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IFI27, a novel epidermal growth factor-stabilized protein, is functionally involved in proliferation and cell cycling of human epidermal keratinocytes

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Abstract

Objectives: IFI27 is highly expressed in psoriatic lesions but its function has not been known. The present study aimed to explore its role in proliferation of epidermal keratinocytes.

Materials and methods: IFI27 knockdown and overexpression in keratinocytes were used to compare their proliferation, by MTT assay, apoptosis (by annexin V binding) and cell cycle progression by flow cytometry. Formation of cyclin A/CDK1 complex was examined by a co-immunoprecipitaion method. Anti-proliferation effects of IFI27 were also examined *in vivo* by topical application of IFI27 siRNA on imiquimod-induced psoriatic lesions, in a mouse model.

Results: Epidermal growth factor was demonstrated to increase IFI27 expression by prolonging half-life of IFI27 protein. The IFI27 knockdown in keratinocytes reduced the proliferation rate, but had no effect on apoptosis nor on apoptosis-related genes. Interestingly, IFI27 knockdown resulted in S-phase arrest that was found to be associated with increased Tyr15 phosphorylation of CDK1, reduced CDC25B and reduced formation of cyclin A/CDK1 complex. In addition, IFI27 knockdown was also shown to activate p53 by Ser15 phosphorylation and increase p21 expression. Topical application of IFI27 siRNA on imiquimod-induced psoriatic lesion in a mouse model reduced epidermal thickness, formation of rete ridges and PCNA expression.

Conclusions: Our study demonstrates for the first time, that cell function of IFI27 is involved in proliferation of skin keratinocytes both *in vitro* and *in vivo*. It suggests that IFI27 might be a suitable target for development of a novel anti-psoriasis therapy.

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Introduction

Psoriasis is a chronic inflammatory skin disease, with cytokines, chemokines, resident skin cells and infiltrating leucocytes creating an inflammatory network that results in scaly, erythematous plaques, in which keratinocyte hyperproliferation and abnormal differentiation cause thickening of the epidermis (1–4). Hyperproliferation of keratinocytes, the hallmark of psoriasis, is considered to be a potential therapeutic target for treating this disease (5–8).

Interferon α-inducible protein 27 (IFI27), up-regulated in psoriatic skin and in some epithelial cancers (9,10), has been suggested to be a putative cell proliferation marker. It maps chromosome 14q32, a region where a psoriasis susceptibility locus has, in several studies (11), been identified. However, cell function of IFI27 in human epidermal keratinocytes has not preiously been elucidated.

In the present study, we aimed to investigate whether expression and function of IFI27 were involved in proliferation of epidermal keratinocytes. The effect of epidermal growth factor (EGF), known to be highly increased in psoriatic patients (12), on regulating the IFI27 expression in keratinocytes was studied. IFI27 knockdown, using a shRNA technique, was carried out to investigate possible function of IFI27, and expression levels of related genes on cell proliferation, cell cycle and apoptosis. Effects of anti-IFI27 siRNA in the psoriasis-like mouse model with daily topical application of imiquimod (IMQ) to the skin (13) were also examined.

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Materials and methods

Cell culture

Human epidermal keratinocytes were isolated from skin of donors by an enzymatic digestion method (14). Samples were obtained after written informed consent and under the approval of the Institutional Review Board of Chang Gung Memorial Hospital in Tao-Yuan, Taiwan. Primary keratinocytes released were grown in keratinocyte growth medium (KGM) (Gibco, Grand Island, NY, USA) and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. HaCaT cells, a spontaneously immortalized human keratinocyte cell line (15), were kindly provided by Dr. Jong-Shyan Wang (Graduate Institute of Rehabilitation Science, Chang Gung University); they were grown as described previously (16).

