of peripheral actin fibers, which is necessary for cell movements. Detailed analysis will be required to clarify the mechanism through which Fussel-15 is involved in the cytoskeleton network and how this mechanism is linked to enhanced cell migration.

We detected an ongoing expression of Fussel-15 in the fibroblasts of keloid and skin sclerosis patients (Figure 6). We are particularly interested in the mechanisms leading to this continuous mode of expression. Comparing the expression levels of Fussel-15 in a free-floating assay over time (at 2, 4, and 8 hours), we observed significant changes in expression after 8 hours. In normal fibroblasts, the level of Fussel-15 expression is reduced, whereas keloid fibroblasts continue to express high levels of Fussel-15. We suggest that, in keloid and skin sclerosis, an unknown regulation mechanism prevents the decrease of Fussel-15 expression.

TGF- β is known to play a major role during fibrogenesis and induces and sustains the activation of keloid and scleroderma fibroblasts.¹⁵ Notably, our data show that the regulation of Fussel-15 expression is TGF- β independent, suggesting that other factors are responsible for the regulation of Fussel-15 expression. Furthermore, we observed an induction of Fussel-15 in 2-D-cultivated fibroblasts stimulated with PDGF (Figure 1C). Haisa et al¹⁶ demonstrated that stimulation by PDGF enhanced cell proliferation and migration of keloid fibroblasts due to elevated levels of PDGF α receptors in keloid, compared with normal fibroblasts.

Future studies will be required to reveal whether the enhanced PDGF response of keloid fibroblasts might be responsible for the permanent induction of Fussel-15 in these cells.

We conclude that the present novel findings, that Fussel-15 is involved in wound healing and is permanently expressed in keloid-derived and skin sclerosis-derived fibroblasts (and so potentially contributes to the pathogenesis of these diseases), are of considerable interest and present a new area for future research.

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