Molecular Medicine

miR-145 Contributes to Hypertrophic Scarring of the Skin by Inducing Myofibroblast Activity

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Hyperthrophic scarring of the skin is caused by excessive activity of skin myofibroblasts after wound healing and often leads to functional and/or aesthetic disturbance with significant impairment of patient quality of life. MicroRNA (miRNA) gene therapies have recently been proposed for complex processes such as fibrosis and scarring. In this study, we focused on the role of miR-145 in skin scarring and its influence in myofibroblast function. Our data showed not only a threefold increase of miR-145 levels in skin hypertrophic scar tissue but also in transforming growth factor β1 (TGF-β1)-induced skin myofibroblasts compared with healthy skin or nontreated fibroblasts ($p < 0.001$). Consistent with the upregulation of miR-145 induced by TGF-β1 stimulation of fibroblasts, the expression of Kruppel-like factor 4 (KLF4) was decreased by 50% and α-smooth muscle actin (α-SMA) protein expression showed a threefold increase. Both could be reversed by miR-145 inhibition ($p < 0.05$). Restoration of KLF4 levels equally abrogated TGF-β1–induced α-SMA expression. These data demonstrate that TGF-β1 induces miR-145 expression in fibroblasts, which in turn inhibits KLF4, a known inhibitor of α-SMA, hence upregulating α-SMA expression. Furthermore, treatment of myofibroblasts with a miR-145 inhibitor strongly decreased their α-1 type I collagen expression, TGF-β1 secretion, contractile force generation and migration. These data demonstrate that upregulation of miR-145 plays an important role in the differentiation and function of skin myofibroblasts. Additionally, inhibition of miR-145 significantly reduces skin myofibroblast activity. Taken together, these results suggest that miR-145 is a promising therapeutic target to prevent or reduce hypertrophic scarring of the skin.

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INTRODUCTION

Pathological skin scarring has a high clinical impact in both developing and industrialized countries. Surgery or trauma of the skin, particularly burn injuries, can trigger excessive fibrotic responses that frequently result in hypertrophic scarring (1). Pathological scars often cause a significant impairment of the patient's quality of life because of functional limitations or aesthetic disfigurement (2). The treatment of pathological scars is difficult because of a lack of effective therapeutic options and frequently involves scar revision surgery, a procedure that itself induces renewed scar formation (1). Therefore, it is of high importance to unravel the molecular mechanisms underlying pathological scarring and identify novel preventive and therapeutic strategies to adequately remedy the problem.

In physiological wound healing, progenitor cells such as fibroblasts are activated and differentiate to myofibroblasts. Fibroblasts are essential in the wound closure process, since they migrate to the defect, where they synthesize and deposit extracellular matrix (ECM) components within granulation tissue and mediate wound contraction. Upon wound closure, myofibroblasts normally disappear from granulation tissue by apoptosis, so that immature scars can proceed to the remodeling and maturation phase. However, in many cases, myofibroblasts persist within the granulation tissue and contribute to pathological scarring by excessive ECM deposition and contractile force generation, leading to irreversible tissue contractures (3). Over the last decades, many studies addressed the molecular mechanisms underlying myofibroblast biology. One of the major growth factors driving fibroblast differentiation and maturation to myofibroblasts is transforming growth factor β1 (TGF-β1), which is present at high concentrations

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in wound granulation tissue (4). TGF-β1 coordinately induces the expression of collagen type I and α-smooth muscle actin (α-SMA), of which the latter has been widely used as a myofibroblast marker (3). *De novo* expression of α-SMA together with other proteins such as nonmuscle myosin or rho-kinase is important for contractile force generation (3,5). Furthermore, myofibroblasts express a group of proteins including lysyl hydroxylase and pro-collagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD2), which are responsible for ECM modulation in fibrotic skin and likely contribute to tissue contraction (5).

In spite of a detailed knowledge of myofibroblast biology and of the wound healing process per se, many attempts using several different proteins as drug or therapeutic targets (such as TGF-β3, interleukin [IL]-10 or mannose-6-phosphate) have shown limited success. It is thought that the manipulation of single molecules in a complex process such as fibrosis may not be sufficient to prevent or treat pathological scarring (1).

As a new therapeutic approach for fibrotic disorders, microRNA (miRNA) gene therapies have been proposed (6). miRNAs are ~22-nucleotide-long, noncoding RNAs that play a pivotal role in posttranscriptional gene regulation. Mature miRNAs integrate into the RNA-induced silencing complex (RISC) to pair with partially complementary mRNAs and, consequently, repress mRNA translation or promote target degradation (7). Imperfect base-pairing between miRNA and target mRNA allows single miRNAs to regulate up to hundreds of genes. This ability makes miRNAs interesting therapeutic targets, especially for pathological settings where miRNAs are prominently deregulated. Several groups are working on the development of miRNA-based therapeutic strategies for different pathological conditions. The most advanced miRNA gene therapy product is the low noise linear amplifier (LNA)-miR-122 antagomir against hepatitis C virus infection, which is currently being tested in Phase II clinical trials (8). Recent studies have also shown that miRNAs play an important role in fibrosis in several tissues and organs. Thum *et al.* (9) reported that inhibition of miR-21 prevented interstitial fibrosis and cardiac hypertrophy in a mouse model of heart infarction. Cheng *et al.* (10) showed that miR-29b is involved in the regulation of collagen type I production by skin fibroblasts (10). In keloid fibroblasts, collagen production was found to be regulated by miR-196a (11).