IFI27 *gene manipulation*

Human IFI27 cDNA (GenBank no.: NM_001130080) was amplified by PCR and subcloned into the pcDNA3.1 eukaryotic expression vector (pcDNA3.1-IFI27). Over-expression of IFI27 gene in HaCaT cells was performed by transfecting the recombinant vector with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) and G418 selection (Mediatech, Herndon, VA, USA) according to the manufacturer's protocols. After 8 weeks, pools of G418-resistant pcDNA3.1-IFI27-transfected and mock-transfected cells were cultured, and stable over-expression of IFI27 was confirmed by RT/real-time PCR and western blotting. Specific IFI27 shRNA constructs cloned into plasmid pLKO.1-puro were obtained from The RNAi Consortium (TRC) (Academia Sinica, Taipei, Taiwan). Lentivirus production and infection were performed based on TRC protocols. The following plasmids were used: TRCN0000115859 (sh-IFI27), 5′-GCTACAGTTGT-GATTGGAGGA-3′; TR CN0000115857 (sh-IFI27-2), 5′-CCCTGCAGAGAAGA GAACCAT-3′. Twenty-four hours after infection, cells were treated with 2 μg/ml puromycin (Invitrogen) to select a pool of puromycin-resistant clones.

MTT assay

Cell viability was determined using colorimetric MTT assay as described previously (7) and cell viability was expressed as a percentage relative to the control.

Western blot analysis

Western blot analysis was performed as previously described (7). The following primary antibodies were used: anti-IFI27 (H00003429-D01P; Abnova, New Taipei City, Taiwan), anti-cyclin A (ab38; Abcam, Taipei, Taiwan), anti-cyclin B (MS-338; Thermo, Waltham, MA, USA), anti-cyclin D (RM-9104; Thermo), anti-cyclin E (14-6714; eBioscience, San Diego, CA, USA), anti-p53 (MS-104; Thermo), anti-phospho-p53 (S15) (9286; Cell Signaling, Danvers, MA, USA), anti-CDK1 (MS-110-P1; Thermo), anti-CDK2 (MS-459-P0; Thermo), anti-phospho-CDK1 (Y15) (ab47594; Abcam), anti-CDC25B (AP7256c; Abgent, San Diego, CA, USA), anti-p21 (MS-891-P1; Thermo) and anti-tubulin (MS-581-P; Thermo). Tubulin was used as the sample loading control.

RT/real-time PCR

Mouse skin tissue was homogenized in a MagNA Lyser system (Roche, Mannheim, Germany) and RT/real-time PCR was performed as described previously (7). All real-time PCRs were performed in triplicate, and changes in gene expression were presented as multiples of increases relative to controls. Sequences of primers used are listed in Table 1.

Flow cytometry

Cells were harvested, re-suspended and stained following techniques previously described (17). Cell cycle distribution was quantified using a FACS Calibur system (Becton-Dickinson, Franklin Lakes, NJ, USA). Percentages of cells in G_0/G_1, S and G_2/M phases were determined and analysed using CellQuest Pro software (Becton Dickinson).

Table 1. Sequences of oligonucleotide primers used for RT/real-time PCR

Primer-ID	Forward primer	Reverse primer
GAPDH	5′-gaggggccatccacagtctt-3′	5′-ttcattgacctcaactacat-3′
IFI27	5′-tgggagcaactggactct-3′	5′-caatgacagccgcaatgg-3′
CASP3	5′-tgacatctcggtctggta-3′	5′-aacatcacgcatcaattcc-3′
Fas	5′-cactcgcaacctctctccc-3′	5′-agagtgtgtgcacaaggctg-3′
FasL	5′-tcaatgaaactgggctgtacttt-3′	5′-agagttcctcatgtagaccttgt-3′
CASP8	5′-gaagataatcaacgactatg-3′	5′-ttcactatcctgttctct-3′
CASP9	5′-cattggttctggaggatt-3′	5′-ccatgctcaggatgtaag-3′
BAX	5′-cttctggagcaggtcaca-3′	5′-taaggaaaacgcattatagacca-3′
Bcl2	5′-tgcctttgtggaactgta-3′	5′-gagcagagtcttcagaga-3′
BAD	5′-atgagtgacgagtttgtggac-3′	5′-catctgcgttgctgtgcc-3′
Cyclin A	5′-ggagctgcctttcatt-3′	5′-tgaaggtccatgagacaaggct-3′
Cyclin B	5′-tttgcacttccttcggagagc-3′	5′-aaggaggaaagtgcaccatgtc-3′
Cyclin D	5′-gctggccatgaactacctgg-3′	5′-atcttagaggccacgaacatgc-3′
Cyclin E	5′-tacaccagccacctccagacac-3′	5′-cctccacagcttcaagcttttg-3′
P53	5′-agtatttggatgacagaa-3′	5′-atgtagttgtagtggatg-3′
p21	5′-tacatcttctgccttagt-3′	5′-tcttaggaacctctcatt-3′
p27	5′-aggaataaggaagcgacc-3′	5′-ggaaccgtctgaaacattt-3′
p57	5′-acgatggagcgtcttgtc-3′	5′-cctgctggaagtcgtaatc-3′

