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MicroRNA-31 Is Overexpressed in Psoriasis and Modulates Inflammatory Cytokine and Chemokine Production in Keratinocytes via Targeting Serine/Threonine Kinase 40

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Psoriasis is a common chronic inflammatory skin disease, which affects 2–3% of the population. It is a lifelong disease with spontaneous remissions and exacerbations that are severely detrimental to the patients’ quality of life (1). Psoriasis skin lesions are typically characterized by keratinocyte hyperproliferation and aberrant differentiation, increased vascularity in the dermis, and infiltration of inflammatory cells, such as macrophages, neutrophils, and lymphocytes into the dermis and epidermis (1). There is a close interdependence between keratinocytes and immune cells in psoriatic skin: the cytokines and chemokines secreted by keratinocytes, such as IL-1β, TNF-α, CXCL1/growth-related oncogene-α, CXCL5/epithelial-derived neutrophil-activating peptide 78, and CXCL8/IL-8 activate and attract immune cells to migrate into epidermis and dermis; immune cell–derived cytokines, in turn, act on keratinocytes to increase the expression of inflammatory genes, promote keratinocyte proliferation, and impair keratinocyte differentiation (reviewed in Ref. 1). Several of these deregulated miRNAs have been shown to act on cellular processes crucial for psoriasis. For example, miR-203 (7), miR-125b (9), miR-424 (4), and miR-99a (6) regulate keratinocyte cellular processes investigated to date (3). We and others have previously identified a distinct miRNA expression profile in psoriasis skin compared with healthy skin (4–8). Several of these deregulated miRNAs have been shown to act on cellular processes crucial for psoriasis. For example, miR-203 (7), miR-125b (9), miR-424 (4), and miR-99a (6) regulate keratinocyte proliferation and differentiation, whereas miR-21 suppresses T cell apoptosis (10). Although being in the infancy, these studies reveal important roles for miRNAs in the biology of psoriasis.

In this study, we identify a function for miR-31 in the context of psoriasis. We show that specific inhibition of miR-31, a miRNA overexpressed in psoriasis keratinocytes, suppresses NF-κB signaling and the production of IL-1β, CXCL1, CXCL5, and CXCL8/IL-8. Serine/threonine kinase 40 (STK40), a negative regulator of NF-κB signaling, was identified as a direct target for miR-31. Silencing of STK40 rescued the suppressive effect of miR-31 inhibition on cytokine/chemokine expression, indicating that miR-31 regulates cytokine/chemokine expression via targeting STK40 in keratinocytes. Finally, we demonstrated that TGF-β1, a cytokine highly expressed in psoriasis epidermis, upregulated miR-31 expression in keratinocytes in vitro and in vivo. Collectively, our findings suggest that overexpression of miR-31 contributes to skin inflammation in psoriasis lesions by regulating the production of inflammatory mediators and leukocyte chemotaxis to the skin. Our data indicate that inhibition of miR-31 may be a potential therapeutic option in psoriasis.

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inflammation by interfering with the cross-talk between keratinocytes and immune cells.

Materials and Methods

Patients

Four-millimeter punch biopsies were taken, after informed consent, from nonlesional (n = 20) and lesional skin (n = 43) of patients with moderate or severe chronic plaque psoriasis and from noninflamed, nonirritated skin of healthy individuals (n = 35). The psoriasis patients had not received systemic immunosuppressive treatment or psoralen + ultraviolet A/solarium/UV for at least 1 mo and topical therapy for at least 2 wk before skin biopsy.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissues and cells using miRNeasy Mini kit (Qiagen). Skin biopsies from human and K5.TGF-β1 mice (11, 12) were homogenized in liquid nitrogen using a Micro-Dismembrator U (Braun Biotech) prior to RNA extraction. Epidermal cells were isolated from punch skin biopsies by dispase treatment and trypsin digestion as described in Ref. 13. CD45-negative cells were subsequently isolated using MACS separation columns (Miltenyi Biotec), according to the manufacturer’s instructions. Quantification of miR-31 by TaqMan Real-Time PCR was performed as described previously (7). Its expression was normalized between different samples based on the values of U48 RNA expression in human and snRNA 251 in mouse. The primary miRNA transcripts were quantified by TaqMan Pri-miRNA assay (Hs00335764; Applied Biosystems) following the manufacturer’s instructions. To quantify miRNAs, 500 ng total RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). The miRNAs of IL-1β, CXCL1, CXCL5, CXCL8/IL-8, STK40, ICAM-1, VCAM-1, and E-selectin were quantified by TaqMan gene expression assays (Applied Biosystems). Target gene expression was normalized based on the expression of the internal positive control 18S RNA: 5'-CGGCTACCCACATCCAAGGA-3' (forward), 5'-GCTGGAAATACCGGGCT-3' (reverse), and 5'-FAM-TGCTGGCACAGACTTGGCCCT-TAMRA-3' (probe).

