TGFβ/SMAD/microRNA-486-3p Signaling Axis Mediates Keratin 17 Expression and Keratinocyte Hyperproliferation in Psoriasis

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Keratin 17 (K17) is strongly expressed in psoriatic lesions but not healthy skin, and plays a crucial role in disease pathogenesis. The mechanism of aberrant K17 expression in psoriasis has not been fully elucidated. MicroRNAs are short, single-stranded, noncoding RNAs that play important roles in regulating gene expression. Psoriasis exhibits a specific microRNA expression profile distinct from that of healthy skin. In this study, we showed that miR-486-3p was markedly reduced in psoriatic epidermis and negatively correlated with the psoriasis area and severity index score. Its expression repressed K17 protein expression and decreased proliferation in a keratinocyte cell line overexpressing K17 (LV K17) compared with controls. Our data indicated that miR-486-3p was regulated by a transforming growth factor-β (TGFβ)/SMAD pathway and possibly mediated the downregulation of K17 protein in TGFβ-treated keratinocytes. Finally, the decreased expression of TGFβ receptor I in psoriatic epidermis inactivated the TGFβ/SMAD pathway, leading to K17 overexpression and cell proliferation. Collectively, our findings demonstrated that a TGFβ/SMAD/miR-486-3p signaling axis in keratinocytes regulated K17 expression and cell proliferation. We conclude that the loss of miR-486-3p in psoriatic epidermis leads to K17 protein overexpression and contributes to the pathogenesis of psoriasis. Overexpression of miR-486-3p may therefore be a therapeutic option for psoriasis.

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INTRODUCTION

Keratins are a family of fibrous structural proteins in epithelial cells, which provide mechanical support and are also associated with proliferation, differentiation, apoptosis, tissue polarity, and epithelial remodeling (Chamcheu et al., 2011; Porter and Lane, 2003). Hyperproliferative keratinocytes in psoriasis exhibit an abnormal keratin expression pattern (Leigh et al., 1995; Thewes et al., 1991). For example, keratin 17 (K17) is not expressed in healthy epidermis, but overexpressed in psoriatic lesions. Its expression level is positively associated with psoriasis severity (de Jong et al., 1991), and thus K17 is considered a hallmark of psoriasis. Moreover, K17 may function as an autoantigen in the immunopathogenesis of psoriasis, which may be a major target for autoreactive T cells (Gudmundsdottir et al., 1999; Johnston et al., 2004). Our previous study showed that K17 stimulates proliferation of T cells and IFN-γ production in psoriasis (Shen et al. 2005, 2006). Furthermore, the inhibition of K17 expression using antisense and RNA interference strategies results in improvement of psoriasis-like symptoms in a severe combined immunodeficiency-human xenogeneic transplantation model for psoriasis (Chang et al., 2011). Taken together, K17 may play an important role in psoriasis pathogenesis and is a potential therapeutic target of the disease. The precise mechanisms of abnormal K17 elevation in psoriasis have not been fully elucidated.

MicroRNAs (miRNAs or miRs) are short noncoding RNA molecules that regulate gene expression posttranscriptionally and play crucial roles in many physiological or pathological processes, including psoriasis. Deregulated miRNA expression has been uncovered in psoriasis (Sonkoly et al., 2007). miRNAs can regulate proliferation and differentiation of keratinocytes (Choi et al., 2014; Ichihara et al., 2011; Xu et al., 2011; Yan et al., 2015), mediate activation and survival of T cells (Fu et al., 2015; Meisgen et al., 2012; Zhao et al., 2014), and propagate crosstalk between immunocytes and keratinocytes through regulating chemokine production (Xu et al., 2013). Circulating miRNAs may serve as biomarkers for diagnosis and reflect the disease severity (Lovendorf et al., 2014). miR-486-3p, which is hosted within the Ank1 gene, plays diverse roles in different tissues and cell types. Deep sequencing of small RNAs from human skin revealed that miR-486-3p is among the top 10 downregulated miRNAs in psoriatic lesions compared with healthy controls (Joyce et al., 2011), suggesting that the loss of miR-486-3p may contribute to psoriasis pathogenesis.

In this study, we confirmed that the expression of miR-486-3p was significantly reduced in psoriatic epidermis. We showed that reduced miR-486-3p associated with increased K17

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Abbreviations: HaCaT, normal human keratinocyte cell line of skin origin; K17, keratin 17; miRNA, microRNA; qRT-PCR, quantitative real-time reverse transcriptase-PCR; TGFβ, transforming growth factor-β; TGFβRI/II, TGFβ receptors I/II

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protein. Expressing miR-486-3p reduced cell proliferation associated with overexpression of K17. Transforming growth factor-β (TGFβ), which has been shown to regulate several miRNAs (Davis et al., 2010), activated miR-486-3p, resulting in the reduction of K17 in a normal human keratinocyte cell line of skin origin (HaCaT). We also identified a SMAD (transducer of TGFβ signaling) binding site on the pri-miR, suggesting the possibility that SMADs regulate miR-486-3p expression. Together, our results reveal a link between miR-486-3p deficiency and overexpression of K17 protein in psoriatic keratinocytes, which opens the possibility of using miR-486-3p overexpression as a potential therapeutic for psoriasis.

RESULTS

Reduced miR-486-3p levels in psoriatic epidermis associated with K17 protein overexpression

To identify possible miRNAs that may lead to K17 overexpression in psoriasis, we predicted candidate miRNAs targeting K17 by resorting to TargetScan and miRanda databases, and then compared them with a microarray database of downregulated miRNAs in psoriatic lesions (Joyce et al., 2011). miR-486-3p was among the top 10 downregulated microRNAs in psoriasis and predicted to target K17 (Figure 1a). To confirm the expression of miR-486-3p in psoriatic epidermis, we separated the epidermis of the psoriatic skin and healthy controls. Quantitative real-time reverse transcriptase-PCR (qRT-PCR) analysis showed that the expression of miR-486-3p was reduced significantly in psoriatic epidermis and adjacent nonlesional skin from patients with psoriasis compared with biopsies from control individuals (n = 25 each group) (Figure 1b) and was negatively correlated with the psoriasis area and severity index score (Figure 1c).