However, the role of miRNAs in the regulation of myofibroblast contractile force generation or migration in pathological skin scarring is not known so far. This study aims to identify miRNAs that are dysregulated in the condition of pathological scarring, particularly in hypertrophic scars, and promote tissue contraction, myofibroblast migration, ECM production and/or myofibroblast survival. Furthermore, candidate miRNAs will be analyzed for their potential use as therapeutic targets to develop novel strategies for hypertrophic scar prevention and treatment.

MATERIALS AND METHODS

Computational Prediction of miRNA Targeting Profibrotic Genes

Screening for miRNA candidates targeting profibrotic genes or their regulators was performed by using the miRecords database, which integrates the results of 11 established miRNA target prediction programs (http://c1.accurascience.com/miRecords/). We selected miRNAs that were predicted to regulate a certain target by at least four different prediction algorithms.

Human Skin Tissue

Tissue samples of healthy skin and hypertrophic scar were collected during plastic surgery with informed consent, as approved by the local ethics committee of the Hannover Medical School. Hypertrophic skin scar samples and healthy control tissue were collected from the same anatomical region of two patients (left shoulder [n = 1] and nasolabial fold [n = 1]) more than 12 months after the initial injury. Hypertrophic scar was defined by manifest skin contracture, moderate itching and redness. Keloids, open wounds and/or infected skin were excluded.

Isolation and Culture of Primary Human Skin Fibroblasts

For the isolation of primary human skin fibroblasts, healthy skin tissue was minced into pieces, and epidermal and dermal layers were separated by dispase II treatment (2 U/mL) (Life Technologies [Thermo Fisher Scientific Inc., Waltham, MA, USA]). Dermal tissue was digested with Collagenase type I (300 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) and strained through a 100-μm cell strainer. Fibroblasts were harvested and grown in Dulbecco modified Eagle medium (DMEM) with 10% heat-inactivated fetal calf serum, penicillin (100 mg/mL), streptomycin (100 mg/mL) and amphotericin B (0.25 mg/mL) (approximately 10^4 cells/cm^2). Fibroblasts at passages 2–5 were used in all experiments conducted in this study. For differentiation into myofibroblasts, fibroblasts were cultured in the presence of TGF-β1 (2 ng/mL; PeproTech, Rocky Hill, NJ, USA).

Real-Time Polymerase Chain Reaction

Total RNA was prepared from skin tissue and cells by using the mirVana miRNA Isolation Kit (Life Technologies [Thermo Fisher Scientific]) according to the manufacturer's instructions. To preserve RNA integrity, skin tissue was soaked in RNA later (Life Technologies [Thermo Fisher Scientific]) before RNA isolation. Reverse transcription (RT) of mRNA and miRNA to cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit and the TaqMan MicroRNA Reverse Transcription kit with specific looped RT primers for each miRNA (Life Technologies [Thermo Fisher Scientific]), respectively, according to the manufacturer's protocol. Transcript levels of α-SMA, Kruppel-like factor 4 (KLF4), α-1 type I collagen (COL1A1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were an-

alyzed by using real-time polymerase chain reaction (PCR) with specific gene expression assays (Life Technologies [Thermo Fisher Scientific]). miRNA transcripts were measured by using specific TaqMan MicroRNA Assays for miR-145, miR-29b, miR-24, miR-27b, miR-101 and miR-133b. Either GAPDH or U6 served as the internal control for mRNA and miRNA quantification, respectively. All real-time PCR analyses were performed in triplicate.

Western Blot Analysis

Total cell lysate was prepared by using NP40 buffer (Invitrogen [Thermo Fisher Scientific]) supplemented with proteinase inhibitor cocktail (Roche, Rotkreuz, Risch, Switzerland). Protein concentrations were measured by bicinchoninic acid (BCA) assay (Interchim, Montluçon, France), and equal amounts of protein (50 μg) were separated by 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blotting to polyvinylidene fluoride (PVDF) membranes by using the iBlot system (Life Technologies [Thermo Fisher Scientific]). PVDF membranes were blocked with phosphate-buffered saline (PBS) containing 2% bovine serum albumin and 0.05 % Tween-20 overnight at 4°C. Membranes were then probed with the following primary antibodies: α-SMA (1:200 dilution), KLF4 (1:500 dilution) (both from Abcam, Cambridge, UK) and GAPDH (1:10,000 dilution; Sigma-Aldrich) for 1 h at room temperature. Membranes were washed and incubated with goat–anti-mouse secondary antibody (1:3,000 dilution; Dako, Glostrup, Denmark) for 1 h at room temperature. The blots were detected by using Roti-Lumin enhanced chemiluminescence detection kits (Carl Roth, Karlsruhe, Germany). Relative expression of detected proteins was quantified and normalized to GAPDH by using the open-source ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

For immunofluorescence staining, fibroblasts were grown in eight-well chamber slides (Life Technologies [Thermo Fisher Scientific]), fixed with Cytofix (BD Biosciences, San Jose, CA, USA) for 20 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 3 min. Cells were stained with Texas Red–conjugated phalloidin (Life Technologies, [Thermo Fisher Scientific]) for 20 min at 37°C, washed with PBS and blocked with PBS containing 10% fetal calf serum for 40 min at room temperature. Subsequently, cells were stained with anti–α-SMA antibody for 1 h at room temperature, washed and incubated with Alexa Fluor 488–labeled secondary antibody (Life Technologies [Thermo Fisher Scientific]) for 1 h. The slides were mounted in ProLong Gold Antifade Reagent mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen [Thermo Fisher Scientific]). Fibroblasts were placed on an Olympus IX81 microscope (Olympus, Hamburg, Germany) with a fluorescein isothiocyanate (FITC), Texas Red or DAPI filter set using 10× and 40× objectives. Images were acquired by using a digital black and white camera (Olympus) and analyzed by using Xcellence Pro image software (Olympus).

