NF-κB-induced microRNA-31 promotes epidermal hyperplasia by repressing protein phosphatase 6 in psoriasis

NF-κB is constitutively activated in psoriatic epidermis. However, how activated NF-κB promotes keratinocyte hyperproliferation in psoriasis is largely unknown. Here we report that the NF-κB activation triggered by inflammatory cytokines induces the transcription of microRNA (miRNA) miR-31, one of the most dynamic miRNAs identified in the skin of psoriatic patients and mouse models. The genetic deficiency of miR-31 in keratinocytes inhibits their hyperproliferation, decreases acanthosis and reduces the disease severity in psoriasis mouse models. Furthermore, protein phosphatase 6 (ppp6c), a negative regulator that restricts the G1 to S phase progression, is diminished in human psoriatic epidermis and is directly targeted by miR-31. The inhibition of ppp6c is functionally important for miR-31-mediated biological effects. Moreover, NF-κB activation inhibits ppp6c expression directly through the induction of miR-31, and enhances keratinocyte proliferation. Thus, our data identify NF-κB-induced miR-31 and its target, ppp6c, as critical factors for the hyperproliferation of epidermis in psoriasis.
Psoriasis is a complex, chronic inflammatory disease affecting the skin among 2–3% of the general population. Psoriasis is manifested as white silvery scales covered with erythematous plaques, and it is a lifelong disorder that severely reduces the quality of life of those affected. Psoriatic lesions are characterized by epidermal hyperplasia with loss of the superficial granular layer, thickening of the cornified envelope, aberrant differentiation of keratinocytes and a dramatic infiltration of the major inflammatory immune cells into the dermis or epidermis. It is now widely accepted that a dysregulated crosstalk between epidermal keratinocytes and immune cells leads to epidermal hyperplasia in psoriasis, and NF-κB may act as a link in this crosstalk.

NF-κB is sequestered by its inhibitor IκB in the cytoplasm of resting cells as a transcriptionally inactive form. Once dissociated from IκB, p65 undergoes phosphorylation, enters the nucleus and initiates transcriptional activity. In sharp contrast to the absence of phosphorylated p65 in the epidermis of normal skin, the epidermis of psoriatic plaques exhibits a high level of phosphorylated p65, closely correlating with the grade of epidermal hyperplasia. Moreover, the tumour necrosis factor-α (TNF-α)-targeting agent etanercept markedly inhibits p65 phosphorylation in the epidermal compartment, which is accompanied with an attenuation of epidermal thickness, restoration of keratinocyte differentiation molecular indicators and favourable clinical outcomes of psoriasis patients. These studies strongly suggest a critical role of epidermal NF-κB activation in the pathophysiology of the disease.

Several factors including A20 of the NF-κB signalling pathway are genetically linked to psoriasis as revealed by genome-wide association studies. Located in the cytoplasm, A20 is a zinc finger protein encoded by the A20 gene. A20 contains a death domain, a CUB (complement C1r/C1s, Kentlytin, and Ubiquitin) domain, a baculovirus IAP repeat (BIR) domain and a caspase recruitment domain (CARD). A20 is an important physiological regulator of inflammatory and immune responses and is known to modulate activation of the NF-κB pathway by targeting and inhibiting NF-κB-regulated genes. The main biological function of A20 is to deubiquitinate NF-κB activators, which is localized within keratinocytes and exerts regulatory effects on NF-κB-induced miR-31.

MicroRNAs (miRNAs) are single-stranded, noncoding short RNA molecules regulating gene expression by binding target(s) of complementary messenger RNAs (mRNAs) and inhibiting their expression via interruption of protein translation and mRNA degradation. Previous studies reported a distinct miRNA expression profile in psoriatic skin compared with healthy skin, and these deregulated miRNAs have been suggested to regulate keratinocyte proliferation and/or differentiation or suppress T-cell apoptosis in psoriasis. More recently, an interesting study showed that overexpressed miR-31 is present in psoriatic keratinocytes and contributes to psoriatic inflammation by modulating inflammatory mediator production and leukocyte infiltration to skin. Nevertheless, the physiological significance and the in vivo function of endogenous miR-31 in basal keratinocytes in the epidermal hyperplasia of psoriasis remain poorly understood.

Here we show that the inflammatory cytokines that activate NF-κB signalling in keratinocytes induce the NF-κB-dependent transcription of miR-31 in the epidermis of lesional skin derived from not only psoriatic mouse models but also patients with psoriasis. We demonstrate a previously unrecognized role of miR-31 in regulating the keratinocyte cell cycle by generating a knockout mouse model with a conditional deletion of miR-31 in epidermal basal keratinocytes. We have revealed that the miR-31 deletion in basal keratinocytes inhibits acanthosis and reduces the disease severity in two mouse models of psoriasis. Moreover, we show that protein phosphatase 6 (PPP6C), an inhibitor of the G1–S phase transition in the cell cycle, is diminished in epidermis derived from human psoriatic skin and is directly targeted by miR-31. PPP6C inhibition is functionally essential for the biological effects mediated by miR-31 in epidermal hyperplasia. Taken together, our results support the existence of a post-transcriptional mechanism through which NF-κB signalling modulates the hyperproliferation of basal keratinocytes and identify NF-κB-induced miR-31 and its target PPP6C as important factors in the development of chronic inflammatory skin disorder.