To determine the effect of miR-486-3p on K17, we transfected HaCaT keratinocytes with miR-486-3p mimics or inhibitors and detected the expression of K17 by western blot and qRT-PCR. The results showed that the K17 protein level was downregulated by miR-486-3p mimics and upregulated by inhibitor (Figure 1d), whereas K17 mRNA levels were not significantly changed (Figure 1e). Furthermore, we treated normal human epidermal keratinocytes with Aldara (Walter et al., 2013) or cytokines (TNF-α, IL17, and IL22) (Bernard et al., 2012) to induce psoriatic-like keratinocytes, followed by transfection of miR-486-3p mimics or control (ctrl) miR. K17 protein was increased (Figure 1f and g) and miR-486-3p was reduced in psoriatic-like keratinocytes compared with normal human epidermal keratinocytes (Supplementary Figure S1 online). The elevated K17 expression was restored by miR-486-3p.

To validate K17 mRNA as a miR486-3p target, we performed a 3′-UTR luciferase-binding assay. Bioinformatic analysis predicted that the seed sequence in miR-486-3p would match the *KRT*17 3′-UTR from 53 to 59 nucleotides (potential binding site); therefore, we cloned the *KRT*17 3′-UTR wild-type sequence as well as a deletion, double repeat, or point mutation of the potential binding site into a luciferase reporter system (Figure 1h). Compared with the vector control, the reporter activity was repressed by 35.7% and 46.4% respectively when miR-386-3p was cotransfected with the potential binding site and double repeat vectors. The miR-486-3p-dependent repression of luciferase activity was abrogated by deletion and point mutation of the potential binding site (Figure 1i). These showed that K17 was directly regulated by miR-486-3p. Together, the miR-486-3p level was reduced in psoriatic epidermis and this decrease may be responsible for the K17 overexpression.

miR-486-3p suppressed proliferation of keratinocytes overexpressing K17

As K17 has been reported to promote cell growth through regulating protein synthesis (Kim et al., 2006), we hypothesized that miR-486-3p would reduce proliferation in keratinocytes overexpressing K17. HaCaT keratinocyte proliferation was significantly enhanced by K17 overexpression (LV K17), compared with mock-infected cells (Figure 2). We then transfected LV K17- or mock-infected cells with miR-486-3p mimics or ctrl miR and analyzed proliferation. The CCK-8 assay revealed that miR-486-3p mimics significantly inhibited proliferation of LV K17 cells (Figure 2a and b). Likewise, the percentage of EdU-positive cells was significantly reduced by miR-486-3p mimics in LV K17 cells compared with controls (Figure 2c). These data confirmed that miR-486-3p can suppress K17-enhanced keratinocyte proliferation.

miR-486-3p was involved in TGFβ-induced K17 downregulation

Because TGFβ has been shown to regulate several miRNAs (Davis et al., 2010), we tested whether TGFβ regulated miR-486-3p expression in keratinocytes. qRT-PCR analysis showed that the level of miR-486-3p was increased in a dose-dependent manner by TGFβ (Figure 3a), correlating with decreased K17 protein expression (Figure 3b). We then incubated HaCaT keratinocytes with a specific antagonist of the TGFβ receptor (SB431542) 2 hours before TGFβ treatment (40 ng/ml). The upregulation of miR-486-3p induced by TGFβ was repressed by SB431542 (Figure 3c). K17 protein was reduced by TGFβ treatment, but restored by SB431542 (Figure 3d). To test whether miR-486-3p was involved in TGFβ-induced K17 downregulation, we transfected miR-486-3p mimics into HaCaT keratinocytes and treated with TGFβ and/or SB431542 (Figure 3e). Expression of miR-486-3p dampened K17 protein even when TGFβ was repressed by SB431542 (Figure 3f). We conclude that TGFβ regulates miR-486-3p expression and that miR-486-3p is involved in TGFβ-induced K17 downregulation.

miR-486-3p processing was enhanced by SMAD2/3 protein

Bioinformatics analysis revealed a potential SMAD (transducers of TGFβ signaling) binding motif in pri-miR-486-3p (http://rna.tbi.univie.ac.at) (Figure 4a). We further investigated the mechanism of miR-486-3p regulation by the TGFβ/SMAD pathway. TGFβ treatment induced nuclear translocation of phosphor-SMAD2/3 as detected by immunofluorescence microscopy (Figure 4b), which provided the possibility of interaction of phosphor-SMAD2/3 and pri-miR-486-3p. Western blot analysis showed peak phosphorylation of SMAD2/3 30 minutes after TGFβ treatment (Supplementary Figure S2 online).

To identify the association between SMAD3 and pri-miR-486-3p, we performed an RNA-chromatin immunoprecipitation