Transfection of miR-145 Inhibitor

A miR-145 inhibitor (mirVana® miRNA inhibitor; Life Technologies [Thermo Fisher Scientific]) was used to inhibit the expression of hsa-miR-145 (5-GUCCAGUUUUCCCAGGAAUCCCU-3) in nonstimulated or TGF-β1–treated fibroblasts. Before transfection, fibroblasts were treated with 2 ng/mL TGF-β1 for 24 h. Transfection of the miR-145 inhibitor was performed by using Lipofectamine 2000 Transfection Reagent (Life Technologies, Thermo Fisher Scientific). Briefly, miR-145 inhibitor or Anti-miR mirVana® miRNA Inhibitor Negative Control #1 (final concentration: 5 nmol/L; Life Technologies [Thermo Fisher Scientific]) were mixed with 120 μL OptiMEM (Life Technologies [Thermo Fisher Scientific]). In a second tube, 7.5 μL Lipofectamine 2000 was mixed with 120 μL OptiMEM. After 5 min of incubation, the OptiMEM/miR-145 inhibitor or Anti-miR control mix was added to the OptiMEM/Lipofectamine 2000 mix and incubated for 20 min at room temperature to form transfection complexes, which were then added drop-wise to the cells.

Collagen Contraction Assay

Collagen contraction assays (Cell Biolabs, San Diego, CA, USA) were performed according to the manufacturer's instructions. Briefly, primary human skin fibroblasts were harvested 48 h after transfection with the miR-145 inhibitor, mixed with 3 mg/mL bovine type I collagen I diluted in 5× DMEM plus neutralizing solution and then seeded into a 12-well plate at 2–4 × 10^5 cells/well. After 1 h of incubation at 37°C, the collagen gels were detached from the wells. Diameters of the gels were measured at 48 h after gel release.

TGF-β1 Secretion Assay

For TGF-β1 secretion analysis, fibroblasts were stimulated with 2 ng/mL TGF-β1 for 24 h and then treated with miR-145 inhibitor for 48 h. Fibroblasts incubated without TGF-β1 and TGF-β1–stimulated fibroblasts treated with a nonspecific Anti-miR were used as control. At 48 h after transfection, cells were rinsed with PBS and incubated for 48 h in growth medium without exogenous TGF-β1. Supernatants of fibroblast and myofibroblast cultures were then collected and analyzed for TGF-β1 secretion using Luminex technology (Milliplex MAP TGFβ3 plex kit; Millipore, Billerica, MA, USA; Luminex 200TM instrument, Invitrogen [Thermo Fisher Scientific]).

Cell Migration Assay

A linear defect was produced in a confluent fibroblast monolayer with a pipette tip, washed with PBS and cultured in complete growth medium. The defect area was marked with dots on the bottom of the plate with a black marker pen for reference. Closure of the defect area was examined by light microscopy and photographed immediately and 72 h after wounding. For statistical analysis, num-

bers of migrating cells were counted in three microscopic fields per experiment.

Lentiviral Vector Transduction

A lentiviral KLF4 expression vector (pMXs-hKLF4; Addgene #17219, S. Yamanaka, Kyoto, Japan) was used to restore KLF4 expression in TGF-β1–treated fibroblasts. Viral vector production and cell transduction was performed as previously described (12). Before transduction, fibroblasts were treated with 2 ng/mL TGF-β1 for 24 h. Cells were then infected with a multiplicity of infection of 10 in the presence of 8 μg/mL protamine sulfate (Sigma-Aldrich). After 8 h, the fibroblasts were washed by using fresh growth medium. At 72 h after transduction, cells were harvested for real-time PCR analysis.

Statistical Analysis

Statistical analyses were performed by using two-tailed *t* tests run on GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Levels of significance were expressed as *p* values (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

RESULTS

Identification of Candidate miRNAs Potentially Involved in Hypertrophic Scarring of the Skin

To identify miRNAs with putative roles in the regulation of myofibroblasts and thus playing a part in hypertrophic scarring of the skin, we used computational target prediction algorithms. The disadvantage of *in silico* target prediction is the potentially high rate of false predictions or the fact that these algorithms may fail to predict the most biologically relevant miRNA–target gene interactions (7). Therefore, we selected only miRNAs that were predicted by at least four different target prediction algorithms to interact with a given target gene of interest. By using the miRecords service that integrates results of different target prediction algorithms, such as miRANDA and TargetScan, we identified miRNAs that putatively regulate proteins with a known role in myofibroblast regulation and function, for example, collagen type I, α-SMA (3) and PLOD2 (13) (Table 1). Furthermore, we chose to analyze miR-101 (14), miR-133b (15) and miR-145 (16,17), which were reported to play a role in fibrosis of tissues other than skin, but might have a similar role in skin fibrosis and scarring.

Table 1. Exemplary results of target prediction algorithms used to identify miRNAs with putative therapeutic potential in skin scarring.