**Results**

**miR-31 expression is elevated in psoriatic skin.** To identify miRNAs that are preferentially expressed in inflamed keratinocytes, we carried out comparative miRNA screening of normal ear skin from control mice and of lesional ear skin from CD18 hypomorphic (CD18<sup>−/−</sup>) mice, which spontaneously develop a T-cell-mediated psoriasiform skin disease. Of the 610 miRNAs analysed, we observed that miR-31 was the miRNA that was most highly expressed in affected skin (4.3-fold increase) (Fig. 1a,b). The increased expression of miR-31 in diseased skin promoted us to assess its in vivo functionality in an imiquimod (IMQ)-induced psoriasis mouse model that closely resembles the human disease phenotype. To perform this assessment, we painted Aldara, a cream containing IMQ on the back of shaved mice for 7 consecutive days. In agreement with other study, both C57BL/6 and BALB/c mice treated with IMQ developed sharply demarcated erythematous lesions covered with white silvery squama (Supplementary Fig. 1a,b). Hyperplasia of the epidermis with regular elongation of rete ridges, hyperkeratosis, increased proliferative basal layer epidermal keratinocytes, dilated capillaries, microabscesses and massive dermal cellular infiltrates were histologically evident in the IMQ-treated mouse skin (Supplementary Fig. 1c). Notably, by quantitative real-time PCR (qPCR), we determined that miR-31 was significantly increased in the lesional skin from IMQ-treated mice compared with the healthy skin (1 ± 0.2276 versus 3.877 ± 0.4426, n = 13, P < 0.0001, two-tailed Student’s t-test) (Fig. 1c), and the upregulated miR-31 was mainly confined to epidermis (Fig. 1d). To confirm this observation, we performed in situ hybridization on skin cryosections from the IMQ-treated mice using miR-31-specific locked nucleic acid-modified (LNA) probes. We found that miR-31 expression was restricted to the basal and suprabasal cell layers of the epidermis in the lesional skin of the IMQ-treated mice (Fig. 1e). Consistent with a published report, we showed that miR-31 expression was significantly upregulated in lesional skin from patients with psoriasis (Fig. 1f). Taken together, these data demonstrate that the expression of miR-31 is abundantly increased in the affected skin of psoriasis patients and mouse model, and support the notion that overexpressed miR-31 in hyperproliferating keratinocytes may be functionally involved in the pathogenesis of psoriasis.

**NF-κB activation induces miR-31 expression.** The expression of Th17 cytokines such as interleukin-17 (IL-17) and IL-22 is elevated in psoriatic skin, and anti-IL-17 agents are clinically effective in the treatment of human psoriasis. We sought to investigate whether the upregulation of Th17 cytokines coincides with the induction of miR-31 in skin inflammation. We compared the miR-31 induction in lesional skin derived from IL-17A-deficient (IL-17A<sup>−/−</sup>) and wild-type (IL-17A<sup>+/+</sup>) mice treated with IMQ. Clearly, miR-31 expression was significantly increased in the lesional skin from IMQ-treated mice compared with the healthy skin (1 ± 0.2276 versus 3.877 ± 0.4426, n = 13, P < 0.0001, two-tailed Student’s t-test) (Fig. 1c), and the upregulated miR-31 was mainly confined to epidermis (Fig. 1d). To confirm this observation, we performed in situ hybridization on skin cryosections from the IMQ-treated mice using miR-31-specific locked nucleic acid-modified (LNA) probes. We found that miR-31 expression was restricted to the basal and suprabasal cell layers of the epidermis in the lesional skin of the IMQ-treated mice (Fig. 1e). Consistent with a published report, we showed that miR-31 expression was significantly upregulated in lesional skin from patients with psoriasis (Fig. 1f). Taken together, these data demonstrate that the expression of miR-31 is abundantly increased in the affected skin of psoriasis patients and mouse model, and support the notion that overexpressed miR-31 in hyperproliferating keratinocytes may be functionally involved in the pathogenesis of psoriasis.
decreased in the psoriatic lesions in the IL-17A−/− mice compared with the IL-17A+/+ mice after the IMQ application (Fig. 2a). To examine the possible involvement of key inflammatory cytokines in the induction of miR-31, we stimulated primary normal human epidermal keratinocytes (NHEK) with IL-1α, IL-6, IL-17A, IL-22, interferon-γ (IFN-γ) and TNF-α. Almost all of the tested inflammatory cytokines were able to stimulate miR-31 expression to different extents; however, IL-6 appeared to be an inducer of miR-31 at low concentrations in NHEK (Fig. 2b). Thus, we chose IL-6 as a stimulus for miR-31 induction. Interestingly, we further demonstrated that IL-17A deletion led to a pronounced decrease of IL-6 expression in the epidermis of the mouse lesional skin induced by IMQ (Supplementary Fig. 2a), suggesting that IL-17A is a proinflammatory cytokine that modulates IL-6 production in an IMQ-induced mouse model. Importantly, we observed that IL-6 expression was closely correlated with miR-31 expression in human psoriatic lesions (Fig. 2c and Supplementary Table 1). Although activation of the STAT3 transcription factor is a classical downstream event of IL-6 stimulation, we found that there was no significant difference of the miR-31 levels in the HaCaT keratinocytes stimulated with IL-6 in the absence and presence of a STAT3 inhibitor (Supplementary Fig. 2b), indicating that the miR-31 expression induced by IL-6 may not be mediated by STAT3 signalling. NF-κB activation plays an essential role in regulating miRNAs27. In the intestinal epithelia,
Figure 2 | Requirement of NF-κB signaling for the induction of miR-31 in epidermal keratinocytes. (a) miR-31 expression in the epidermis of skin samples derived from either IL-17A+/- or IL-17A-/- mice without treatment or after treatment with IMQ. (b) miR-31 expression in NHEK stimulated with 0 ng ml⁻¹, 10 ng ml⁻¹, 20 ng ml⁻¹ and 50 ng ml⁻¹ of IL-1a, IL-6, IL-17, IL-22, IFN-γ and TNF-α for 24 h. (c) Correlation between IL-6 and miR-31 expression in 29 psoriatic skin samples. (d) The NF-κB-driven luciferase activity was monitored at day 0 and day 7 after the application of IMQ in NF-κB reporter mice. (e) NHEK were stimulated with IL-6 for 0 and 1 h. The p65 nuclear translocation was imaged at 0 and 1 h. Scale bar, 50 μm. (f) The schematic diagram showed one potential binding sites of p65 in the putative promoter element of human miR-31. (g) Phosphorylated p65 was immunoprecipitated from NHEK stimulated with IL-6, Immunoprecipitates were assayed for the expression levels of miR-31 promoter. (h) NHEK were transfected with scramble siRNA (Ctr) or p65 siRNA (siRNA p65). Cell lysates were immunoblotted with anti-p65 or anti-actin. Values were expressed as fold change relative to controls and normalized to β-actin. (i) miR-31 expression in NHEK transfected with scramble siRNA (Ctr) or p65 siRNA (siRNA p65), and stimulated with IL-6 for 24 h. (j) Luciferase activity in lysates of HaCaT keratinocytes transfected with luciferase reporter plasmids of pGL3-basic empty vector (basic), miR-31 promoter (p31-wt) or miR-31 promoter with mutation on the predicted NF-κB binding site (p31-mut), unstimulated or stimulated with indicated cytokines. Results are presented as the ratio of firefly luciferase to renilla luciferase activity, relative to that of unstimulated HaCaT keratinocytes transfected with pGL3-basic empty vector. Black or grey dotted line indicates the mean of relative luciferase activity in unstimulated HaCaT keratinocytes. Results (a,b,i) are presented as the ratio of miRNA to the small nuclear RNA U6, relative to that in untreated IL-17A+/- mice (a) or relative to that in non-stimulated keratinocytes (b) or relative to that in siRNA control-treated keratinocytes (i). *P<0.05, **P<0.01, ***P<0.001, NS, not significant, two-tailed Student’s t-test. Data are representative of at least two independent experiments with four to five samples per group in each (mean and s.e.m.).
IL-6 has also been reported to induce NF-κB activation. We sought to investigate whether IL-6 could trigger the expression of miR-31 mediated by NF-κB signalling in keratinocytes. We applied IMQ to the back skin of NF-κB luciferase reporter mice thus enabling in vivo real-time imaging of NF-κB activity. Indeed, NF-κB-driven luciferase activity was markedly increased in the exposed areas applied with IMQ (Fig. 2d), suggesting that the NF-κB signalling pathway is activated in affected skin in the IMQ-induced mouse model. Flow cytometry analysis confirmed that the IMQ-induced NF-κB activation was in keratinocytes rather than CD45+ leucocytes (Supplementary Fig. 3c). The magnitude of this increase is similar to what we observed in psoriatic lesions. (Supplementary Fig. 2c). Moreover, the nuclear translocation of the p65 NF-κB subunit was observed in NHEK stimulated with IL-6 for 1 h (Fig. 2e). A database analysis identified one potential p65-binding site in the promoter element at −130 upstream from the transcription start site of human miR-31 (Fig. 2f). To verify the binding of the p65 component of NF-κB to the putative binding site in the promoter element of miR-31, we performed chromatin immunoprecipitation (CHIP) assays. These assays showed that IL-6 stimulation resulted in the recruitment of p65 to the putative miR-31 promoter (Fig. 2g). Using a specific short interfering RNA (siRNA), we further silenced p65 in NHEK (Fig. 2h). In contrast to the controls, the knockdown of p65 led to a significant decrease in miR-31 expression in keratinocytes stimulated with IL-6 (Fig. 2i). To further confirm whether IL-1α, IL-6, IL-17A, TNF-α, IFN-γ and IL-22 are able to induce the expression of miR-31 through NF-κB signalling, we used luciferase reporter constructs driven by miR-31-specific promoter response in HaCaT keratinocytes. We found that IL-1α, IL-6, IL-17A, TNF-α, IFN-γ and IL-22 stimulation increased p65 binding to the putative binding site at −130 of the putative promoter of miR-31, and mutation of the binding site at −130 blocked the luciferase activity induced by these cytokines (Fig. 2j). Thus, we suggest that IL-1α, IL-6, IL-17A, TNF-α, IFN-γ or IL-22 directly or indirectly activates the NF-κB signalling pathway mediating miR-31 expression in keratinocytes.