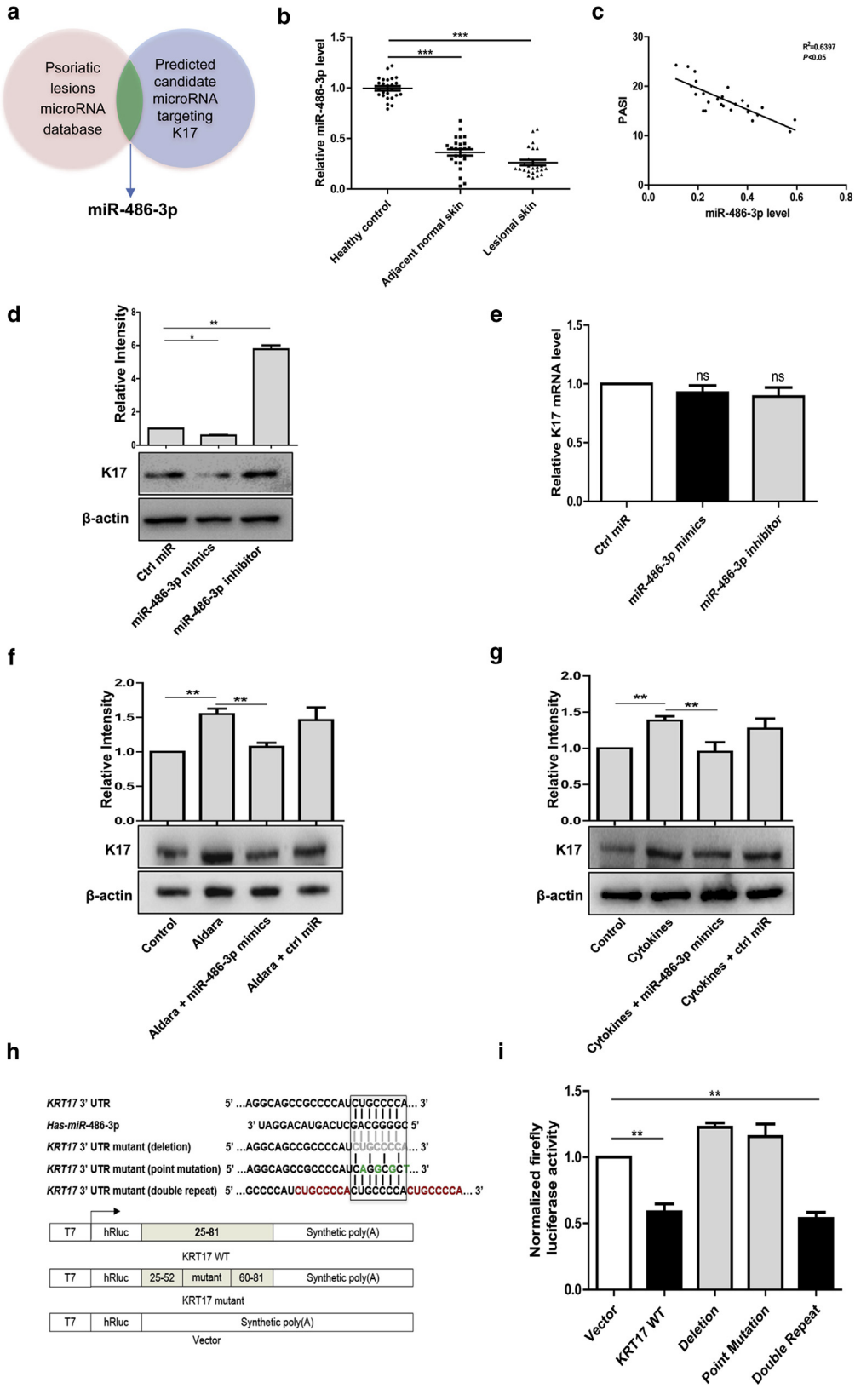

Figure 1. Reduced miR-486-3p expression levels in psoriatic epidermis and the regulation of K17 by miR-486-3p. (**a**) Venn diagram depicting potential miRNA candidates that may regulate K17 expression in psoriasis. (**b**) Expressions of miR-486-3p in normal, psoriatic nonlesional, and lesional skin samples (n = 25 in each group). (**c**) Correlation between miR-486-3p and PASI score in psoriasis (n = 25). Expression of K17 (**d**) protein and (**e**) mRNA in HaCaTs transfected with miR-486-3p mimics or inhibitor. Expression of K17 in NHEKs treated with (**f**) Aldara or (**g**) cytokines followed by miR-486-3p mimics or ctrl miR. (**h**) Predicted binding sites of miR-486-3p to K17 3′-UTR: the deletion (gray), point (green), and double repeat (red). (**i**) HaCaTs transfected with firefly luciferase reporter construct containing empty vector, K17 3′-UTR wild type, or K17 3′-UTR with mutations. n = 3. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. HaCaT, normal human keratinocyte cell line of skin origin; K17, keratin 17; miRNA, microRNA; NHEK, normal human epidermal keratinocyte; PASI, psoriasis area and severity index.

analysis of HaCaT keratinocytes cotransfected with pCMV-pri-miR-486-3p and pCMV5B-Flag-SMAD3, followed by TGFβ treatment. The data showed that pri-miR-486-3p was significantly increased in the Flag-SMAD3/RNA complexes when TGFβ/SMAD3 signaling was activated (Figure 4e). In addition, higher levels of endogenous phosphor-SMAD2 and the miRNA processing protein DROSHA could be coimmunoprecipitated with Flag-SMAD3 in TGFβ-treated cells versus mock-treated

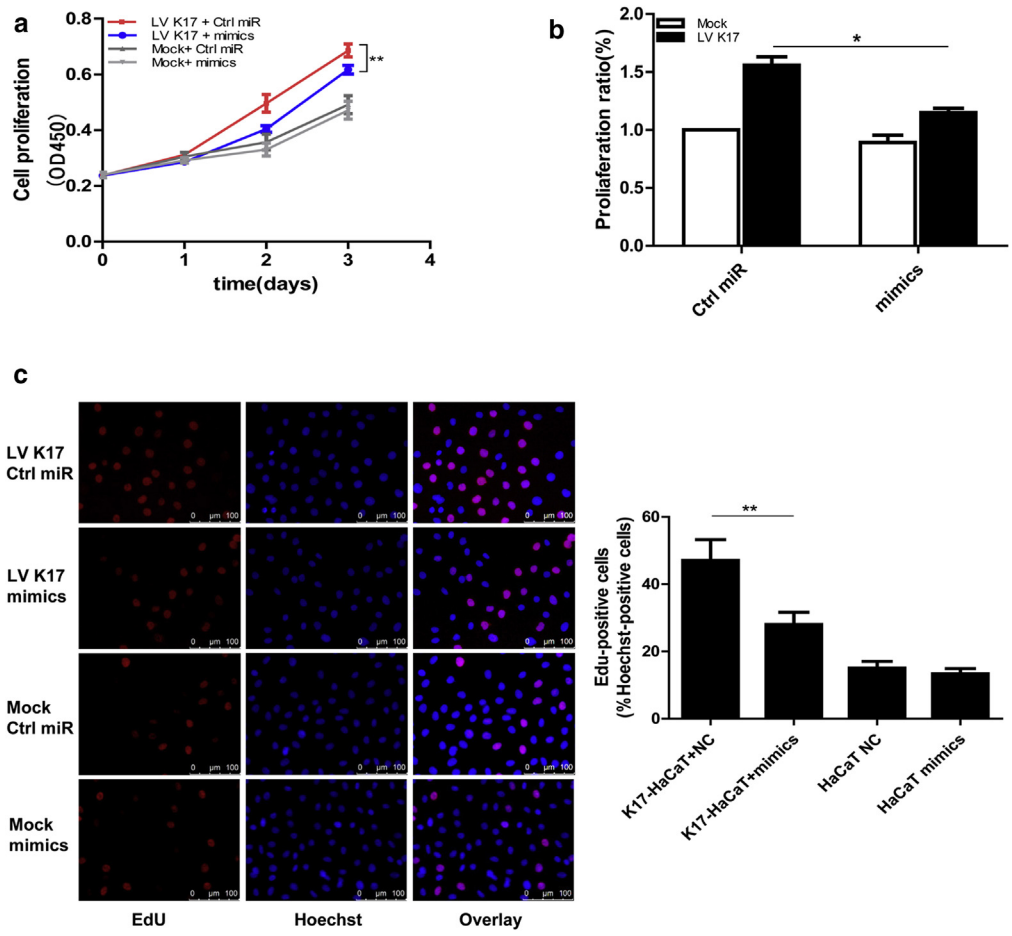

Figure 2. miR-486-3p suppressed proliferation of keratinocytes overexpressing K17. (**a**) Cell proliferation of mock- and LV K17-infected cells analyzed using the CCK-8 assay at 24, 48, and 72 hours after transfection of miR-486-3p mimics or Ctrl miR. (**b**) Proliferation ratio of mock- and LV K17-infected cells analyzed by the CCK-8 assay at 48 hours after the transfection of miR-486-3p mimics or Ctrl miR. (**c**) Representative images (left) and graphical representation (right) of the Edu proliferation assay performed on mock- and LV K17-infected cells 48 hours after the transfection of miR-486-3p mimics or Ctrl miR. Graphical representation of data were derived after counting five microscopic fields for each sample. Bars = 100 μm, n = 3, $*P < 0.05$; $**P < 0.01$. K17, keratin 17.

cells (Figure 4c). Together, these data suggest that SMAD3 can interact with pri-miR-486-3p and may enhance DROSHA recruitment to promote miR-486-3p processing.