Target gene	miRNA	Predicting algorithms
Collagen type 1	miR-29b	PicTar, TargetScanS, PITA, RNAhybrid
α-SMA	miR-27b	TargetScanS, miRanda, PITA, RNAhybrid
PLOD2	miR-24	PicTar, TargetScanS, miRanda, PITA, RNAhybrid, miRTarget2

miRNA Expression in Healthy Human Skin and Hyperthrophic Scar Tissue

To assess if the selected miRNA candidates might play a role in fibrotic processes of the skin, we isolated total RNA from healthy skin and hypertrophic scar tissues. We then performed miRNA specific real-time PCR assays to analyze expression profiles of different miRNAs between healthy skin and pathological scar. Our preliminary data show that for most candidate miRNAs, there was no apparent difference in expression levels between healthy skin and skin scar (Table 1). miR-133b, which was reported to be downregulated in mouse corneal scar tissue (15), was slightly upregulated in skin scar tissue compared with healthy skin. miR-29b, which was previously reported to be downregulated in skin scar (10), did not show a significant change between healthy and scarred skin. Interestingly, the expression of miR-145, which has been recently shown to indirectly regulate α-SMA in lung fibroblasts (16), showed a threefold increased expression in hypertrophic scar samples compared with healthy skin tissues (Table 2).

miR-145 Is Upregulated in TGF-β1–Stimulated Skin Fibroblasts

For experimental evaluation of candidate miRNAs, we used a cell culture model of fibroblast-to-myofibroblast differentiation. Therefore, primary fibroblasts were isolated from healthy skin tissue of patients undergoing reconstructive surgery. Isolated fibroblasts were then cultured in the presence or absence of TGF-β1 for 24, 48 and 72 h. The induction of fibroblast-to-myofibroblast differentiation in TGF-β1–stimulated fibroblasts was demonstrated by the *de novo* expression of α-SMA (Figure 1), a commonly used myofibroblast marker (3).

This *in vitro* model was then used to analyze the differential expression of miRNAs between nonstimulated skin fibroblasts and differentiated myofibroblasts. miRNA-specific real-time PCR showed that miR-29b is significantly downregulated in myofibroblasts compared with fibroblasts (Figure 2A) as previously reported by Cheng *et al.* (10). These data show that our *in vitro* model is suitable for the experimental evaluation of miRNA expression in fibroblasts and myofibroblasts. In addition, the expression of miRNA-145 in (myo)-fibroblasts was analyzed. Consistent with the results obtained for complete skin and hypertrophic scar tissue, our data showed that miR-145 is significantly upregulated in skin myofibroblasts by up to 3.7-fold compared with

Table 2. miRNA expression in healthy human skin versus hyperthrophic scar tissue.

miRNA	Healthy skin	Hypertrophic scar
miR-29b	1.00 ± 0.0	0.96 ± 0.01
miR-24	1.00 ± 0.0	1.05 ± 0.12
miR-27b	1.00 ± 0.0	0.99 ± 0.03
miR-101	1.00 ± 0.0	0.97 ± 0.01
miR-133b	1.00 ± 0.0	1.19 ± 0.10
miR-145	1.00 ± 0.0	3.01 ± 0.48

miRNA levels were assessed using specific gene expression assays and normalized to U6 snRNA levels (mean ± SD; n = 2).

nonstimulated fibroblasts ($p < 0.001$; Figure 2B). Expression levels of miR-24, miR-27b, miR-101 and miR-133b, which were also analyzed in fibroblasts and myofibroblasts, did not show significant changes (data not shown).

miR-145 Regulates the Expression of α-SMA in Primary Human Fibroblasts

To determine if the upregulation of miR-145 is required for skin myofibroblast function, we used a specific Anti-miR targeting miR-145 to downregulate its expression in TGF-β1–induced myofibroblasts. A nonspecific Anti-miR was used as control in all experiments. By using real-time PCR, we showed that the miR-145 inhibitor indeed downregulated the expression of miR-145 in TGF-β1–stimulated fibroblasts to background levels (Figure 3A). To analyze if the induction of miR-145 expression by TGF-β1 was important for the function of skin myofibroblasts, we assessed the expression of α-SMA, which is important for myofibroblast contractility. Our results showed that myofibroblasts transfected with the miR-145 inhibitor had significantly lower α-SMA mRNA levels compared with myofibroblasts treated with the control Anti-miR ($p < 0.001$; Figure 3C). On protein level, α-SMA expression was reduced to background levels in myofibroblasts upon miR-145 inhibition, whereas myofibroblasts treated with control Anti-miR showed a 3.4-fold upregulation of α-SMA expression in comparison to nonstimulated fibroblasts ($p < 0.05$; Figures 3D, E). These data indicate that miR-145 is an important intermediate for the TGF-β1–induced expression of α-SMA.

miR-145 Targets KLF4 in Skin Fibroblasts

Previous studies demonstrated that miR-145 directly targets KLF4 (18,19), a known negative regulator of α-SMA (16). Therefore, we analyzed if KLF4 plays a role in TGF-β1–induced α-SMA expression in skin fibroblasts. Our data showed that KLF4 levels were significantly downregulated in skin fibroblasts on