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Determination of protein stability

Cells, with or without stimulation with 20 ng/ml EGF (R&D Systems, Minneapolis, MN, USA) for 8 h, were treated with 50 μM cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) (inhibitor of protein synthesis), then were harvested at the indicated times. Protein samples were subsequently processed for western blot analysis to determine expression levels of remaining proteins.

Detection of apoptosis

Levels of apoptosis were assessed using the Annexin V-FITC Apoptosis Detection kit (Strong Biotech, Taipei, Taiwan). Cells were stained with FITC-conjugated annexin V and PI for 10 min at 37 °C and examined using a Nikon DXM1200 microscope; Nikon ACT-1 image analysis software was used for data processing. Positive control cells were prepared by treating with UV (254 nm) irradiation for 6 h; this is acknowledged to induce keratinocyte apoptosis (18).

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed as previously described (19). Samples were then analysed by western blotting with a specific antibody targeting the binding partner of the complex.

IMQ-induced psoriasis-like mouse model

Groups of five male C57BL/6 mice, 8 weeks of age, were provided with food and water *ad libitum* and maintained on a 12-h light/dark cycle. They received a daily topical dose of 62.5 mg 5% IMQ cream (Aldara; 3M Pharmaceuticals, Maplewood, MN, USA), on the shaved back, for 10 consecutive days to induce psoriasis-like changes. IFI27 siRNA or negative control siRNA were topically applied on IMQ-treated animals three times, once every 3 days. Two different IFI27 siRNA and negative control siRNA were synthesized by MDBio, Inc; IFI27 siRNA sequences (dsRNA) were IFI27-mus (s), 5′-AGUUGUAAGAAGCAAGAUUTT-3′, and IFI27-mus (as), 5′-AAUCUUGCUUCUUACAACU TT-3′; IFI27-2-mus (s), 5′-CCACAAUAACCAACAUC AUTT-3′ and IFI27-2-mus (as), 5′- AUGAUGUUGGUU AUUGUGGTT -3′. The siRNA sequences (dsRNA) for negative controls were 5′-UUCUCCGAACGUGUCAC GUTT-3′(s) and 5′-ACGUGACACGUUCGGAGAATT-3′ (as). Before topical treatment, 15 μg (5 μl) siRNA or negative control siRNA was coated with 30 μl of liposome in a 25-μl emulsion vehicle (5% glycerine, 2% Tween 80 and water); this was instantly and topically applied to the skin lesions. At the end of experiments, mice were anaesthetized by intraperitoneal injection of 100 mg/kg sodium pentobarbital and sacrificed. All animal studies were performed in compliance with guidelines of the Institutional Animal Care and Use Committee (IACUC) under approval from the IACUC, at the Chang Gung University (Permit Number CGU 12-170).

Histopathological analysis

On experimental day 10, back skins of mice in each group were excised and fixed in 10% formaldehyde. Paraffin wax-embedded sections were cut, then stained with haematoxylin and eosin. Tissue images were captured using a Zeiss Primo Star light microscope (Carl Zeiss Microimaging GmbH). Epidermal hyperplasia was quantified by measuring its thickness in more than six fields, per section, at intervals of 100 μm.