Inactivation of the TGFβ/SMAD pathway and overexpression of K17 in psoriatic epidermis

We observe expressions of TGFβ receptors I/II (TGFβRI/II), SMAD2/3, miR-486-3p, and K17 in psoriatic and healthy control epidermis. TGFβRI was decreased in psoriatic epidermis compared with healthy controls, whereas there was no apparent change in TGFβRII (Figure 5a). There were no significant changes in fluorescence intensity of SMAD2/3 in psoriatic epidermis compared with healthy controls (Figure 5c), consistent with the lack of changes in mRNA levels of these SMADs detected by qRT-PCR (Supplementary Figure S3 online). Both SMAD2 and SMAD3 have more cytoplasmic localization and less nuclear localization in psoriatic keratinocytes compared with healthy controls (Figure 5c). Western blot analysis confirmed the decrease of TGFβRI in psoriatic epidermis (Figure 5b) and also showed reduction in phosphor-SMAD2/3 in psoriatic epidermis (Figure 5d). Fluorescence in situ hybridization showed that miR-486-3p was decreased in psoriatic epidermis compared with healthy controls (Figure 5e). Together, K17 was overexpressed in psoriatic epidermis, correlated with TGFβRI decrease, SMAD2/3 cytoplasmic translocation, and miR-486-3p loss.

DISCUSSION

In this study, we showed that miR-486-3p was reduced in psoriatic epidermis and negatively correlated with the psoriasis area and severity index score. miR-486-3p repressed K17 protein expression and suppressed proliferation in LV K17. We identified TGFβ as a regulator of miR-486-3p in HaCaT keratinocytes and showed that TGFβ activates the binding of SMADs to pri-miR-486-3p, which may enhance processing to the mature form of the miRNA. An aberrant activity of the TGFβ/SMAD2/3 pathway correlated with K17 overexpression in psoriatic epidermis. Our study thus suggests that the aberrant TGFβ/SMAD2/3 activity may reduce miR-486-3p, allowing K17 overexpression in psoriasis, thus contributing to disease pathogenesis (Figure 6).

K17 is a keratin family member that plays a key role in psoriasis, connecting aberrant keratinocyte proliferation, production of psoriatic-related cytokines, and autoreactive T cells in the disease (Jin and Wang 2014). The known regulators of K17 to date are mainly cytokines produced by psoriatic T cells, leading to K17 overexpression. K17 is aberrantly overexpressed in psoriatic epidermis, driven by psoriasis-related cytokines IFN-γ (Bonnekoh et al., 1995), IL17 (Shi et al., 2011), and IL22 (Zhang et al., 2012), and its expression is positively associated with psoriasis severity. K17 shares the ALEEAN amino-acid sequence with streptococci M protein, an exogenous antigen of psoriasis (Gudmundsdottir et al., 1999). Our previous study found