In situ hybridization

In situ hybridization was performed on frozen sections (10 μm in thickness) of skin biopsy specimens as described previously (9). Briefly, after incubated in acetylation solution (0.06 M HCl, 1.3% triethanolamin, and 0.6% acetic anhydride in diethyl pyrocarbonate-treated water) for 10 min at room temperature, sections were incubated in permeabilization buffer (1% of Triton X-100) for 30 min at room temperature, washed, and prehybridized for 1 h at 50°C. Hybridization with digoxigenin-labeled miCRURY locked nucleic acid (LNA) probes (Exiqon) was performed overnight at 50°C. Slides were then washed four times with 2× SSC buffer followed by one time with 0.1× SSC buffer at 67°C. The probe binding was detected by incubating the sections with alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments (1:2500 [Roche]) for 1 h at room temperature. Sections were visualized by adding BM purple alkaline phosphatase substrate (Roche), according to the manufacturer’s instructions.

Cells culture and treatments

Human adult epidermal keratinocytes used in miR-31 function study were purchased fromCascade Biologies and cultured in EpiLife serum-free keratinocyte growth medium including Human Keratinocyte Growth Supplement at a final Ca2+ concentration of 0.06 mM (Cascade Biologies) at 37°C in 5% CO2. To study the biological effects of miR-31 on keratinocytes, third passage keratinocytes at 30–50% confluence were transduced from MatTek. TGF-β1 (50 ng/ml; Immunotools), TGF-β2 (3 ng/ml; R&D Systems) at the indicated time points and stored at −80°C. The protein levels of CXCL1, CXCL5, and CXCL8/IL-8 were analyzed by ELISA (R&D Systems) following the manufacturer’s instructions.

Leukocyte chemotaxis

Human leukocytes were isolated from 0.2% EDTA antigenuated whole blood collected by venipuncture from healthy donors. Erythrocytes were removed using dextran sedimentation (1 mixture of blood: 6% dextran/0.9% NaCl), followed by one or two rounds of hypotonic lysis using dH2O. Purified leukocytes were suspended in EpiLife serum-free keratinocyte growth medium, and 3 × 105 cells were added to the inner chamber of a BD Falcon Cell Culture Insert. Leukocytes migrate through a 3-μm porosity polyethylene terephthalate membrane toward the outer chamber containing culture medium from keratinocytes, which were transfected with anti–miR-31 or anti–miR-CTRL for 72 h. After incubation for 3 h at 37°C in 5% CO2, the migrating cells in the medium of the outer chamber were quantified by CyQUANT GR dye (Life Technologies) staining.

Endothelial cell activation

HUVECs were isolated as previously described (17) and maintained in Medium 199 (Invitrogen) containing 20% FCS, 2.5 μg/ml amphotericin B, 1 ng/ml epidermal growth factor, and 1 μg/ml hydrocortisone (all from Sigma-Aldrich). Second-passage HUVECs were treated with keratinocytes culture medium for 4 h and then harvested.

Luciferase reporter assays

Renilla luciferase reporter plasmids containing synthetic sequence repeats that are fully complementary to miR-31 (miR-31 sensor) or 3'-UTR of the STK40 gene were obtained from SwitchGear Genomics. The mutations were generated with the predicted target site of STK40 3'-UTR of the STK40 gene cloned downstream of the reporter gene and empty luciferase vector were obtained from SwitchGear Genomics. The mutations were generated with the predicted target site of STK40 3'-UTR using the QuickChange XL site-directed mutagenesis kit (Stratagene), according to the manufacturer’s instructions. NF-κB reporter plasmid pGL4.32[luc2P/NF-κB/RE/Hygro] Vector was obtained from Promega, which contains five copies of an NF-κB response element that drives transcription of the luciferase reporter gene luc2P (Phoitus pyralis). Human primary keratinocytes growing in 24-well plates were cotransfected with the luciferase reporters (25 ng/ml) together with 10 nM anti-miR-31 or anti-miR-CTRL using FuGENE HD transfection reagent (Promega). Luciferase activity was analyzed 24 h posttransfection using LightSwitch Luciferase Assay reagent (SwitchGear) or Dual-Luciferase Reporter Assay System (Promega).