miR-31 conditional deletion reduces the disease severity. To further study the potential role of miR-31 in the aetiology and pathogenesis of psoriasis, we created miR-31 transgenic mice (miR-31TG) using a viral vector carrying enhanced green fluorescent protein (EGFP) and miR-31 under the control of the cytomegalovirus (CMV) promoter (Supplementary Fig. 3a,b). Compared with the wild-type (WT) littermates, the miR-31TG mice increased miR-31 by 4.0-fold (±0.4895 versus 4.078 ± 1.082, n = 4–5, p = 0.0498, two-tailed Student’s t-test) (Supplementary Fig. 3c). The magnitude of this increase is similar to what we observed in psoriatic lesions.

We next investigated the role of miR-31 in the development of IMQ-induced psoriasiform skin disease, and found that the miR-31TG mice displayed a more severe form of the disease than the control mice (Supplementary Fig. 4a–d). Moreover, we detected that NHEK exhibited an enhanced proliferation after overexpressing miR-31 (Supplementary Fig. 5). To further investigate the functional relevance of miR-31 with psoriasis, we induced mouse model. For at least 32 weeks. We then applied IMQ to both the miR-31 control (miR-31fl/fl) and cKO mice, and found that miR-31 deficiency led to a pronounced decrease in plaque formation (Fig. 3e). The splenomegaly and lymphadenopathy are primarily caused by a large expansion of inflammatory cells. We observed that the conditional knockout of miR-31 in basal keratinocytes dramatically decreased splenomegaly and lymphadenopathy in the IMQ-treated mice (Fig. 3f). Strikingly, we demonstrated that the miR-31-specific ablation in the epidermis resulted in a pronounced decrease in skin thickness in the cKO mice treated with IMQ (Fig. 3g). Moreover, histological analysis of the inflammation revealed a marked decrease in epidermal hyperplasia (acanthosis) and dermal cell infiltration in the cKO mice compared with the miR-31fl/fl controls (Fig. 3h–j). Notably, we detected that the expression of Ki67, a marker strictly associated with cell proliferation, was significantly decreased in the cKO mice when compared with the controls, indicating that the excessive proliferation of basal keratinocytes induced by IMQ was reduced in the absence of miR-31 (Fig. 3k). Furthermore, we observed that the miR-31 deletion in the mice treated with IMQ significantly restored the expression of the terminal differentiation markers Keratin 10, Loricrin and Filaggrin close to that of the untreated miR-31fl/fl mice (Fig. 3m). Consistently, we found that miR-31 deletion in the epidermis led to an obvious decrease in plaque formation accompanied with a significant decrease in ear thickness and acanthosis in an IL-23-mediated psoriasis mouse model (Supplementary Fig. 6a–d). However, in addition to IL-1α there was no significant difference of the inflammatory genes between the miR-31fl/fl and cKO mice in both the IMQ-induced and IL-23-mediated mouse models of psoriasis (Supplementary Fig. 7), suggesting a unique role of miR-31 in epidermal hyperplasia. In addition, miR-31 expression in epidermis even decreased in the cKO mice treated with IMQ compared with the untreated cKO controls (Supplementary Fig. 8a), indicating that infiltrated leucocytes did not contribute to the elevated levels of miR-31 in the epidermis. Moreover, the
deletion of miR-31 in keratinocytes did not decrease miR-31 expression in splenocytes in the cKO mice treated with IMQ (Supplementary Fig. 8b). Together, these data imply that intrinsic miR-31 plays a pivotal role in keratinocyte proliferation and differentiation, and in epidermal hyperplasia.