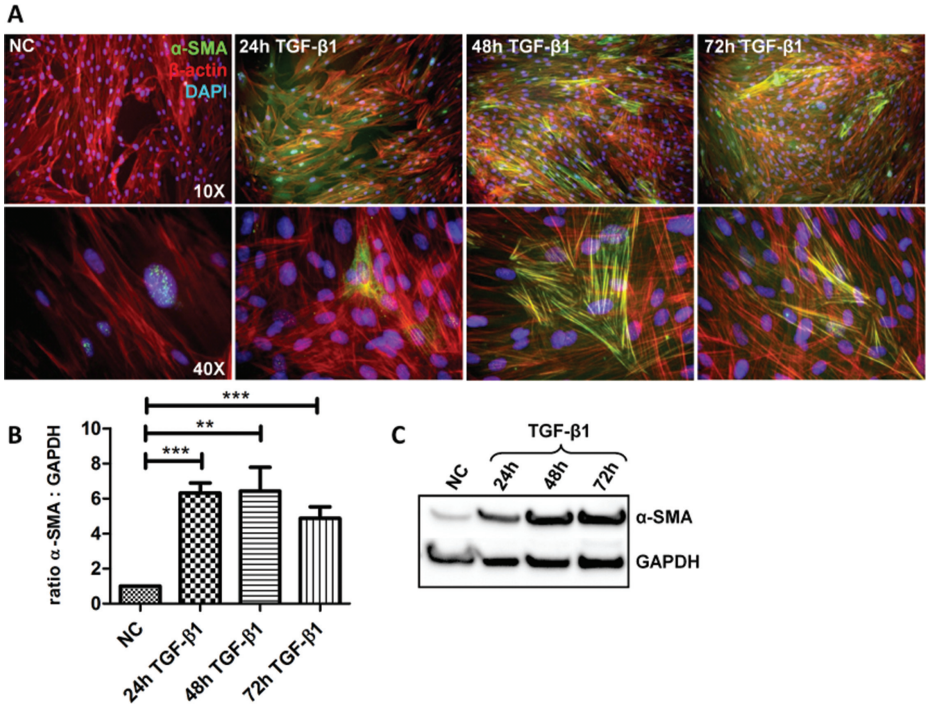

Figure 1. Fibroblast-to-myofibroblast differentiation *in vitro*. Skin fibroblasts were stimulated with TGF-β1 (2 ng/mL) for 24, 48 and 72 h. (A) Representative immunofluorescence pictures. Cells were stained with Phalloidin-Texas Red to label F-actin (red), DAPI to visualize the nucleus (blue) and anti–α-SMA antibody conjugated to Alexa Fluor 488 to visualize α-SMA (green) as a marker for myofibroblast differentiation. (B) α-SMA mRNA levels were assessed by real-time PCR and normalized to GAPDH mRNA levels. Graph depicts mean ± standard deviation (SD) (n = 4, $^{**}p < 0.01$, $^{***}p < 0.001$). (C) Representative Western blot showing α-SMA protein levels; GAPDH was used as an internal control.

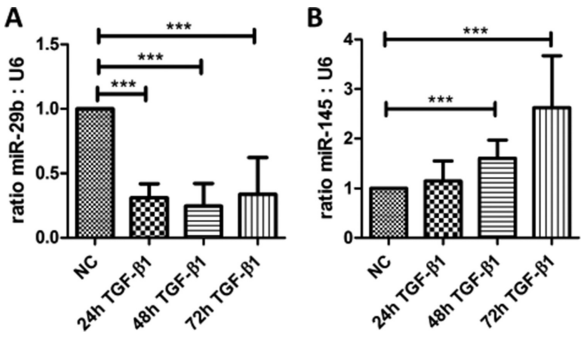

Figure 2. miR-29b is downregulated and miR-145 is upregulated on TGF-β1 stimulation of skin fibroblasts. Primary skin fibroblasts were stimulated with TGF-β1 for 24, 48 and 72 h. miRNA levels were assessed by using specific gene expression assays and normalized to U6 snRNA levels. miR-29b expression (A) and miR-145 expression (B) in fibroblasts and TGF-β1–induced myofibroblasts. Mean ± SD (n = 4, $^{***}p < 0.001$).