Immunohistochemistry

STK40 protein expression was analyzed in both frozen and formalin-fixed paraffin embedded skin sections (7 μm in thickness) using rabbit anti-human STK40 Ab (1:200) (Sigma-Aldrich) and the avidin-biotin-peroxidase complex staining system (Vector Laboratories) following the manufacturer’s instructions. Replacement of the primary Ab with rabbit Ig fraction (DakoCytomation) in the staining process was used as negative control (Supplemental Fig. 3).

Statistics

Statistical significance for experiments was determined by Mann–Whitney U test or Student’s t test. Correlation between the expression of different genes in the same samples was made using Pearson’s correlation test on log-transformed data. p < 0.05 was considered to be statistically significant.

Study approval

The clinical materials were taken after patients’ consent and the study was approved by the Stockholm Regional Ethics Committee and conducted according to the Declaration of Helsinki’s principles. All procedures in-
volving mice were approved by Institutional Animal Care and Use Committee at Colorado School of Public Health (University of Colorado Denver, Aurora, CO).

Results

miR-31 is upregulated in psoriasis keratinocytes

Earlier expression profiling data from our and other groups showed that miR-31 is one of the miRNAs overexpressed in psoriasis skin (5, 7, 8). To confirm these data, we measured mature miR-31 expression in skin biopsies from healthy donors (n = 14), nonlesional (n = 10), and lesional skin (n = 25) from psoriasis patients using quantitative real-time PCR (qRT-PCR) (Fig. 1A). miR-31 expression was found to be dramatically increased in psoriasis lesional skin compared with healthy skin (33-fold change; p = 4.1 × 10⁻⁷) and compared with psoriasis nonlesional skin (14-fold change; p = 8.1 × 10⁻⁷). In addition, we found that the expression of primary miR-31 transcript (pri-miR-31), from which the mature miR-31 is processed, was also upregulated in psoriasis lesional skin (n = 9) compared with healthy skin (n = 7) (Fig. 1B). The expression of pri-miR-31 correlated positively (R = 0.7659; p = 0.0014) with the expression of mature miR-31 in these skin biopsies, suggesting that the upregulation of miR-31 in psoriasis skin may occur at the transcriptional level, rather than during processing of the primary transcript.

To investigate which cell type(s) in the skin expresses miR-31, we surveyed miR-31 expression in a panel of isolated primary human cell types. qRT-PCR results showed that miR-31 was mainly expressed by keratinocytes, fibroblasts and melanocytes isolated from the skin (Supplemental Fig. 1). To identify which cell type(s) in the skin are primarily responsible for the increased expression of miR-31 in psoriasis, we performed in situ hybridization on skin sections from healthy individuals (n = 11), nonlesional (n = 6), and lesional skin (n = 11) from psoriasis patients using miR-31–specific LNA-modified probes (Fig. 1C). The expression of miR-31 was low in healthy skin and restricted to the basal cell layer of the epidermis. In contrast, miR-31 expression was higher in psoriasis nonlesional skin and appeared at both basal and suprabasal cell layers. In lesional skin from the same psoriasis patients, miR-31 expression was further upregulated and mainly detected in the suprabasal layers.
To quantify the change of miR-31 level in epidermal cells, we measured miR-31 expression in sorted epidermal CD45 (common leukocyte Ag)-negative cells from healthy (n = 10), psoriasis nonlesional (n = 4), and lesional skin (n = 7) (Fig. 1D). In line with the results of in situ hybridization, qRT-PCR analysis revealed a 13-fold (p = 0.0001) higher miR-31 expression in CD45\textsuperscript{-} cells sorted from psoriasis lesional skin compared with those obtained from healthy skin and a 9-fold (p = 0.0061) increase compared with psoriasis nonlesional skin. Collectively, our results demonstrate that miR-31 is upregulated in keratinocytes in psoriasis skin lesions.