miR-31 directly targets Ppp6c. To identify putative target miRNAs of miR-31, four bioinformatics tools, TargetScan, miRDB, miRWALK and RNA22, were used to predict the potential targets of miR-31 (Supplementary Fig. 9a), and 20 potential target genes were identified (Supplementary Fig. 9b). We compared the
mRNA expression profile of the predicted targets in the WT and miR-31fl/fl mice. Only one predicted target of miR-31, ppp6c, was significantly downregulated at the mRNA levels both after the application of IMQ and on the overexpression of miR-31 (Supplementary Fig. 9c). Consistent with our qPCR results, western blot analysis further demonstrated that the ppp6c expression was increased by ∼3.7-fold in the epidermis of the cKO mice treated with IMQ compared with that of miR-31fl/fl controls. A modest expression of ppp6c in the epidermis of the miR-31fl/fl mice was observed in the absence of IMQ treatment; however, no apparent difference of ppp6c expression was found between the IMQ-treated and untreated cKO mice (Fig. 4a,b). In an IL-23-mediated mouse model of psoriasis, the ppp6c expression was increased more than ∼8.0-fold at the mRNA levels and 12.0-fold at the protein levels in the ear epidermis of the cKO mice injected with IL-23 compared with that of the miR-31fl/fl controls (Supplementary Fig. 10a,b). These data indicate that ppp6c is a potential miR-31 target. In mammalian cells, the majority of mature miRNAs are loaded into Argonaute 2 (Ago2)-associated complexes through which mRNA silencing is conducted29. To confirm whether ppp6c is a direct target of miR-31, Ago2 immunoprecipitates of the epidermis derived from

![Figure 4](image-url)

**Figure 4 | miR-31 directly targets ppp6c.** (a,b) qPCR and western blot analysis of ppp6c expression in the epidermis of cKO and miR-31fl/fl mice treated with IMQ or vehicle. Results (a) are presented as the ratio of miRNA to the small nuclear RNA U6, relative to that in vehicle-treated miR-31fl/fl controls. Values (b) were expressed as fold changes relative to controls and normalized to β-actin. One representative blot out of four independent experiments is shown. (c,d) Ago2 was immunoprecipitated from epidermis lysates derived from untreated controls or mice treated with IMQ. Immunoprecipitates were assayed for ppp6c and miR-31. (e,f) Ago2 was immunoprecipitated from epidermis lysates derived from miR-31fl/fl or cKO mice treated with IMQ. Immunoprecipitates were assayed for ppp6c and miR-31. (g) U1 positive control was tested in Ago2 immunoprecipitates from normal epidermis lysates. (h) WT and point-mutated 3' UTR reporter constructs. TS, target site. (i) Luciferase activity was determined in NIH3T3 cells that were transfected with miR-31 mimics and the indicated 3' UTR reporter construct or with the indicated WT or point-mutated 3' UTR reporter construct (WT UTR or mutant UTR). Results (c–g) are presented as the ratio of mRNA or miRNA to the β-actin or the small nuclear RNA U6, relative to that in controls. *P < 0.05, **P < 0.01, ***P < 0.001, NS, not significant, two-tailed Student’s t-test. Data are representative of at least two independent experiments with four to six samples per group in each (mean and s.e.m.).
untreated and IMQ-treated mice or from miR-31fl/fl and cKO mice treated with IMQ were assayed for ppp6c and miR-31. The levels of ppp6c and miR-31 detected in the psoriatic epidermal immunoprecipitates were 11.2- and 49.1-fold greater, respectively, than those in untreated controls (Fig. 4c,d). Furthermore, we determined that ppp6c and miR-31 expression in the miR-31fl/fl epidermal immunoprecipitates were 5.0- and 9.1-fold greater, respectively, than those in the cKO mice (Fig. 4e,f). Correspondingly, as a technique control we found that a 232.2-fold increase in U1 expression in the Ago2 immunoprecipitates compared with the immunoglobulin-G (IgG) control (Fig. 4g). Unlike ppp6c there was essentially no difference of the expression levels for a non-related miRNA (IgG) control (Fig. 4g). Unlike ppp6c there was essentially no difference of the expression levels for a non-related miRNA (IgG) control (Fig. 4g). Unlike ppp6c there was essentially no difference of the expression levels for a non-related miRNA (IgG) control (Fig. 4g).

9.1-fold greater, respectively, than those in the cKO mice (Fig. 4c,d). In contrast to a control construct lacking the target sequence, miR-31 overexpression led to significantly decreased luciferase activity derived from the construct expressing the target sequence, and a 88.12% reduction of the target ppp6c mRNA was achieved (Fig. 4i). Thus, our results demonstrate that miR-31 and its target gene ppp6c are specifically associated with Ago2-containing complexes. We next generated a reporter construct that includes the 3′ untranslated region (UTR) of the ppp6c mRNA (Fig. 4h). In contrast to a control construct lacking the target sequence, miR-31 overexpression led to significantly decreased luciferase activity derived from the construct expressing the target sequence, and a 88.12% reduction of the target ppp6c mRNA was achieved (Fig. 4i). Thus, our results demonstrate that miR-31 and its target gene ppp6c are specifically associated with Ago2-containing complexes. We next generated a reporter construct that includes the 3′ untranslated region (UTR) of the ppp6c mRNA (Fig. 4h). In contrast to a control construct lacking the target sequence, miR-31 overexpression led to significantly decreased luciferase activity derived from the construct expressing the target sequence, and a 88.12% reduction of the target ppp6c mRNA was achieved (Fig. 4i). Thus, our results demonstrate that miR-31 and its target gene ppp6c are specifically associated with Ago2-containing complexes. We next generated a reporter construct that includes the 3′ untranslated region (UTR) of the ppp6c mRNA (Fig. 4h). In contrast to a control construct lacking the target sequence, miR-31 overexpression led to significantly decreased luciferase activity derived from the construct expressing the target sequence, and a 88.12% reduction of the target ppp6c mRNA was achieved (Fig. 4i). Thus, our results demonstrate that miR-31 and its target gene ppp6c are specifically associated with Ago2-containing complexes. We next generated a reporter construct that includes the 3′ untranslated region (UTR) of the ppp6c mRNA (Fig. 4h). In contrast to a control construct lacking the target sequence, miR-31 overexpression led to significantly decreased luciferase activity derived from the construct expressing the target sequence, and a 88.12% reduction of the target ppp6c mRNA was achieved (Fig. 4i). Thus, our results demonstrate that miR-31 and its target gene ppp6c are specifically associated with Ago2-containing complexes.