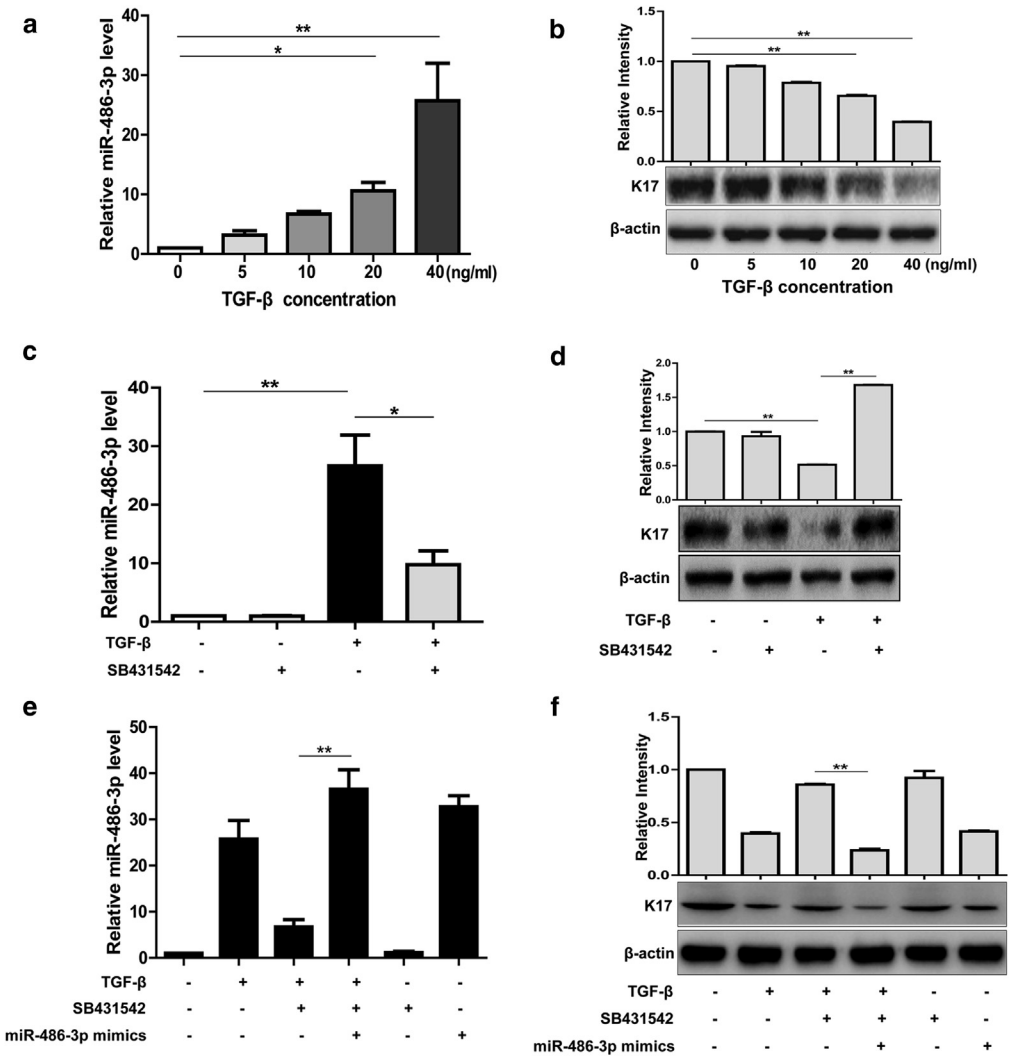

Figure 3. MiR-486-3p was involved in TGFβ-induced K17 downregulation. (**a**, **b**) HaCaT keratinocytes treated with TGFβ at concentrations of 5, 10, 20, and 40 ng/ml for 48 hours. (**a**) Real-time PCR analysis of miR-486-3p and (**b**) western blot analysis of K17. (**c, d**) HaCaT keratinocytes treated with 40 ng/ml TGFβ for 48 hours or pretreated with SB431542 followed by 40 ng/ml TGFβ. (**c**) Real-time PCR analysis of miR-486-3p and (**d**) western blot analysis of K17. (**e**, **f**) HaCaT keratinocytes transfected with miR-486-3p mimics or negative control, and then pretreated with SB431542 followed by the addition of 40 ng/ml TGFβ for 48 hours. (**e**) Real-time PCR analysis of miR-486-3p and (**f**) western blot analysis of K17. n = 3, $*P < 0.05$; $**P < 0.01$. HaCaT, normal human keratinocyte cell line of skin origin; K17, keratin 17; TGFβ, transforming growth factor-β.

several other restricted T-cell epitopes that can promote proliferation of psoriatic T cells and induce IFN-γ production (Shen et al., 2006). Together, this constitutes a K17/T-cell/cytokine autoimmune positive feedback loop (Bockelmann et al., 2005; Shen et al., 2006), which is critical in the maintenance, aggravation, and relapse of psoriasis.

Little is yet known about the regulatory mechanisms behind K17 overexpression, particularly in the initiation of psoriasis. In this study, we demonstrated the direct association between loss of miR-486-3p in psoriasis and overexpression of K17 protein. miR-486-3p is a highly conserved miRNA among mammals and widely expressed in different tissues and cell types. However, its role in skin diseases, in particular, psoriasis, had not previously been explored. Our study suggests an important role for miR-486-3p in skin homeostasis. miR-486-3p is located within the last intron of the Ank1 gene (Oh et al., 2011), which encodes an Ankyrin repeat domain protein that links the cytoskeleton to the plasma membrane (Gallagher et al., 2000). This miR has been shown to maintain muscle growth and homeostasis by targeting the phosphate and tension homology deleted on chromsome ten (PTEN)/protein kinase B (PKB/Akt) pathway and Foxo1 (Alexander et al., 2014; Small et al., 2010; Xu et al., 2012), to participate in hematopoiesis by regulating fetal hemoglobin in human erythroid cells (Lulli et al., 2013), and may function as a potent tumor suppressor in lung cancer (Peng et al., 2013). Although its roles are diverse, miR-486-3p tends to exert protective functions in tissues. Our data also support a protective function of miR-486-3p in skin homeostasis.

miRNAs begin as pri-miRNAs, which are processed within the nucleus to precursor miRNAs by an RNase III enzyme DROSHA. Precursor miRNAs are then transported to the cytoplasm and further processed to become mature miRNAs by another RNase III enzyme Dicer (Wahid et al., 2010). The transcription of pri-miR-486-3p can be regulated by myocardin-related transcription factor (Small et al., 2010) in muscle cells and GATA binding protein 1 in human leukemic cells (Shaham et al., 2015). However, little is known about the regulation mechanism in keratinocytes. In this study, we demonstrated that phospho-SMAD3, a transducer of the TGFβ pathway, directly interacted with pri-miR-486-3p and that there was an increase in DROSHA in complex with the pri-miR-486-3p after TGFβ treatment. This is consistent with studies by Davis et al. (2008, 2010), which identified that SMAD proteins can bind a set of miRNAs with a conserved RNA sequence to promote miRNA maturation.