**miR-31 regulates the production of inflammatory mediators in keratinocytes**

To study the biological role of miR-31 in keratinocytes, we transfected miR-31 hairpin inhibitor (anti–miR-31) into primary human keratinocytes to inhibit endogenous miR-31. Inhibition of miR-31 was confirmed by qRT-PCR analysis of miR-31 expression and by luciferase assay using a synthetic miR-31-target as sensor (Supplemental Fig. 2A, 2B). We performed a global transcriptome analysis of keratinocytes upon suppression of endogenous miR-31 using Affymetrix arrays, which identified 234 genes that were significantly changed (Supplemental Table I). Of note, the expression of the host gene of miR-31, encoding a long noncoding RNA, LOC554202 (18), was not affected by miR-31 inhibition, neither that of the adjacent IFN\textgreek{e} (IFNE1) gene (Supplemental Fig. 2C).

Interestingly, we found that several mediators of key importance in psoriasis pathogenesis, such as IL-1\textgreek{b}, CXCL1, CXCL5, and CXCL8/IL-8, were downregulated by anti–miR-31 in keratinocytes (Supplemental Table I), and this was validated by qRT-PCR on keratinocytes transfected with anti–miR-31 or anti–miR-Ctrl for 24, 48, 72, and 96 h (Fig. 2A). Consistently, the amount of these chemokines secreted into the culture medium was decreased by anti–miR-31, shown by ELISA (Fig. 2B). Notably, IL-1\textgreek{b} was not detectable in the culture medium, because the cultured human keratinocytes produce but do not process the IL-1\textgreek{b} precursor into its biologically active form because of the lack of IL-1 convertase (19).

Next, we aimed to test whether inhibition of miR-31 can efficiently suppress the production of IL-1\textgreek{b}, CXCL1, CXCL5, and CXCL8/IL-8 under inflammatory conditions. To this end, we measured the effect of miR-31 inhibition on mRNA and protein levels of these cytokine/chemokines in keratinocytes treated with the proinflammatory cytokine TNF-\textgreek{a} (Fig. 2C, 2D). Consistent with earlier reports, TNF-\textgreek{a} induced the expression of IL-1\textgreek{b}, CXCL1, CXCL5, and CXCL8/IL-8. Inhibition of endogenous miR-31

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**FIGURE 2.** Inhibition of miR-31 suppresses the production of cytokines/chemokines by keratinocytes. The expression and secretion of CXCL1, CXCL8, CXCL5, and IL-1\textgreek{b} were analyzed in keratinocytes transfected with anti–miR-31 or anti–miR-Ctrl for 24–96 h by qRT-PCR (A) and ELISA (B) or analyzed in keratinocytes transfected with anti–miR-31 or anti–miR-Ctrl for 48 h and then treated with TNF-\textgreek{a} for 6 h by qRT-PCR (C) and ELISA (D). *p < 0.05, **p < 0.01, ***p < 0.001; Student t test.
miR-31 regulates the endothelial cell–activating and leukocyte-attracting capacity of keratinocytes

Because CXCL1, CXCL5, and CXCL8 have the ability to activate endothelial cells and recruit polymorphonuclear leukocytes into inflamed tissues (20), we examined whether the capacity of keratinocytes to attract leukocytes was affected by miR-31. The first step of recruitment of leukocytes to the skin is the attachment of circulating cells to vascular endothelial cells, which is mediated by cell adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin (21). It has been shown that the expression of these cell adhesion molecules is increased on dermal vessels of psoriatic skin (22–25). To test the effect of miR-31 on endothelial cell-activating capacity of keratinocytes, we measured the expressions of ICAM-1, VCAM-1, and E-selectin in primary HUVECs incubated with culture medium from miR-31 inhibitor–treated keratinocytes. qRT-PCR results demonstrated that inhibition of endogenous miR-31 in keratinocytes decreased their capacity to induce the expression of these cell adhesion molecules in endothelial cells (Fig. 3A).