Immunohistochemical staining

Immunohistochemical staining was performed as previously described (17) by using anti-PCNA antibody (MS-106; Thermo). PCNA-positive cells were counted by an expert blind to the treatment protocol, in three different areas per skin, and the mean was calculated.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis of group differences was performed using Student's *t*-test. $^{**}P < 0.01$, $^{*}P < 0.05$.

Results

EGF increased IFI27 gene expression and protein stability

To further comprehend the functional role of IFI27 in cell proliferation, the spontaneously immortalized HaCaT cell line was used to mimic hyperproliferation of psoriatic keratinocytes. HaCaT cells share some other characteristics with psoriatic keratinocytes and are often employed as surrogate "psoriatic" keratinocytes (20–24). More importantly, as shown in Fig. S1, we demonstrated that IFI27 mRNA expression was highly up-regulated in HaCaT cells compared to primary human keratinocytes. HaCaT cells could thus serve as an excellent cell model to study whether knockdown of IFI27 would interfere with proliferation, in the following experiments.

It has previously been demonstrated that serum levels of EGF are higher in patients with psoriasis compared to

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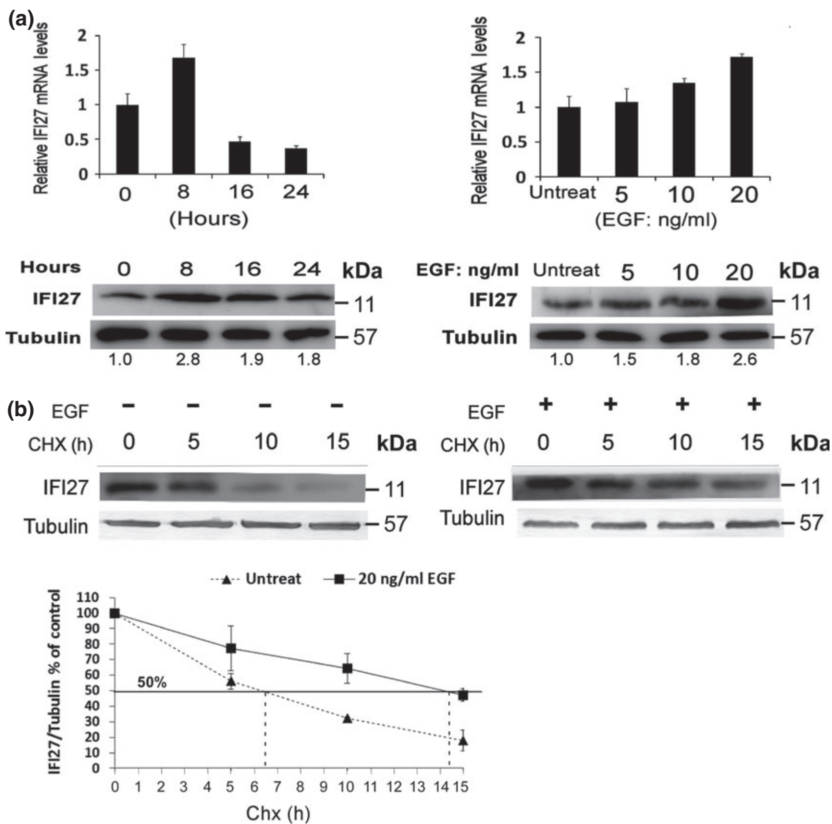

Figure 1. Epidermal growth factor (EGF) up-regulated IFI27 mRNA expression and increased IFI27 protein stability. (a) HaCaT cells were serum-deprived for 2 days and stimulated by 20 ng/ml EGF for 0, 8, 16 and 24 h, or by selected concentrations of EGF for 8 h. mRNA and protein expressions of IFI27 were analysed by RT/real-time PCR (upper panel) and western blotting (lower panel) respectively. (b) HaCaT cells stimulated without or with EGF for 8 h were then treated with 50 μg/ml cycloheximide (CHX). The remaining IFI27 protein level was determined by western blotting. Upper panel shows representative immunoblotting results from three independent experiments. Expected molecular weights are indicated on the right. Results quantified by densitometry analysis are shown as mean ± SD in lower panel. Similar results were obtained from three independent experiments.