Ppp6c inhibition is required for miR-31-mediated effects. We next investigated the ppp6c expression in the epidermis derived from lesional skin of human patients with psoriasis. Of note, we found that the levels of ppp6c protein were significantly decreased in the epidermis of psoriatic lesions compared with the healthy controls (Fig. 5a,b). In contrast to normal skin, a diminished expression of ppp6c was revealed in the psoriatic lesions by immunohistochemistry staining (Fig. 5c). To explore the biological function of ppp6c, we depleted ppp6c in keratinocytes with specific siRNA (Fig. 5d). Strikingly, in contrast to the controls, the dampening of ppp6c expression in keratinocytes resulted in an increased percentage of cells in S phase (Fig. 5e). However, silencing ppp6c did not alter the miR-31 expression in NHEK (Supplementary Fig. 11c). To silence ppp6c in vivo, we generated a lentivirus that expressed green fluorescent protein and ppp6c short hairpin RNA (shRNA) driven by the CMV immediate early promoter and the RNA Pol III-dependent, human U6 promoter (U6), respectively. Lentiviral shRNA-ppp6c downregulated ppp6c protein in epidermis in vivo (Fig. 5f). Moreover, ppp6c silencing led to an increase in the epidermis thickness and an enhanced proliferation of keratinocytes as reflected by the elevated levels of Ki67 (Fig. 5g–i). These data suggest that ppp6c is possibly functionally important in the regulation of epidermal hyperplasia in psoriasis.

Anti-miR-31 administration decreases epidermal hyperplasia. Antagomirs are modified antisense oligonucleotides, and exhibit superior miRNA-inhibiting properties when applied in mice. We administered antagomirs to block the miR-31 seed sequence (anti-miR-31) and to test the inhibitory effects of anti-miR-31 on the disease development in the IMQ-induced psoriasis mouse model. As expected, there was a pronounced decrease in both acanthosis and dermal cellular infiltration after the anti-miR-31 treatment (Fig. 7a–c). Correspondingly, we found enhanced ppp6c mRNA and protein levels in the epidermis after the administration of anti-miR-31 (Fig. 7d,e). The increased ppp6c expression after the anti-miR-31 treatment in epidermis was further confirmed by immunohistochemistry analysis (Fig. 7f). Moreover, the anti-miR-31 administration markedly reduced the keratinocyte hyperproliferation as indicated by the Ki67 levels (Fig. 7g). Thus, our data again indicate that miR-31 and its target ppp6c are critical factors in epidermal hyperplasia in psoriasis.

Discussion

Increased NF-κB activation was demonstrated in lesional psoriatic skin compared with non-lesional psoriatic skin. Major advances highlighting important roles for NF-κB in the aetiology of psoriasis have been achieved. In contrast, very little is known about the intrinsic factor(s) induced by NF-κB activation in keratinocytes in the promotion of epidermal hyperplasia. In this study, we demonstrate that inflammatory cytokines activate NF-κB signalling and induce miR-31, which represses ppp6c, a negative regulator of the cell cycle, thereby contributing to basal keratinocyte proliferation and epidermal hyperplasia. Our data provide the mechanistic evidence for miRNA-mediated regulation involved in the hyperproliferative keratinocytes in psoriasis. These findings also suggest potential therapeutic targets for psoriasis treatment. Although the initial events triggering a psoriatic lesion are still not defined, a complex interplay between dysfunctional keratinocytes and abnormal activation of the innate and the adaptive immune system may drive epidermal hyperproliferation and aberrant keratinocyte differentiation in psoriasis. Epidermal keratinocytes are responsive to dendritic cell-derived and
T-cell-derived cytokines such as IFNs, TNF, IL-6, IL-17 and the IL-20 family of cytokines and vice versa, they are able to release proinflammatory cytokines and chemokines to sustain or even amplify the chronic inflammatory disease loop in lesional skin in psoriasis. Using the ChIP assay, the siRNA-mediated knockdown of NF-κB subunit p65 or luciferase reporter constructs driven by the specific promoter, we demonstrate that inflammatory cytokines are capable of triggering miR-31 transcription directly or indirectly through NF-κB signalling, which plays an essential role both in cell cycle regulation and inflammatory response, and critically connects keratinocytes with lymphocytes in the pathogenesis of psoriasis. Thus, the overexpression of miR-31 in psoriatic keratinocytes, likely as a consequence of the production of excess inflammatory cytokines in both skin lesion and plasma of patients, may therefore contribute to the epidermal hyperplasia that occurs in psoriasis. In our study NF-κB acts as an upstream enhancer of miR-31 in keratinocytes, while another report shows that miR-31 targets Ppp6c.
serine/threonine-protein kinase 40 (STK40), a negative regulator of the NF-κB signalling pathway. These findings suggest different target genes of miR-31 in fact indicate that the miR-31-mediated positive feedback loop may amplify NF-κB activity to pathological levels in epidermal hyperplasia.

miR-31 is universally expressed in a variety of tissues, and has been shown to negatively regulate lymphatic vascular lineage-specific differentiation, to sensitize breast cells to apoptosis by targeting protein kinase c-ε, to enhance vascular smooth muscle cell proliferation via inhibiting its target gene, the large tumor suppressor homologue 2 (ref. 36), to regulate keratinocyte differentiation with a targeting effect on factor-inhibiting hypoxia-inducible factor 1 (ref. 37) and to control hair cycle-associated genes in mouse skin. To our knowledge, no prior studies have addressed the possible direct intrinsic role of miR-31 in keratinocyte proliferation or differentiation and in the pathogenesis of psoriasis. Here we demonstrate that the conditional knockout of miR-31 leads to decreased keratinocyte hyperproliferation mediated by NF-κB signalling, prevents Ki67 expression, inhibits anacrotion and reduces the disease severity in

![Figure 6](image.png)