Next, we considered whether altered expression of miR-31 in keratinocytes could affect their capacity to attract leukocytes. To address this question, we performed migration assays with peripheral blood leukocytes using conditioned supernatant from miR-31 inhibitor–treated keratinocytes (Fig. 3B). The results revealed that supernatant from keratinocytes with inhibited miR-31 expression attracted less leukocytes compared with medium from control treated cells. Taken together, these data show that inhibition of miR-31 in keratinocytes results in decreased endothelial cell activation and leukocyte chemotaxis.

miR-31 regulates the NF-κB pathway in keratinocytes

The NF-κB pathway is one of the signaling pathways commonly regulating the expression of IL-1β, CXCL1, CXCL5, and CXCL8/IL-8. We therefore investigated whether other target genes of the NF-κB pathway are also regulated by miR-31 in keratinocytes. To answer this question, we evaluated the enrichment for known target genes of the NF-κB pathway (summarized on the Web site: http://bioinfo.lifl.fr/NF-KB/) in our microarray data of keratinocytes transfected with anti–miR-31 or anti–miR-Ctrl (Fig. 4A). Results of the GSEA (14, 15) revealed that NF-κB pathway target genes were significantly enriched among the genes downregulated by anti–miR-31, and a negative enrichment score curve was generated by GSEA (p < 0.001). These data indicated that inhibition of miR-31 might affect the activity of the NF-κB signal transduction pathway.

To test this hypothesis, we determined the effect of miR-31 inhibitor on NF-κB–dependent promoter luciferase reporter gene activity in human primary keratinocytes (Fig. 4B). Results of luciferase assays demonstrated that inhibition of miR-31 suppressed both the basal and TNF-α–induced NF-κB–dependent luciferase activity, indicating that miR-31 regulates the activity of NF-κB pathway in keratinocytes.

miR-31 targets STK40, a negative regulator of the NF-κB pathway

Next, we aimed to identify the molecular mechanism by which miR-31 modulates the NF-κB pathway. miRNAs exert biological functions by regulating their target genes. To predict target genes of miR-31, we used three different public algorithms, TargetScan

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** miR-31 regulates the endothelial cell–activating and leukocyte-attracting capacity of keratinocytes. (A) HUVECs were treated with medium from cultured keratinocytes transfected with anti–miR-31 or anti–miR-Ctrl, and the expression of endothelial cell activation markers (ICAM-1, VCAM-1, and E-selectin) was analyzed by qRT-PCR. (B) Human leukocytes chemotaxis toward the unused medium (med) or the medium from cultured keratinocytes transfected with anti–miR-31 or anti–miR-Ctrl for 54 h and then treated with or not with TNF-α for 18 h. The migrating cells were quantified by CyQUANT GR dye staining. *p < 0.05, **p < 0.01; Student t test.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** miR-31 regulates the NF-κB pathway in keratinocytes. (A) Microarray analysis was performed in independent biological triplicates for primary human keratinocytes transfected with either anti–miR-31 or anti–miR-Ctrl. Genes represented in the profile data set were ranked by fold change (anti–miR-31/anti–miR-Ctrl). GSEA evaluated enrichment within the profile data set for the reported target genes of NF-κB signaling pathway. Vertical bars along the x-axis of the GSEA plot denote the positions of NF-κB target genes within the ranked list. NES, normalized enrichment score. (B) Keratinocytes were transfected with NF-κB luciferase reporter plasmid 48 h after the transfection of anti–miR-31 or anti–miR-Ctrl. Eighteen hours later, the cells were treated with or not with TNF-α for 5 h, and luciferase activity was measured. **p < 0.01, ***p < 0.001; Student t test.
miR-31 targets STK40, a negative regulator of the NF-κB signaling pathway. (A) GSEA evaluated the enrichment of predicted miR-31 targets, as determined by the given algorithms (TargetScan, miRanda, and PicTar), within the microarray profiling data set of keratinocytes transfected with anti–miR-31 or anti–miR-Ctrl. (B) Nucleotide resolution of the predicted miR-31 binding site in 3′-UTR of STK40 mRNA: seed sequence (green letters); and mutated miR-31 binding sites (red letters). (C) The miR-31 mediated regulation of STK40 was verified by qRT-PCR in keratinocytes transfected with anti–miR-31 or anti–miR-Ctrl for 24–96 h. (D) Keratinocytes were transfected with luciferase reporter plasmid containing WT or mutant (Mut) STK40 3′-UTR or empty vector (Vector) together with anti–miR-31 or anti–miR-Ctrl. **p < 0.01, ***p < 0.001; Student t test. (E) Human skin from the same donors was used for the detection of miR-31 by in situ hybridization (left panel) and STK40 expression by immunohistochemistry (right panel). Red-brown color indicates STK40 expression. Scale bar, 50 μm.
we performed 3′-UTR luciferase reporter assays with luciferase reporter gene constructs containing the full-length 3′-UTR of STK40 mRNA in human primary keratinocytes (Fig. 5D). The wild-type (WT) STK40 3′-UTR luciferase activity was increased 8-fold ($p = 0.004$) by anti–miR-31 in comparison with anti–miR-Ctrl. Mutation of one of the predicted target sites (Mut1) markedly decreased luciferase activity upon miR-31 inhibition, although the luciferase activity was still affected by anti–miR-31. Mutation of target site #2 (Mut 2) or both target sites (Mut1+2), however, completely abolished the effect of miR-31 inhibition on reporter gene expression. These data demonstrate that miR-31 directly regulates STK40 expression through binding to the two predicted target sites in its 3′-UTR.