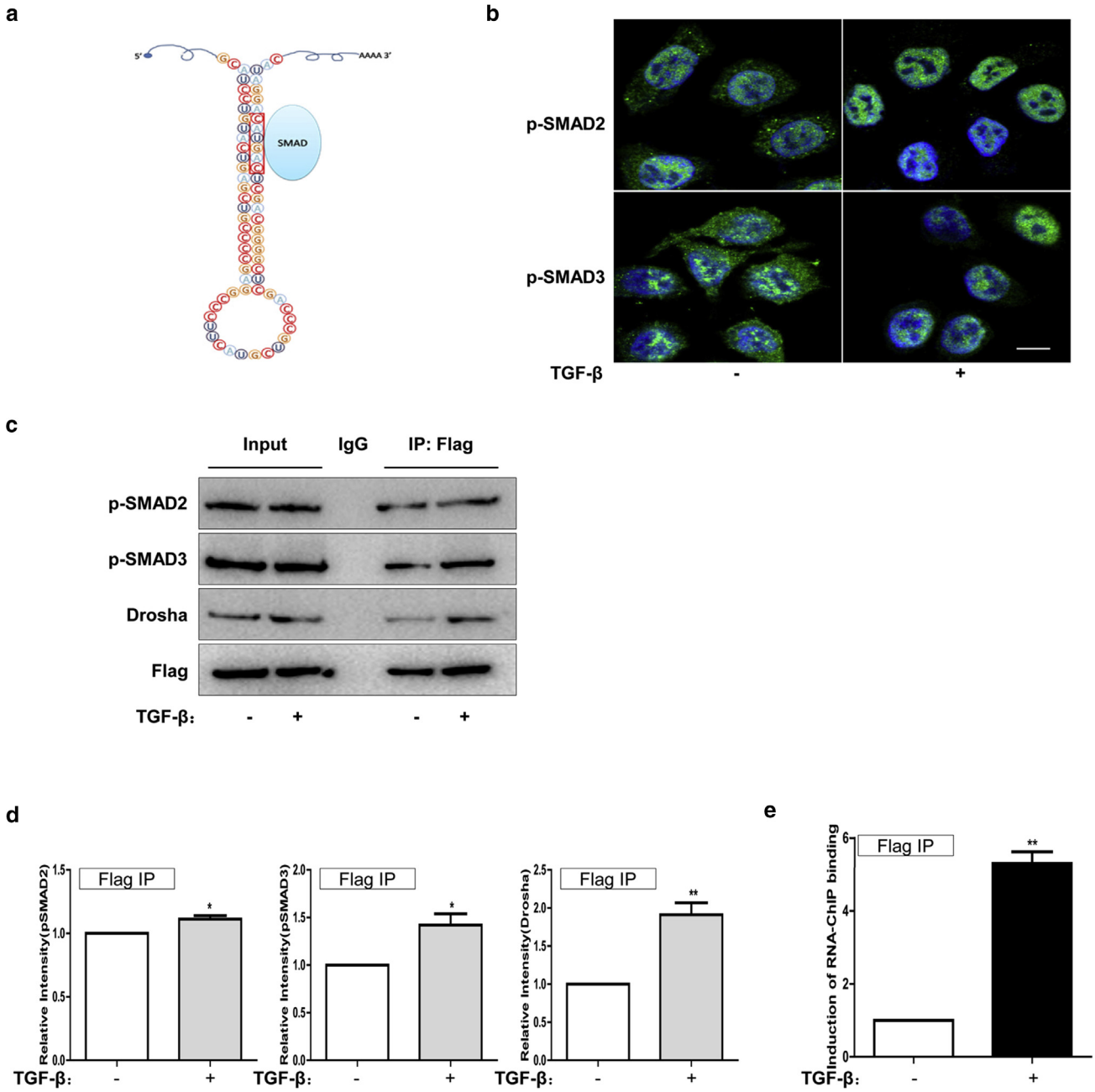

Figure 4. MiR-486-3p processing was enhanced by SMAD2/3 expression. (**a**) A potential SMAD binding motif in pri-miR-486-3p predicted by bioinformatics analysis at http://rna.tbi.univie.ac.at. (**b**) Representative immunofluorescence images of phosphor-SMAD2 or 3 in HaCaTs 30 minutes after TGFβ (40 ng/ml) or mock treatment. Nuclei were counterstained with DAPI. Bar = 10 μm. (**c**) HaCaTs were cotransfected with pCMV-miR-486-3p and pCMV5B-Flag-tagged SMAD3 and then treated with TGFβ for 2 hours. RNA-IP assay using an anti-Flag antibody was performed, followed by western blot of SMAD2, SMAD3, DROSHA, and Flag in input samples and immunoprecipitated complexes. **(d)** Relative intensity of SMAD2, SMAD3, and DROSHA in **(c)**. **(e)** PCR amplification of pri-miR-486-3p (normalized to β-actin) in the immunoprecipitated complexes of **(c)**. n = 3, $*P < 0.05$; $**P < 0.01$. HaCaT, normal human keratinocyte cell line of skin origin; TGFβ, transforming growth factor-β.

TGFβ negatively regulates keratinocyte proliferation. When TGFβ binds to a TGFβRI-TGFβRII complex, SMAD2 and SMAD3 are phosphorylated. Phosphorylated SMAD2 and SMAD3 form heteromeric complexes with SMAD4 and translocate into the nucleus to regulate TGFβ-responsive genes (Macias et al., 2015). Despite the increased expression of TGFβ in the skin and serum of psoriatic patients (Litvinov et al., 2011), psoriatic keratinocytes still stay hyperproliferative. We observed that psoriatic epidermis displayed decreased TGFβRI expression compared with healthy controls, which was consistent with the study of Doi et al. (2003). It is reported that TNF-α converting enzyme mediates TGFβRI cleavage (Huang and Chen, 2012) and is activated by extracellular signal-regulated kinase signaling (Liu et al., 2009). This reminds us of the activation of extracellular signal-regulated kinase by Th1/Th17 cytokines in psoriatic epidermis (Peric et al., 2008; Zhang et al., 2012), which may explain the TGFβRI decrease. We also