Figure 6 | NF-κB signaling inhibits ppp6c expression by inducing miR-31. (a) Undifferentiated NHEK proliferation induced by various concentrations of IL-6 for 24 h, and analysed by BrdU incorporation assay. (b) NHEK were transfected with scramble siRNA (Ctr) or with p65 siRNA (p65 siRNA). Cell cycle analysis of NHEK or transfected NHEK treated without or with IL-6 for 24 h. (c) In vitro wound healing rate of NHEK treated without or with IL-6 for 16 h. (d,e) Three-dimensional organotypic culture of HaCaT keratinocytes treated without or with IL-6. Scale bar, 100 μm. (f) 1 μg recombinant mouse IL-6 (in 25 μl PBS) or PBS was injected i.d. in ears of C57BL/6J mice. Ear sections were prepared for Ki67 staining 3 days after IL-6 administration. Scale bar, 100 μm. (g) NHEK were transfected with scramble siRNA (Ctr) or with p65 siRNA (p65 siRNA). Western blotting of ppp6c expression in NHEK with or without IL-6 treatment for 24 h. (h) Cell cycle analysis of primary mouse keratinocytes derived from miR-31fl/fl or cKO mice in absence or presence of IL-6. Values were expressed as fold changes relative to non-stimulated HaCaT keratinocytes (e) or to non-stimulated NHEK (g) and normalized to β-actin. IL-6 was used at the concentration of 50 ng ml⁻¹ (b-e,g,h). **P<0.01, ***P<0.001, two-tailed Student’s t-test. Data are representative of at least two independent experiments.
two psoriasis mouse models. Our data identify miR-31 as a downstream target of NF-κB and highlight the critical role of NF-κB-mediated post-transcriptional regulation for epidermal hyperplasia in psoriasis. The critical role of miR-31 in hyperproliferative keratinocytes is further indicated by the observation that repeated intradermal (i.d.) injection of anti-miR-31 in the IMQ-induced mouse model results in a significant improvement of the psoriasiform phenotype.

Although miR-31 was reported to modulate inflammatory cytokine and chemokine expression in keratinocytes by suppressing STK40 (ref. 21), the in vivo function of miR-31 and the underlying mechanism by which it regulates cell proliferation and differentiation in psoriasis has been poorly explored. Here we identify that miR-31 induced by NF-κB activation directly targets pp6c to promote keratinocyte hyperproliferation. We present several lines of evidence to support that pp6c is one of the primary targets for miR-31 in keratinocytes. First, four separate bioinformatics tools predict that miR-31 targets a sequence in the 3′ UTR of pp6c mRNA. Second, pp6c expression is significantly decreased in diseased epidermis of miR-31 flo/flo control mice but not in the epidermis of cKO mice treated with IMQ or IL-23. Third, pp6c and miR-31 expression in Ago2 immunoprecipitates of lesional epidermis derived from miR-31 flo/flo animals is markedly enriched compared with that in immunoprecipitates from cKO mice, suggesting that pp6c and miR-31 associate within Ago2, the effector element of the miRNA-induced silencing complex, which directly binds to miRNAs and subsequently mediates mRNA repression39. Fourth, miR-31 overexpression in NIH3T3 cells results in significantly decreased luciferase activity after the transfection of the cells with the construct expressing the target sequence in the 3′ UTR of pp6c. Fifth, the administration of anti-miR-31 blocks miR-31 function, and enhances the pp6c mRNA and protein levels in vivo.

A marked increase in the percentage of normally quiescent basal keratinocytes during cell cycle progression is one of the essential characteristics of epidermal hyperproliferation in psoriasis40. However, post-transcriptional evidence by which NF-κB regulates the aberrant cell cycle of epidermal basal

Figure 7 | Administration of antimir-31 decreases epidermal hyperplasia and dermal cellular infiltration induced by IMQ. Mice were injected subcutaneously with an irrelevant antimir (NC) or an antimir to miR-31 (anti-miR-31). The first injection was administered 3 days before the application of IMQ and thereafter was performed every other day until the end of the experiment. (a) H&E staining of the back skin derived from mice injected with NC (upper panel) or anti-miR-31 (lower panel). Scale bar, 100 μm. (b, c) Acanthosis and dermal cellular infiltrates were quantitated for mice treated with NC or anti-miR-31. (d, e) Pp6c mRNA and protein levels in NC- or anti-miR-31-treated mice. (f, g) Immunohistochemical staining of pp6c or Ki67 in skin sections derived from NC- or anti-miR-31-treated mice after induction of skin phenotype by IMQ (n = 8–9). Scale bar, 50 μm (f) or 100 μm (g). For all measurements (c), the median number of specifically stained dermal nucleated cells was counted in three high-power fields per section. Results (d) are presented as the ratio of mRNA to the β-actin, relative to that in NC-treated mice. *P < 0.05, ***P < 0.001, two-tailed Student’s t-test. Data (a–g) are representative of at least two independent experiments with four to nine samples per group in each (mean and s.e.m.).
keratinocytes is lacking. The data presented here show that NF-kB induces miR-31, which directly inhibits pp6pc, thereby increasing basal keratinocyte proliferation. Pp6pc has been shown to regulate mitotic spindle formation by dephosphorylating Aurora A bound to its activator TPX2 (ref. 41). The role of pp6pc in keratinocyte hyperproliferation and in the pathogenesis of psoriasis is not known. We showed that pp6pc expression is diminished in the epidermis of lesional skin from patients, and that knockdown of pp6pc by siRNA or shRNA results in an enhanced percentage of proliferating keratinocytes in vitro and promotes psoriasiform skin disease in the IMQ-induced mouse model. These data noted above indicate that the conditional deletion of NF-κB in keratinocytes increases the basal keratinocyte proliferation. Pp6pc has been shown to be a negative regulator of the epidermis from the dermis. Complementary DNA was synthesized using the epidermis RNA extraction, an overnight incubation of skin samples at 4 °C, and reverse transcription and qPCR through these experimental studies, mice were euthanized by CO2 inhalation. For the Care and Use of Laboratory Animals with the approval (SYXK-2003-0026) of the Scientific Investigation Board of Shanghai Jiao Tong University School of Medicine, Shanghai, China. Age- and sex-matched mice at 8–12 weeks of age were kindly provided by Dr Yoichiro Iwakura (Research Institute for Biomedical Sciences, Tokyo University of Science, Tokyo, Japan). NF-kB luciferase reporter mice were kindly provided by Dr Jiong Deng (Shanghai Jiao Tong University School of Medicine, Shanghai, China)44. Age- and sex-matched mice at 8–12 weeks of age were used for all experiments. All mice were kept under specific pathogen-free (SPF) conditions in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with the approval of the Second Affiliated University School of Medicine, Shanghai, China. To ameliorate any suffering of mice observed throughout these experimental studies, mice were euthanized by CO2 inhalation.