Next, we analyzed the expression of STK40 in healthy ($n = 11$), psoriasis nonlesional ($n = 9$), and lesional skin ($n = 11$) by immunohistochemistry. miR-31 expression in the same donors was detected by in situ hybridization (Fig. 5E, Supplemental Fig. 3). STK40 expression was mainly detected in the cytoplasm and the plasma membrane of epidermal keratinocytes. In healthy skin, STK40 was strongly expressed in the granular layers and a weaker signal of STK40 was detected also in the basal and spinous layers of the epidermis. In psoriasis nonlesional skin, the strong signal of STK40 in granular layers was similar as in healthy skin, whereas the expression of STK40 in basal and spinous layers was slightly decreased. In psoriasis lesional skin, the STK40 signal was absent in the lower spinous layers but strongly expressed in the upper spinous layers, which is opposite to the pattern of miR-31 expression in psoriasis lesional skin. No STK40-positive leukocytes were observed in the dermis. The reciprocal expression of miR-31 and STK40 further supports that STK40 is regulated by miR-31 in psoriasis skin.

**miR-31 regulates cytokine/chemokine expression via targeting STK40**

To determine whether the observed effects of miR-31 on keratinocyte cytokine/chemokine production are, at least partially, mediated through STK40, we analyzed the effects of silencing of STK40 expression by siRNA in keratinocytes (Fig. 6). STK40 expression in keratinocytes was significantly increased by anti–miR-31, which was prevented by simultaneous transfection with

![FIGURE 6. MiR-31 regulates cytokine/chemokine expression via targeting STK40. Keratinocytes were cotransfected with siRNA and anti-miR for 48 h and then treated with TNF-α for 6 h. STK40 expression was detected by qRT-PCR (A). The expression and secretion of IL-1β, CXCL1, CXCL5, and CXCL8/IL-8 were analyzed by qRT-PCR (B) and ELISA (C). (D) Keratinocytes were transfected with an NF-κB reporter plasmid 48 h after the transfection of anti-miR and siRNA. Eighteen hours later, the cells were treated with TNF-α for 5 h, and the luciferase activity was measured. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student $t$ test.](http://www.jimmunol.org/)[http://www.jimmunol.org/](http://www.jimmunol.org/)
miR-31 is upregulated by TGF-β1 in keratinocytes both in vitro and in vivo

To understand the mechanism of miR-31 overexpression in psoriasis keratinocytes, we systematically surveyed the effect of cytokines, growth factors, and cell differentiation on miR-31 expression in keratinocytes. Cells were treated with cytokines relevant for psoriasis pathology (TNF-α, IL-22, TGF-β1, IL-6, IL-20, IFN-γ, and GM-CSF), growth factors (epidermal growth factor and keratinocyte growth factor) and keratinocyte differentiation-driving factors (1.5 mM CaCl2, 12-O-tetradecanoylphorbol-13-acetate, and cell confluence) and miR-31 expression was analyzed by qRT-PCR. miR-31 was not significantly changed by these factors (data not shown) with the exception of TGF-β1, a cytokine previously reported to be upregulated in the epidermis and serum of psoriasis patients (31). TGF-β1 significantly induced miR-31 expression in keratinocyte cultures (Fig. 7A). Furthermore, upregulation of miR-31 by TGF-β1 was also observed in three-dimensional reconstructed epidermal equivalents, which is a multilayered artificial epidermis built with human primary keratinocytes on an air-liquid interface (Fig. 7A).