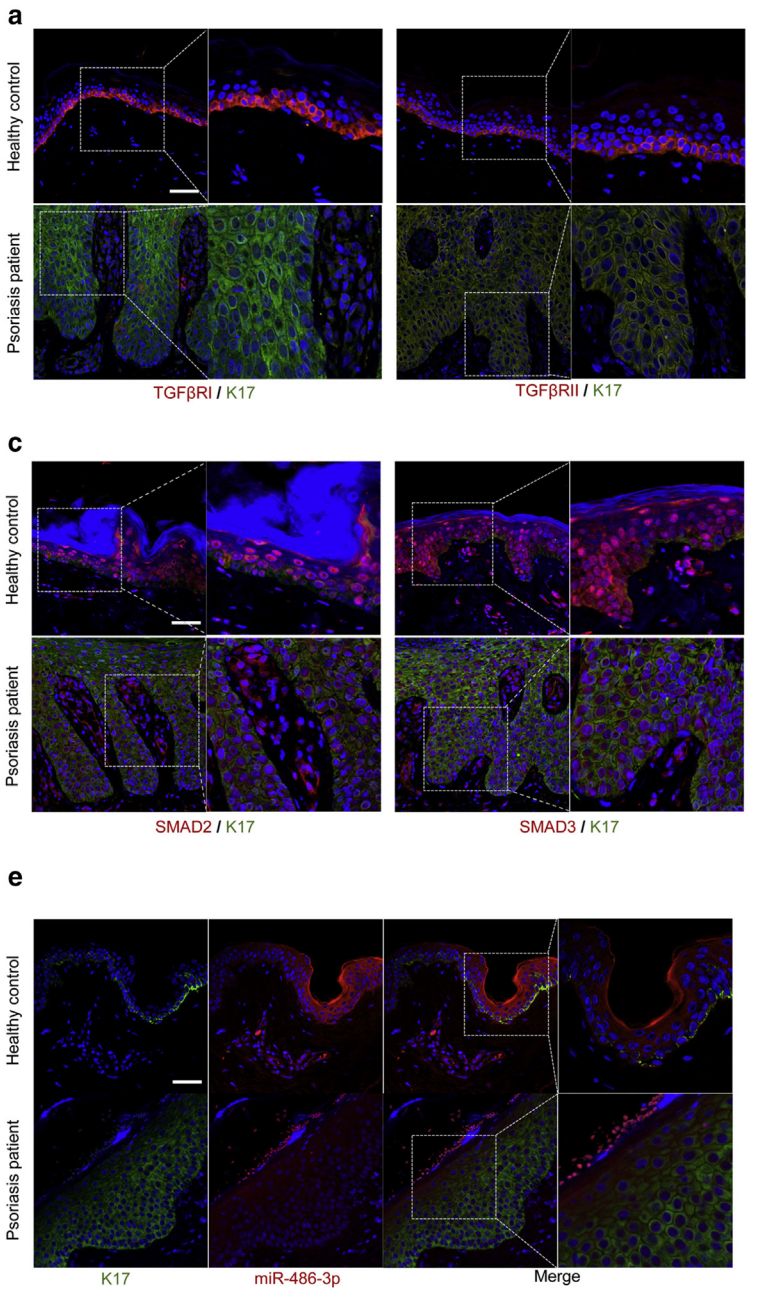

Figure 5. Inactivation of the TGFβ/SMAD pathway and overexpressed K17 in psoriatic epidermis. (**a**) Representative immunofluorescence images of TGFβRI/II and K17 in psoriatic epidermis and healthy controls. (**b**) Western blot analysis of TGFβRI/II in psoriatic epidermis and healthy controls. (**c**) Representative immunofluorescence images of SMAD2/3 and K17 in psoriatic epidermis and healthy controls. (**d**) Western blot analysis of SMAD2/3 and K17 in psoriatic epidermis and healthy controls. **(e)** Fluorescence in situ hybridization analysis of miR-486-3p expression in psoriatic epidermis and healthy controls. K17 were revealed by immunofluorescence with an antibody to K17. Nuclei were counterstained with DAPI. Bars = 50 μm. K17, keratin 17; TGFβ, transforming growth factor-β; TGFβRI/II, TGFβ receptors I/II.

observed lower phosphorylation of SMAD2/3 in psoriatic epidermis compared with healthy controls. Yu et al. (2009) showed that the mRNA levels of SMAD2, 4, and 6 were downregulated in lesional and nonlesional psoriatic skin, whereas our data found no significant change in the mRNA levels of SMAD2, 4, 6, and 7 in psoriatic epidermis compared with healthy controls. This discrepancy may be due to samples from psoriatic patients being from different stages and severities. However, our data showed cytoplasmic localization of SMAD2/3 in keratinocytes within psoriatic skin, suggesting aberrant TGFβ/SMAD signaling in psoriatic epidermis. We further verified the positive correlation between aberrant TGFβ/SMAD activity and the overexpression of K17 in psoriatic epidermis, which may drive cell hyperproliferation.

In conclusion, from our results we propose that aberrant TGFβ/SMAD signaling in psoriatic epidermis downregulates miR-486-3p, allowing overexpression of K17, driving keratinocyte proliferation, and thus contributing to the development of psoriasis. Activating miR-486-3p may therefore be a potential therapeutic approach for psoriasis.

MATERIALS AND METHODS

Patients

This study was approved by the local medical research ethics committee at Xijing Hospital, the Fourth Military Medical University, Xi'an, China. All study participants gave written informed consent. Psoriatic lesions and the adjacent nonlesional skin were collected from 25 patients diagnosed clinically and histologically with active chronic plaque psoriasis (age range: 24–35 years; psoriasis area and

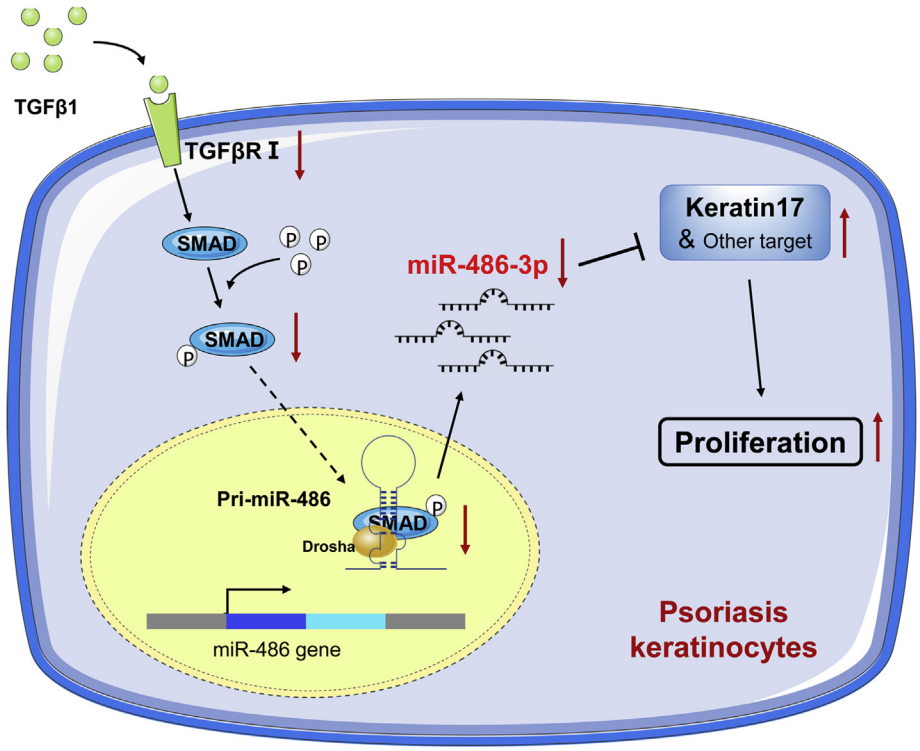

Figure 6. Proposed mechanism by which miR-486-3p modulates K17 overexpression in psoriatic keratinocytes. TGFβRI, which is significantly reduced in psoriatic epidermis, represses the nuclear translocation of SMAD2/3 and downregulates the expression of miR-486-3p. Loss of miR-486-3p allows expression of K17 in psoriatic keratinocytes. K17 overexpression may promote keratinocyte proliferation, contributing to the pathogenesis of psoriasis. K17, keratin 17; TGFβ, transforming growth factor-β; TGFβRI/II, TGFβ receptors I/II.

severity index > 10). These patients received no treatments for 1 month before biopsy. Normal skin harvested from 25 healthy participants (age range: 27−38 years) during cosmetic surgery served as controls.

For epidermis isolation, skin biopsies were incubated in 0.25% dispase (Invitrogen, Carlsbad, CA) at 4°C overnight. Then the epidermis was separated from dermis by forceps and minced for RNA or protein extraction.

Cell culture and stable cell line establishment

HaCaT keratinocytes were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Gibco-Invitrogen) in a humidified atmosphere containing 5% CO_2 at 37°C. To obtain the LV K17 stable cell line, 100 μl of pLenti6/V5-KRT17 plasmids were transfected into HaCaT keratinocytes (1×10^6) with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were then selected in medium containing 100 μg/ml Blasticidin S (Invitrogen) for 3 weeks to obtain a stable cell line. The expression level of K17 was analyzed to identify the transfection efficiency.