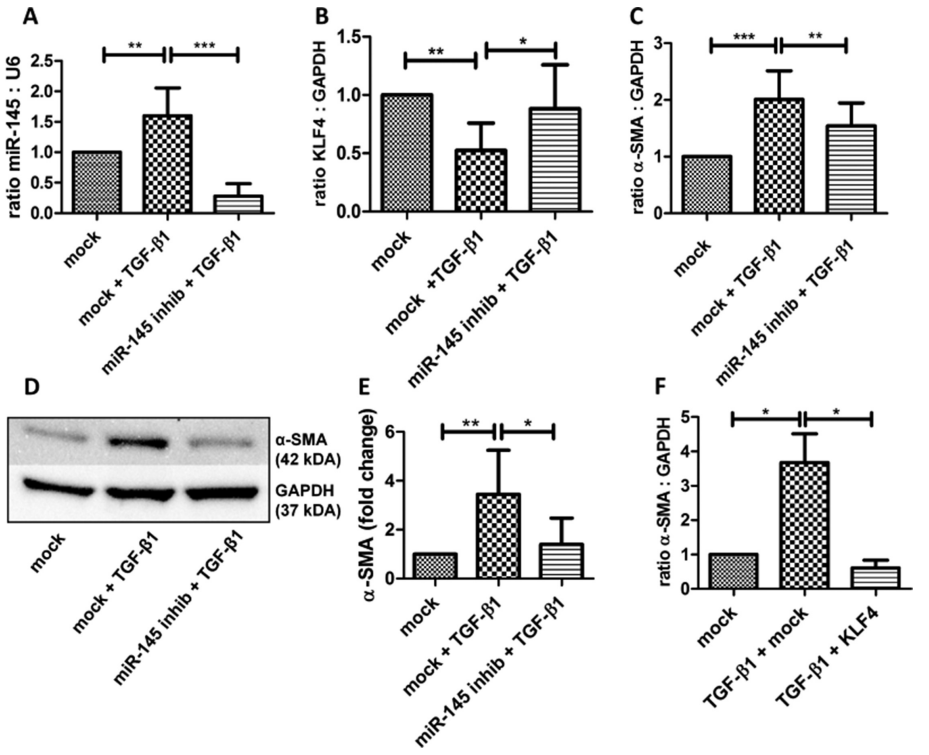

Figure 3. Inhibition of miR-145 expression decreases α-SMA expression in TGF-β1–stimulated skin myofibroblasts. (A–E) Primary skin fibroblasts were stimulated with TGF-β1 for 24 h and then treated with a specific miR-145 inhibitor for 48 h. A nonspecific Anti-miR was used as the control (mock). (A) miR-145 levels normalized to U6 snRNA levels. KLF4 (B) and α-SMA mRNA (C) levels normalized to GAPDH are shown. Representative Western blot (D) and densitometric analysis (E) of three Western blots showing α-SMA protein levels are shown. (F) Fibroblasts were stimulated with TGF-β1 for 24 h and then transduced with a KLF-4 encoding lentiviral vector. Nontransduced cells with and without TGF-β1 stimulation were used as controls. Graph depicts α-SMA mRNA levels normalized to GAPDH. Graphs show mean ± SD (n = 4, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

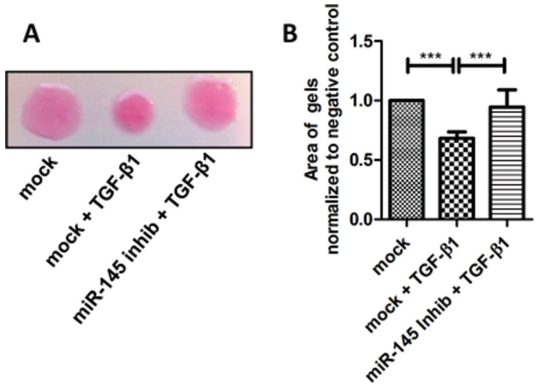

Figure 4. miR-145 inhibition abrogates contractile force generation in TGF-β1–induced skin myofibroblasts. Primary skin fibroblasts were stimulated with TGF-β1 for 24 h and then treated with a specific miR-145 inhibitor for 48 h. A nonspecific Anti-miR was used as the control (mock). Cells were then seeded into collagen gels, and gel contraction was assessed after 48 h. A representative picture of collagen gels (A) and densitometric analysis of gels from three independent experiments (B) are shown. Mean ± SD (***$p < 0.001$).

TGF-β1 stimulation, but were restored in cells transfected with the miR-145 inhibitor ($p < 0.05$), suggesting that miR-145 may indirectly regulate α-SMA expression via KLF4 silencing (Figure 3B). To verify this mechanism, we restored KLF4 levels in TGF-β1–stimulated fibroblasts by KLF4 overexpression. Restoration of KLF4 levels was confirmed by real-time PCR (data not shown). Analysis of TGF-β1–stimulated fibroblasts with restored KLF4 levels showed no induction of α-SMA, whereas TGF-β1–treated control fibroblasts showed the typical increase of α-SMA expression during myofibroblast differentiation (Figure 3F). These data show that the downregulation of KLF4 by miR-145 is responsible for the induction of α-SMA expression in skin fibroblast on TGF-β1 stimulation.

Contractility of Myofibroblasts Can Be Controlled by miR-145 Inhibition

Tissue contraction mediated by myofibroblasts is one of the major problems in hypertrophic scarring (20). Therefore, we used collagen contraction assays to assess if the downregulation of α-SMA by the miR-145 inhibitor was sufficient to reduce contractility in myofibroblasts. Skin fibroblasts were stimulated with TGF-β1 for 24 h to induce myofibroblast differentiation and then transfected with miR-145 inhibitor or Anti-miR control and seeded into collagen gels 48 h after transfection. Nonstimulated fibroblasts transfected with the Anti-miR control were used as negative control. After 48 h, gel contraction was assessed. Our results clearly show that the inhibition of miR-145 significantly abrogated myofibroblast contractile force generation compared with Anti-miR control–treated myofibroblasts ($p < 0.01$; Figures 4A, B).

miR-145 Inhibition Downregulates Collagen Type I and TGF-β1 Production

In addition to α-SMA expression and contractility, we analyzed the effect of miR-145 inhibition on other myofibroblast functions, in particular, ECM production and TGF-β1 secretion. There-

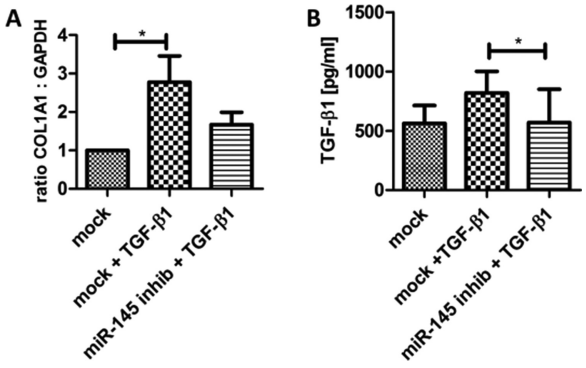

Figure 5. Inhibition of miR-145 decreases collagen 1A1 expression and TGF-β1 secretion by skin myofibroblasts. Skin fibroblasts were stimulated with TGF-β1 for 24 h and then treated with a specific miR-145 inhibitor. A nonspecific Anti-miR was used as the control (mock). At 48 h after transfection, cells were rinsed with PBS and analyzed by real-time PCR or incubated for another 48 h in growth medium without exogenous TGF-β1 for analysis of TGF-β1 secretion. (A) Collagen 1A1 mRNA level normalized to GAPDH. (B) TGF-β1 secretion level. Graphs show mean ± SD (n = 4, *$p < 0.05$).