To investigate whether miR-31 is regulated by TGF-β1 in vivo, we measured miR-31 expression in the skin of K5.TGF-β1 transgenic mice, which overexpress this cytokine in an epidermis-specific manner and develop a psoriasis-like skin disorder (11, 12). qRT-PCR results showed that the expression of miR-31 was increased 35-fold (p = 0.01) in the skin of K5.TGF-β1 mice compared with WT littermates (Fig. 7B). Treatment with etanercept, which blocks TNF-α activity and is one of the current therapies for psoriasis, efficiently alleviated the psoriasis phenotype of K5.TGF-β1 mice (11) and resulted in a 4-fold (p = 0.005) reduction of miR-31 expression compared with the K5.TGF-β1 mice treated with normal saline (Fig. 7B). In line with this, in situ hybridization showed that miR-31 was upregulated in the hyperplastic epidermis of K5.TGF-β1 mice compared with WT littermates, whereas treatment with etanercept reduced epidermal hyperplasia and miR-31 expression (Fig. 7C). Taken together, our results suggest that increased levels of TGF-β1 in psoriatic skin may contribute to the upregulation of miR-31 in psoriasis.

Discussion

In this study, we show that miR-31 is upregulated in psoriasis and identify it as a novel regulator of NF-κB activity. We demonstrate that inhibition of endogenous miR-31 in keratinocytes suppresses the production of inflammatory mediators and the capability of keratinocytes to activate endothelial cells and to attract leukocytes. Moreover, we identify STK40, a negative regulator of the NF-κB pathway, as a novel target for miR-31 in keratinocytes and demonstrate that silencing of STK40 can rescue the effect of miR-31 on inflammatory mediators. Finally, we identify TGF-β1 as a regulator of miR-31 expression in vitro and in vivo. Our results thus suggest a model in which TGF-β1 induces miR-31 in psoriasis.
Psoriasis keratinocytes that leads to increased NF-κB activity partially through suppression of STK40. In turn, inflammatory cytokine/chemokines are induced, which contributes to endothelial cell activation, leukocyte attraction and clinically, skin inflammation (Fig. 8).

miR-31 is widely expressed and plays diverse roles in different tissue and cell types. This miRNA has been shown to positively regulate corneal epithelial glycan metabolism (32), to inhibit fibrogenesis and pulmonary fibrosis (33), to regulate lymphatic vascular lineage–specific differentiation (34), and to reduce TNF-induced expression of E-selectin on human endothelial cells as a feedback control of inflammation (35). In pathological conditions, miR-31 has been mainly studied in cancer and was identified as a critical and pleiotropic regulator of tumor metastasis and growth (reviewed in Ref. 36). In mouse skin, miR-31 has been shown to control hair cycle–associated gene expression programs (37). However, its role in skin diseases and in particular, psoriasis, was unknown. We found that inhibition of miR-31 in keratinocytes decrease the production of IL-1β, CXCL1, CXCL5, and CXCL8, suggesting that miR-31 has an immunomodulatory function in keratinocytes. Keratinocytes in psoriasis lesions overexpress several proinflammatory cytokines and chemokines, which contribute to the maintenance of inflammation in psoriasis skin lesions and represent a crucial element in psoriasis pathogenesis (1). IL-1β is a proinflammatory cytokine, which is essential for Th17 differentiation (38) and potentiates immune cell activation by regulating T cell–targeting chemokine production in keratinocytes (39). Keratinocyte-derived CXCL1, CXCL5, and CXCL8/IL-8 can potently activate endothelial cells and are chemotactants for multiple subsets of leukocytes, especially neutrophil granulocytes, by binding to the cognate receptors CXCR1 and CXCR2 (20). These chemokines play prominent roles in recruitment and retention of inflammatory cells into psoriasis skin, as well as regulation of the formation of new blood vessels.

**FIGURE 8.** Proposed mechanism by which miR-31 modulates IL-1β, CXCL1, CXCL5, and CXCL8/IL-8 production by keratinocytes in psoriasis skin. TGF-β1, which is highly expressed in psoriatic skin, upregulates the expression of miR-31 in keratinocytes. miR-31 directly suppresses STK40, a suppressor of NF-κB signaling activation induced by TNF-α. The activation of NF-κB signaling contributes to the elevated expression and secretion of IL-1β, CXCL1, CXCL5, and CXCL8/IL-8, which promote vascular endothelial cell activation and attract leukocytes via chemotaxis into the skin.