Methods

Mice. The Keratin 5-Cre transgenic mice were kindly provided by Dr Xiao Yang (State Key Laboratory of Proteomics, Genetic Laboratory of Development and Disease, Institute of Biotechnology, Beijing, China). IL-17A−/− mice were kindly provided by Dr Yoichiro Iwakura (Research Institute for Biomedical Sciences, Tokyo University of Science, Tokyo, Japan). NF-kB luciferase reporter mice were kindly provided by Dr Jiong Deng (Shanghai Jiao Tong University School of Medicine, Shanghai, China)43. Age- and sex-matched mice at 8–12 weeks of age were used for all experiments. All mice were kept under specific pathogen-free (SPF) conditions. The mice were applied to a daily topical treatment of IMQ for 14 days. The immunofluorescence staining was performed on 10-μm-thick frozen sections of IMQ-induced mouse psoriasiform specimens and control specimens. Bacterial colonies were grown on 1/10th strength Trypticase Soy Broth (Trypticase Soy Base, Becton Dickinson, Franklin Lakes, NJ) supplemented with 3% (v/v) fetal bovine serum (FBS) and 1 μg/ml gentamicin. Two days after incubation, colonies from bacterial cultures were picked and subjected to DNA extraction using the Qiagen DNeasy Blood & Tissue Kit. The obtained DNA was used as template for PCR amplification of the HIV-1 LTR using the primers listed in Table 1. The bands were excised from 1% agarose gels and recovered using the Nipp_snap3 kit. For Bacterial cultivation, aliquots from stock cultures were inoculated in Bacterial Culture Medium (BMM) and grown at 37 °C. The colonies were detected by incubating sections with anti-DIG-alkaline phosphatase antibody (Ab) and NBT/BCIP substrate. The bands were excised from 1% agarose gels and DNA was recovered using the Nipp_snap3 kit. DNA was purified using the Nippter Kit according to the manufacturer’s instructions. In vivo activation of NF-kB by IMQ. NF-kB luciferase reporter mice with C57BL/6 background in which luciferase expression is driven by the NF-kB-dependent portion of the HIV-1 long terminal repeat42 were treated with IMQ. For bioluminescence imaging, mice were given 150 μg⋅kg−1 of D-luciferin in PBS by i.p. injection. Ten minutes after injection, bioluminescence imaging was captured with a charge-coupled device camera (IVIS; Xenogen).

Immunofluorescence staining. NHEKs were seeded on glass coverslips, cultured for 36 h and stimulated with IL-6 for 1 h. Cultured cells were washed three times in PBS and fixed for 15 min at RT. NHEKs were incubated in BSA (0.3% (v/v)) and DAB (0.3% (v/v)) for 1 h. After washing in PBS, NHEKs were snap frozen in liquid nitrogen and stored at −80 °C. All individuals provided informed consent. The study was performed in accordance with the declaration of Helsinki Principles and approved by the Research Ethics Board of the Second Affiliated Hospital of Xi’an Jiaotong University, Xi’an, China.

In situ hybridization. In situ hybridization was performed on 10-μm-thick frozen sections of IMQ-induced mouse psoriasiform specimens and control specimens. Bacterial colonies were grown on 1/10th strength Trypticase Soy Broth (Trypticase Soy Base, Becton Dickinson, Franklin Lakes, NJ) supplemented with 3% (v/v) fetal bovine serum (FBS) and 1 μg/ml gentamicin. Two days after incubation, colonies from bacterial cultures were picked and subjected to DNA extraction using the Qiagen DNeasy Blood & Tissue Kit. The obtained DNA was used as template for PCR amplification of the HIV-1 LTR using the primers listed in Table 1. The bands were excised from 1% agarose gels and DNA was recovered using the Nipp_snap3 kit. DNA was purified using the Nippter Kit according to the manufacturer’s instructions.
blocking Ab (BioLegend, #10301) treatment. Anti-phosphorylated-p65 rabbit Ab (Cell Signaling Technology, #50303) were used to stain the cells for 60 min in RT. Thereafter, cells were washed with PE-anti-rabbit IgG Ab (Life Technologies, #A11054) for 30 min at 4°C protected from light. Finally, cells were washed, resuspended and analysed with a BD FACS Canto II Flow Cytometer.

Keratinocyte isolation and culture. Newborn mouse back skin was incubated in dispase II for overnight at 4°C. The epidermis was gently scraped and washed with cold HBSS ( Gibco, #11470-090). The epidermal cells were separated following incubation with 5 ml 0.05% Trypsin (Gibco, #25200-056) at 37°C for 10 min, and subsequently the digestion was neutralized with 10 ml cold TNS (HBSS containing 5% FBS chelated with Chelex 100 Resin; #143–2832, Bio-Rad). After centrifugation at 500 x g for 10 min, 10 ml Medium 154 CF (Gibco, #M-154CF-500) was added to the pellet. Cells were seeded in 24-well plate, which was coated with 0.5 ml coating matrix buffer before using. Primary murine keratinocytes were grown in medium 154 CF containing 0.05 mM CaCl2, HKGS (Gibco, #001-5) and Pen Strep/Fungizone (Invitrogen, #15240-096) at 37°C, 5% CO2. Normal human epidermal keratinocytes (NHEK, Lifeline Cell Technology, #FC-0007) in liquid nitrogen were defrosted and cultured in serum-free basal medium with growth factors (Lifeline Cell Technology, #L30134). After 4 days, cells were stained with PE-anti-CD45 Ab (BioLegend, #103106) and blocking Ab (BioLegend, #101301) treatment. Anti-phosphorylated-p65 rabbit Ab (Invitrogen, #11668-019). Twenty-four hours post transfection, cells were stained by removing serum from the medium for 24h. Then cells were treated with ice-cold 7% Paraformaldehyde (PFA, 0.125% w/v) for another 24h before being lysed, and luciferase activity was measured on a microplate reader (Berthold, TriStar LB941) by using the Dual-Luciferase Reporter Assay System (Promega, #E1910). The ratio of firefly luciferase to renilla luciferase was calculated for each well.