fore, we measured mRNA levels of collagen type I, which is the major component of ECM produced by myofibroblasts (10). Interestingly, our results showed that the inhibition of miR-145 significantly downregulated collagen type I expression in TGF-β1–stimulated fibroblasts in comparison to myofibroblasts treated with a nonspecific Anti-miR (Figure 5A). Furthermore, we assessed the secretion of TGF-β1 by using Luminex technology. Endogenous TGF-β1 production was increased to 820 ± 181 pg/mL in differentiated myofibroblasts compared with unstimulated fibroblasts (562 ± 151 pg/mL). After exposing myofibroblasts to the miR-145 inhibitor, no upregulation of TGF-β1 secretion was observed (568 ± 282 pg/mL, $p < 0.05$; Figure 5B).

miR-145 Controls the Migration Capacity of Primary Human Skin Myofibroblasts

Fibroblast activation and subsequent migration into the scarring area is another important issue in hypertrophic scarring (21). Therefore, we analyzed the influence of miR-145 inhibition on the migratory capacity of skin myofibroblasts using a scratch wound assay. Interestingly, our data showed that myofibroblast migration was strongly inhibited in miR-145 inhibitor–treated cells compared with myofibroblasts treated with the control Anti-miR (Figures 6A, B).

DISCUSSION

Currently, there are few effective therapeutic options to prevent or treat hypertrophic scarring of the skin (1,22). Nevertheless, there is a great need for effective treatment, since hypertrophic scars have a high incidence, are disfiguring and, in some cases, lead to functional disability due to contractures (22). Gene therapies based on miRNA have been proposed as a new therapeutic approach for fibrotic disorders (6). In the present study, we identified miR-145 as a promising therapeutic target for prevention and treatment of hypertrophic scarring of the skin. Our results demonstrated that Anti-miR–based inhibition of miR-145 expression allows reduction of skin myofibroblast activity, the key effector cells of tissue fibrosis and scarring.

The role of miRNAs in skin development and pathology is well established (11,23). However, little is known about their function in the process of hypertrophic scarring of the skin. To identify

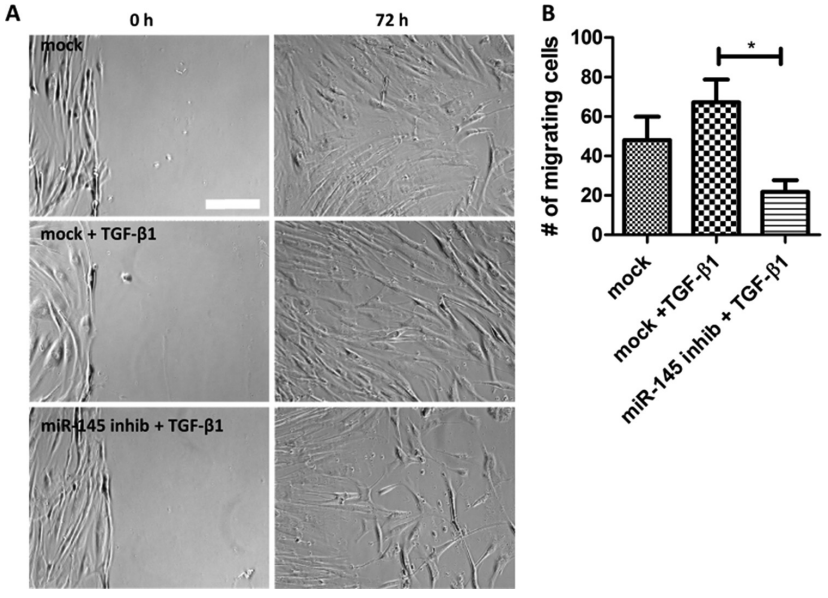

Figure 6. Myofibroblast migration is downregulated upon inhibition of miR-145. Skin fibroblasts were stimulated with TGF-β1 for 24 h and then treated with a specific miR-145 inhibitor. A nonspecific Anti-miR was used as control (mock). At 48 h after transfection, a linear defect was produced in a confluent fibroblast monolayer. After 72 h, closure of the wound area was examined by light microscopy. Representative pictures (white bar: 200 μm) (A) and counts of cells that migrated into the wound area (B) are shown. Graphs show mean ± SD (n = 4, *$p < 0.05$).

miRNAs that could play a role in hypertrophic skin scarring, in particular, in the regulation of myofibroblasts, we initially performed computational target prediction together with an extensive literature research. Part of the results of the *in silico* target prediction corresponded with protein-miRNA interactions known from recently published studies, such as miR-29b and collagen type I (10). Furthermore, we identified several miRNA-target gene pairs with a potential role in skin scarring that needed experimental confirmation (Table 1). In addition, we analyzed miR-101 (14) and miR-133b (15), which were reported to play a role in fibrosis of tissues other than the skin, and miR-145, which was shown to be deregulated in lung and heart fibrosis as well as keloid fibroblasts (16,17,24).

Experimental evaluation of candidate miRNAs showed that miR-145 was highly upregulated, not only in skin hypertrophic scar tissue, but also in TGF-β1–induced myofibroblasts compared with healthy skin and nontreated fibroblasts. These results indicate a role of miR-145 in skin myofibroblast function and scarring. Consistent with previous data (10), miR-29b was significantly downregulated in differentiated myofibroblasts compared with nonactivated fibroblasts. Other tested miRNAs candidates (that is, miR-24, miR-27b, miR-101 and miR-133b) did not show significant differences between healthy skin and hypertrophic scar tissue or between fibroblasts and myofibroblasts, indicating that they are less relevant in the complex processes associated with hypertrophic scarring.