In accordance with known functions of CXCL1, CXCL5, and CXCL8/IL-8, inhibition of endogenous miR-31 in keratinocytes impaired the capability of conditioned supernatant to activate endothelial cells or to attract leukocytes, suggesting that miR-31 may be involved in the cross-talk between keratinocytes and immune cells in psoriasis. Because miR-31 expression is not confined to keratinocytes, its deregulation in other cell types may also contribute to psoriatic skin inflammation. It was previously shown that low levels of miR-31 in human natural regulatory T cells (Tregs) contributes to high expression of FOXP3, which is a master transcription factor crucial for Treg function and identified as the direct target of miR-31 (40). Psoriasis has been associated with impaired immune suppressive capacity of Tregs (41) and their enhanced propensity to differentiate into inflammatory IL-17A–producing cells (42). Thus, it would be interesting to further investigate the effect of miR-31 on the function and differentiation of Tregs in the context of psoriasis.

Our findings show that miR-31 regulates the activity of NF-κB pathway in keratinocytes, which is a key signaling pathway regulating cytokine/chemokine expression (43) and which is activated in psoriasis lesional skin (1, 44, 45). Importantly, activation of NF-κB in both keratinocytes and lymphocytes is required to develop the psoriasis-like phenotype in transgenic mice, indicating the importance of this pathway in psoriasis (46). In this study, we demonstrate that in keratinocytes miR-31 directly targets STK40, which was previously shown to inhibit TNF-induced NF-κB activation (29). Interestingly, the regulation of STK40 expression by miR-31 was also observed in ovarian cancer cells (30). In the skin, STK40 showed a reciprocal expression pattern with miR-31, which further supports a functional miRNA:mRNA interaction in vivo. Derepression of STK40 through miR-31 may, at least partially, explain the reduced activity of NF-κB signaling by anti–miR-31 in keratinocytes. miR-31 was recently shown to downregulate noncanonical pathway of NF-κB activation via targeting NF-κB–inducing kinase in leukemic T cells (47). On the contrary, the expression of NF-κB–inducing kinase was not significantly altered by miR-31 inhibition in human primary keratinocytes, as shown in our microarray profiling data. The seemingly paradoxical findings with regard to the regulatory role of miR-31 in the NF-κB signaling pathway indicate that the biological function of miR-31 is highly cell-type dependent.

By a systematic screen using cytokines, growth factors, and factors modulating cell differentiation, we identified TGF-β1 as a potent regulator of miR-31 expression in primary keratinocytes, in reconstructed human epidermal equivalents, and in a transgenic mouse model. TGF-β1 is a cytokine with increased levels in the epidermis and serum of psoriasis patients (9, 29, 30), and its involvement in psoriasis pathogenesis is supported by the finding that mice overexpressing TGF-β1 driven by a keratin 5 (K5) promoter in keratinocytes (K5.TGF-β1), exhibit a psoriasis-like phenotype (11, 12). The infiltration of inflammatory cells observed in K5.TGF-β1 mice may partially be due to the upregulation of miR-31 by TGF-β1 in keratinocytes, leading to increased production of chemotactants. Importantly, TGF-β1, along with IL-1β, is critical for the differentiation of Th17 cells (38), which play essential pathogenic role in psoriasis. TGF-β1–induced miR-31 can lead to increased IL-1β levels and thus can contribute to the amplification of Th17-type inflammation in psoriasis. Notably, we found that in keratinocytes miR-31 was not induced by TNF-α, although this cytokine has been shown to upregulate miR-31 expression in endothelial cells (27), further underlining that miR-31 is regulated in a cell-type dependent manner.

Taken together, we show that miR-31 is overexpressed in psoriasis keratinocytes and identify STK40, a negative regulator of
NF-kB as a direct target. We further show that the expression of miR-31 in keratinocytes is regulated by the important psoriasis-associated cytokine, TGF-β1. Overexpression of miR-31 in psoriasis keratinocytes may contribute to skin inflammation by enhancing leukocyte migration into the skin. These findings suggest that targeting miR-31 in psoriasis skin may alleviate inflammation by reducing the activity of NF-kB signaling and interfering with the cross-talk between keratinocytes and immune cells.

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Associated cytokine, TGF-

Disclosures
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miR-31 IN PSORIASIS