Normal human epidermal keratinocytes were isolated from adolescent foreskin from urinary surgery patients (age range: 6−15 years) and cultured in serum-free keratinocyte growth medium (Invitrogen). To mimic psoriasis-like keratinocytes, normal human epidermal keratinocytes were treated with Aldara (100 nM, InvivoGen) or cytokines (50 ng/ml TNF-α, 20 ng/ml IL17, and 20 ng/ml IL22; Peprotech, Rocky Hill, NJ).

Quantitative RT-PCR

Total RNAs were isolated using Trizol (Takara, Ohtsu, Japan) and subsequent chloroform/isopropanol/ethanol purification. A PrimeScript RT Master Mix Kit (Takara) or an All-in-one miRNA qRT-PCR Detection Kit (GeneCopoeia, San Diego, CA) was used to convert RNAs into cDNA. Quantitative RT-PCR was performed using SYBR Premix Ex Taq (Takara) on a Chromo4 continuous fluorescence detector, with a PTC-200 DNA Engine Cycler (Bio-Rad, Hercules, CA). *ACTB* (β-actin) was used as an internal control for pri-miRNA (TaqMan Pri-miRNA Assays Kit, ABI, Carlsbad, CA) and gene mRNA (Supplementary Table S1 online). RNU6-2 was used as an internal control for miRNA (All-in-one miRNA qRT-PCR Detection Kit, GeneCopoeia). The specific primers for each gene and cycling conditions are shown in Supplementary Table S1. Results were from three independent experiments.

Western blot analysis

Total proteins in cell lines or epidermis from psoriatic patients and healthy controls were prepared in radioimmunoprecipitation assay lysis buffer for western blot analysis following standard procedures. Anti-human K17, TGFβRI, TGFβRII, SMAD2, phosphor-SMAD2, SMAD3, phosphor-SMAD3, anti-β-actin (Abcam, Cambridge, UK), anti-Flag (Sigma, MO), and anti-DROSHA (Santa Cruz, CA) were used as primary antibodies. The appropriate horseradish peroxidase-conjugated secondary antibody (KPL, Gaithersburg, MD) was then incubated. Antibodies that bound peroxidase were detected by a chemiluminescence detection kit (KPL), documented (ChemiDoc MP imager, Bio-Rad), and quantitated (ImageLab software, Bio-Rad).

Immunohistochemistry

HaCaT keratinocytes mock treated or treated with TGFβ were first fixed in 4% polyoxymethylene and stained with anti-human phosphor-SMAD2 or phosphor-SMAD 3, followed by an appropriate FITC-conjugated secondary antibody (Abcam) and DAPI staining of nuclei.

Human skin specimens were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Tissues were sectioned (4 μm) and stained with anti-human K17 and one of anti-human TGFβRI, TGFβRII, SMAD2, and SMAD3 antibodies. An appropriate Cy3- or

FITC-conjugated secondary antibody (Abcam) and DAPI staining of nuclei were followed.

Samples were analyzed by confocal microscopy using an FV-1000/ES confocal microscope (Olympus, Tokyo, Japan) and images for comparison captured using the same exposure parameters.

Fluorescence in situ hybridization

Fluorescence in situ hybridization was performed on paraffin-embedded sections of skin biopsy specimens from patients with psoriasis and healthy individuals. Briefly, after dewaxing, sections were treated with proteinase K (5 μg ml) at 37°C for 10 minutes. Hybridization with Cy3-labeled hsa-miR-486-3p locked nucleic acid-modified probes (BersinBio, Guangzhou, China) was performed overnight at 42 °C. K17 was then costained with an anti-human K17 antibody followed by an appropriate FITC-conjugated secondary antibody and DAPI staining of nuclei. Samples were analyzed by confocal microscopy using an FV-1000/ES confocal microscope (Olympus).

Plasmid construction and 3′-UTR luciferase-binding assay

The human KRT17 3′-UTR (NM 022893) (from 252 to 1,858 bps) segments, containing the potential binding site for miR-486-3p, were amplified by PCR from genomic DNA and cloned into the PSICHECK-2 Control vector (Promega, Madison, WI, USA) between the NotI and XhoI site downstream the stop codon of the luciferase gene.

For the luciferase assay, cells were cotransfected with 0.8 mg of PSICHECK-2-3′-UTR plasmid, 0.1 mg of Renilla expressing vector, and 40 pmol of either a stability-enhanced nontargeting dsRNA control oligonucleotide (Ambion-ThermoFisher, Waltham, MA) or a stability-enhanced miRNA-486-3p (Ambion), all combined with Lipofectamine3000 (Invitrogen). After 48 hours, cells were washed and lysed according to the manufacturer's protocol (Promega) and luciferase activity was measured. Relative luciferase activity was obtained by normalizing the activity with the Renilla luciferase.

Cell proliferation assay

Cells were seeded in 96-well plates at a density of 10^3 cells/well in a total volume of 100 μl and then transfected with miR-486-3p mimic (200 pmol/μl each well) or negative control by Lipofectamine 3000 (Invitrogen). For the CCK-8 assay, the medium was replaced with serum-free medium on the indicated day and then CCK-8 (10 μl, Cell Counting kit-8, Beyotime Institute of Biotechnology, Haimen, China) was added to each well and incubated for 2 hours at 37°C. The optical density was measured at 450 nm in an SM-3 automatic enzyme-linked immune analyzer (TianShi, Beijing, China). For the Edu assay, the cells were fixed in 4% paraformaldehyde, and stained by Apollo567 and then Hoechst33342 (Cell-light Edu Kit, Ribobio, Guangzhou, China). Images were captured by confocal microscopy using an FV-1000/ES confocal microscope.

RNA-chromatin immunoprecipitation

HaCaT keratinocytes cotransfected with pCMV-miR-486-3p and pCMV5B-Flag-tagged SMAD3 were mock treated or treated with TGFβ for 24 hours. Cells were then collected and lysed in cold complete RIPA Lysis Buffer (Magna RIP Kit, Merke Millipore, MA). Lysates were centrifuged and supernatants were subjected to immunoprecipitation with an anti-Flag antibody, followed by stringent washing, and elution according to the manufacturer's instruction. RNA was isolated from the precipitates by Trizol (Invitrogen). Quantitative PCR reactions for pri-miRs were performed using the pri-miRNA assay kit (TaqMan Pri-miRNA Assays, Applied Biosystems, CA). Results shown were from three independent experiments.

Statistical analysis

All results represent the mean + standard deviation of at least three separate experiments, and each experiment was performed in triplicate. Statistical analyses were performed using Student's t test or one-way analysis of variance followed by Bonferroni corrections for post hoc comparisons. All P values < 0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.06.005.

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