IL-23 UTR reporter assay. 3' UTR fragments of pppc were cloned into pcCHECK-2 vector (Promega, #E2191) and each fragment was inserted into pGL3 vector generated by PCR. The following primers were used: Pppec, forward primer, 5'-AATTCTGAA GGGTTTCCGATCCCAT-3'; reverse primer, 5'-TCGGGCGGCACTTATAG TCCAAAAGAAACAGTA-3'; site-specific mutant, 5'-GGGGCGCGGAGTT AAAAAGAAGTGTCAGGACATTTC-3'. NT673 cell (BioLegend, #107016) was transiently transfected with 0.25 µg of reporter plasmid and 10 pmol miR-31 mimics (GenePharma) using TurboFect (Thermo Scientific, #I053), Twenty-four hours post transfection, the luciferase activity was measured using Dual-Luciferase Reporter Assay System.
Protein Immunoprecipitation Kit (Millipore, #92590) according to manufacturer’s instructions. In brief, epidermal cells were isolated after mice were treated with DMQ or water/vehicle. Keratinocytes were then lysed by RIPA lysis buffer and sonicated at ~80 °C. Magnetic beads were incubated with Ab (5 μg) with rotation for 30 min at RT. Anti-Ago2 (Abcam, #ab32381) was used as the Ab of interest, while anti-SNRP70 and negative control rabbit IgG served as controls. After thawed quickly and centrifuged at 14,000 r.p.m. for 10 min, the supernatant was incubated with beads–Ab complex for overnight at 4 °C. Immunoprecipitated RNA was analysed by qPCR. The primer sequence of ppp6c and miR-31 were 5’-CCGCTGT CAGTTGCAAGAATGTC-3’, 5’-ACACTGGCTGACATTGGC-3’; 5’-GCAGT GGAAAGGTTCAGTG-3’, 5’-TATCTCCAACCTCTGTC-3’, respectively.

Cell cycle analysis of keratinocytes. Primary murine keratinocytes were transfected with siRNA-40 and siRNA-NC (20 μM) by TurboFect or NHEK were treated with different recombinant cytokines (all from BD) as indicated. Cells were harvested after 24 or 48 h and cold 70% ethanol was gently added drop-wise for fixation, followed by resuspension in a blocking solution (2% BSA, 5% FBS, 0.2% Triton X-100 and 0.1% sodium azide) and incubated at 4 °C for 10 min. Cells were then pelleted and resuspended in propidium iodide solution (0.1 mg ml⁻¹, Calbiochem/EMD). Flow cytometry was used to analyse cell cycle.

In vivo administration of ppp6c shRNA lentivirus. DNA oligonucleotides containing mouse ppp6c shRNA sequences designed with the TRC shRNA Design online tool from the RNAi Consortium (http://www.broadinstitution.org/raai/public/ resources/) were synthesized then blended into pLVX-shRNA2 vector (Clontech, #632179). Tested in NIH3T3 cell line, the shRNA sequences with the highest knockdown efficiency were as follows: 5’-GATCCGCCAAAGTTATTCCGAGGGTTTACCTGCTTGCTGGTGTTTTTCCGAGCATCTGC GGAAAGGTTCAGTG-3’. Production of lentivirus, 293FT cells were seeded at a density of 4 x 10⁶ per 10-cm dish 1 day before transfection. Cells were transfected by calcium phosphate treatment with 5 μg pMD2-G, 10 μg pSPAX2 and 15 μg pLVX-shppp6c or pLVX-shRNA2 empty vector. Sixteen hours post transfection, cells were treated with 10 mM sodium butyrate for 12 h. Another 24-h cell incubation in complete medium was performed before the viral supernatant was harvested, passed through 0.45 μm filters to remove cellular debris and ultracentrifuged to concentrate the virus. Viruses were titered by infecting 293FT cells with serial dilutions in medium supplemented with 5 μg ml⁻¹ Polybrene, and the green fluorescent protein-positive cells were counted by flow cytometry 48 h post infection. The transduction titres were between 1 x 10⁸ and 2 x 10⁷ TU ml⁻¹ for concentrated virus preparations. A single dose of 100 μl shRNA-ppp6c or control lentivirus preparation was injected i.d. into the shaved dorsal skin of 6-week-old C57BL/6J female mice. These mice were treated with IMQ for 7 days before being assessed to kill the severity of psoriasis lesions histologically.

BrdU incorporation assay. BrdU (Sigma, #B5002) was directly added to the culture medium to achieve a final concentration of 10 μM and cells were incubated for 1 h in CO₂ incubator at 37 °C. Cells were then harvested and fixed with 5 μl 4% paraformaldehyde for 10 min. Cells were then pelleted and resuspended in 1 ml PBS, and a cholesterol-moiety at 3’-end was synthesized by RIBOBIO (Guangzhou, China). Antigomir-31 or NC (10 nmol) was injected i.d. for 3 consecutive days. Mice were then treated with IMQ. After treatment, skin samples were removed and preserved in liquid nitrogen.

Statistical analysis. The data were analysed with GraphPad Prism 5 and are presented as the mean ± s.e.m. Student’s t-test was used when two conditions were compared, and analysis of variance (ANOVA) with Bonferroni or Newman–Keuls correction was used for multiple comparisons. Simple linear regression model was used to analyse the correlation between RNA levels of IL-6 and miR-31. Probability values of <0.05 were considered significant; two-sided Student’s t-tests or ANOVA were performed. *P<0.05; **P<0.01; ***P<0.001; NS, not significant. Error bars depict s.e.m.

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Author contributions
S.Y., Z.X., F.L. and H.W. designed the research and analysed the data. H.W. supervised the research and wrote the manuscript. S.Y., Z.X. and F.L. conducted most of the experiments. L.Z. helped with mouse breeding and flow cytometry. F.K. offered technical and materials support. S.Y., Z.X., F.L. and H.W. designed the research and analysed the data. H.W. supervised the research and wrote the manuscript. B.S., X.-Z.Y. and Y.E.C. offered help in research design and manuscript revision.

Additional information
Accession codes. Microarray data has been deposited in the GEO database under accession code GSE50099.

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