normal subjects (12), and hyperproliferation of keratinocytes is known to be stimulated mostly by EGF (25), indicating its important role in pathophysiology of psoriasis. To investigate whether EGF could regulate IFI27 expression in keratinocytes, HaCaT cells were serum-deprived for 2 days and stimulated with 20 ng/ml EGF for 0, 8, 16 and 24 h, or by selected concentrations of EGF for 8 h prior to RT/real-time PCR and western blot analysis. Results showed that IFI27 mRNA expression was slightly higher at 8 h after EGF stimulation; IFI27 protein expression was significantly increased by EGF treatment in a time- and dose-dependent manner (Fig. 1a). We hypothesized that EGF could prolong the half-life of IFI27 protein and therefore resulted in increased IFI27 protein level in keratinocytes. To test this possibility, HaCaT cells were serum-deprived for 2 days and treated with 20 ng/ml EGF for 8 h, and then 50 μg/ml cycloheximide was added to the cells. Cells were harvested at 0, 5, 10 and 15 h after cycloheximide treatment, and IFI27 protein levels were analysed by western blotting. Clearly, half-life of IFI27 protein was prolonged from 6.5 h in control keratinocytes to around 14.5 h in EGF-treated keratinocytes (Fig. 1b). For the first time, we demonstrated that IFI27 protein in keratinocytes could be stabilized in the presence of EGF.

IFI27 knockdown reduced keratinocyte proliferative rate and resulted in their S-phase arrest

We demonstrated in Fig. 1 that half-life of IFI27 protein was prolonged by EGF treatment which seemed to provide one of the potential mechanisms for increase in IFI27 expression in the psoriatic lesions. However, we know that IFI27 gene expression in psoriatic lesions is also up-regulated at the transcriptional level (possibly by other mechanisms), as demonstrated in previously published studies by cDNA microarray analysis and *in situ* hybridization (9,10). To explore the potential role of IFI27 in cell proliferation of keratinocytes, the proliferation rate (in which IFI27 expression was either knockdown or over-expressed by transfecting with IFI27-shRNA or pcDNA3.1-IFI27 respectively) was measured by MTT assay. Results were compared to those of keratinocytes transfected with control vectors as described in the Materials and methods section. As analysed by RT/real-time PCR and western blotting, IFI27 expression was confirmed to be knocked-down or over-expressed (Fig. 2a). Results from MTT assay measured from first to the 3rd day, demonstrated that IFI27 knocked-down keratinocytes exhibited slower rates of proliferation compared to controls. On the other hand, IFI27

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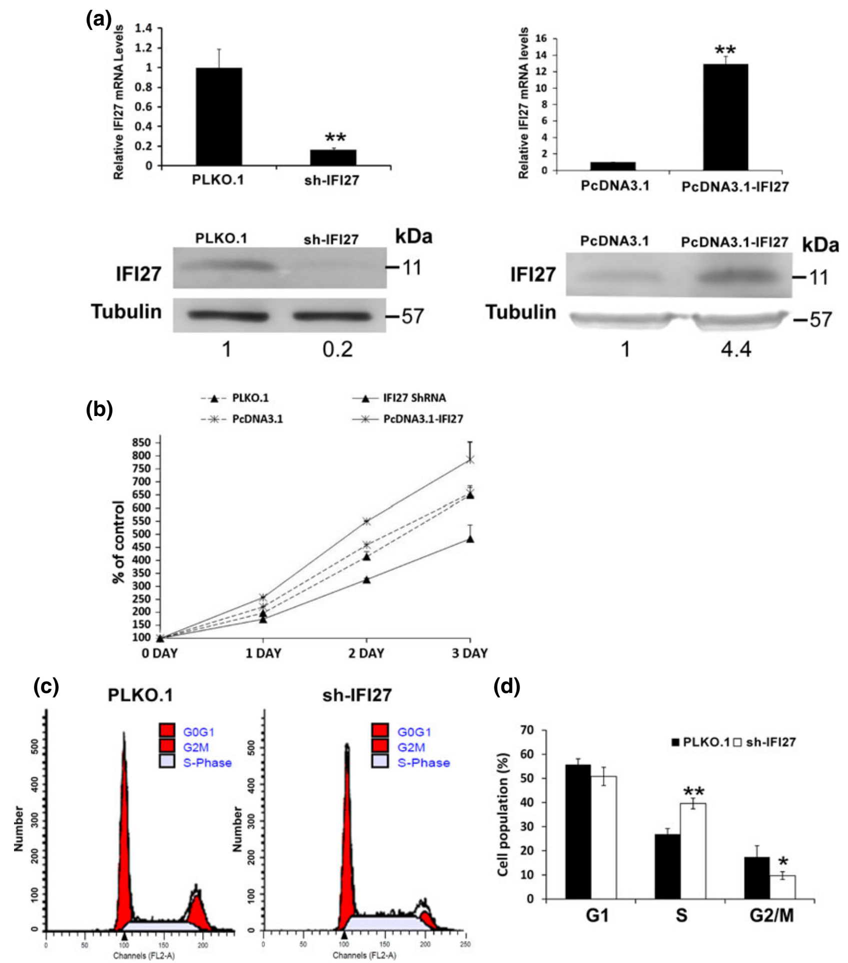