Previously, miR-145 was shown to play a role in fibroblast-to-myofibroblast differentiation in lung and heart tissue (16,17). However, it is known that fibroblasts from internal organs have dramatic differences in gene expression patterns compared with dermal fibroblasts (25,26). In addition, a recent study reported miR-145 to be downregulated in keloid fibroblasts compared with normal skin fibroblasts (24), whereas our results show an upregulation of miR-145 in the condition of hypertrophic scarring. Therefore, we extensively characterized the role of miR-145 upregulation in the function of skin scar myofibroblasts, which was unknown so far. Previously, miR-145 targeted KLF4 expression in lung fibroblast (16). KLF4 is a negative regulator of α-SMA expression and myofibroblast differentiation (5). Our results show that KLF4 expression is significantly decreased and α-SMA expression is increased in skin scar tissue versus healthy skin and in TGF-β1–induced myofibroblasts compared with nontreated fibroblasts. These data suggest KLF4 as a possible link between miR-145 upregulation and α-SMA upregulation in skin scar tissue. Inhibition of miR-145 expression in skin myofibroblasts caused a significant upregulation of KLF4 expression and decreased α-SMA expression. Consistently, the restoration of KLF4 levels in skin fibroblasts after TGF-β1 stimulation completely abrogated the induction of α-SMA expression. Taken together, these observations show that miR-145 indeed induces α-SMA expression via downregulation of KLF4 expression in skin myofibroblasts. In addition, these data demonstrate that the induction of α-SMA expression can be abrogated by inhibition of miR-145.

α-SMA is the main contributor to contractile force generation in myofibroblasts. In hypertrophic scarring, myofibroblast contractility is one of the major problems, since it causes irreversible tissue contracture and contributes to the release of latent TGF-β1 from the ECM, which in turn continuously stimulates myofibroblast differentiation and activity (20). Therefore, it is desirable to inhibit tissue contraction in skin scars after wound healing is complete. Notably, by using collagen gel contraction assays, we demonstrated that inhibition of miR-145 expression in TGF-β1–induced myofibroblasts reduced contractile force generation to the background levels observed in nontreated fibroblasts. The observed reduction in myofibroblast contractility is likely caused by the downregulation of α-SMA expression upon miR-145 inhibition. These data show that miR-145 is a promising therapeutic target for the treatment of hypertrophic skin scarring, since reduction in myofibroblast contractility would not only reduce or prevent tissue contracture but also inhibit the release of latent TGF-β1 from the ECM.

In addition, we assessed ECM production and TGF-β1 secretion by fibroblasts and myofibroblasts. Collagen type I is the major component of ECM produced by myofibroblasts, and its excessive production and disorganized accumulation largely contributes to the formation of scar tissue (10). Furthermore, autocrine TGF-β1 signaling in myofibroblasts is thought to promote hypertrophic scarring (27). Our results show that miR-145 inhibitor treatment of TGF-β1–induced myofibroblasts strongly decreases their ability to produce collagen or secrete TGF-β1 compared with myofibroblasts treated with a nonspecific control Anti-miR. These data show that miR-145 does not only influence α-SMA expression and contractility but also seems to play a role in ECM production and TGF-β1 secretion by myofibroblasts. However, the mechanisms behind these effects still need to be elucidated.

Another important aspect of wound healing and scarring is the migration of activated fibroblasts into the wound area. Therefore, we also analyzed the effect of miR-145 inhibition on myofibroblast migration. Our data show that in addition to all other important myofibroblast functions also, the fibroblasts' migratory capacity is strongly inhibited when we decrease the expression of miR-145 using an Anti-miR.

In a recent study, Zhao *et al.* (28) presented a therapeutic approach based on the use of an siRNA directed against TGF-β1 transcripts in combination with pressure-sensitive adhesive hydrogels to decrease TGF-β1 production in skin scar tissue (28). Their results demonstrate the feasibility of using small nucleic acid molecules (such as siRNAs or Anti-miRs) for the treatment of hypertrophic scars. Furthermore, their strategy can also be

used for the application and delivery of the miR-145 inhibitor into skin scar tissue. Regarding the efficacy of both approaches, we speculate that the use of a miR-145 inhibitor will be more efficient in targeting existing myofibroblasts and therefore may require shorter treatment periods. In addition, the use of siRNA-TGF-β1-337 cannot prevent the release of latent TGF-β1 from the ECM, which continuously stimulates myofibroblast differentiation and activity (20), whereas miR-145 inhibition will prevent TGF-β1–induced myofibroblast differentiation. For a final conclusion, further studies are required to compare the efficacy of miR-145 inhibition and siRNA-TGF-β1-337 or even a combination thereof for the treatment of hypertrophic skin scarring.

CONCLUSION

Taken together, this study shows that miR-145 is upregulated in the condition of hypertrophic scarring of the skin and in TGF-β1–induced myofibroblasts. Our data strongly suggest that upregulation of miR-145 is an essential part of TGF-β1–induced myofibroblast differentiation and activity. Furthermore, we demonstrate that inhibition of miR-145 expression does not only influence one function of myofibroblasts, but it downregulates all functions (that is, contractility, ECM production, TGF-β1 secretion and migration) that act in synergy during the pathological process of hypertrophic scarring. Hence, we suggest miR-145 as a promising therapeutic target for the therapy of pathological scarring of the skin.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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