Figure 2. IFI27 regulated cell proliferation and cell cycling of keratinocytes. (a) IFI27 protein was knocked down or over-expressed in HaCaT cells as described in the Materials and methods section, and mRNA and protein levels were analysed by RT/Real-time PCR (upper panel) and western blot analysis (lower panel) respectively. Experiments were performed in triplicate and data were presented as mean ± SD. Densitometric data of IFI27 in (a) were normalized by their tubulin levels and presented under the corresponding bands. (b) Cell population growth rates were analysed and compared by MTT assay for 3 days as indicated. (c) Cell cycle distribution was analysed by flow cytometry. (d) Percentages of cells in different phases of the cell cycle were calculated. $^{**}P < 0.01$, $^{*}P < 0.05$.

over-expression promoted cell proliferation rate (Fig. 2b). To determine whether growth inhibition of IFI27 knockdown cells was due to changes in cell cycle distribution, flow cytometric analysis was performed. Results showed that knockdown of IFI27 resulted in an accumulation of keratinocytes in S phase of IFI27 knockdown cells, while cells in G_2/M was reduced, suggesting a critical role for IFI27 in transition of cells from S to G_2 phase during the cell cycle (Fig. 2c,d). Similar results obtained from IFI27 knockdown keratinocytes by the use of sh-IFI27-2 are shown in Fig. S3.

Although IFI27 expression level is relatively low in normal human keratinocytes compared to HaCaT cells, knockdown of IFI27 expression was also performed in normal human keratinocytes, the results being shown in Fig. S2. Knockdown of IFI27 expression (Fig. S2a) also retarded their cell proliferation (Fig. S2b). Interestingly, percentage inhibition of cell proliferation in human normal keratinocytes by IFI27 knockdown was not as significant as in HaCaT cells, further providing indirect evidence to support the potential role of IFI27 in cell proliferation.

IFI27 knockdown did not trigger keratinocyte apoptosis

In addition to reduced rate of proliferation, apoptosis could also contribute to reduction in viable cells after IFI27 knockdown. Thus, we analysed expressions of apoptosis-related genes including those coding for caspase 3, 8 and 9, Fas, FasL, BAX, Bcl-2 and BAD, by RT/real-time PCR and made comparisons between keratinocytes transfected with PLKO.1 and sh-IFI27. Results demonstrated no significant change induced by IFI27 knockdown (Fig. 3a). Apoptosis was further detected by staining keratinocytes directly with FITC-conjugated annexin V and propidium iodide. Results again showed that neither early stage apoptosis (green fluorescence), nor late stage apoptosis and other forms of cell death such as necrosis (orange fluorescence) were detected in control or IFI27 knockdown epidermal keratinocytes (Fig. 3b), demonstrating that IFI27 knockdown did not trigger cell apoptosis. Positive controls for apoptosis were prepared by irradiating HaCaT cells with 254 nm UV for 6 h then stained under the same conditions. Typical staining

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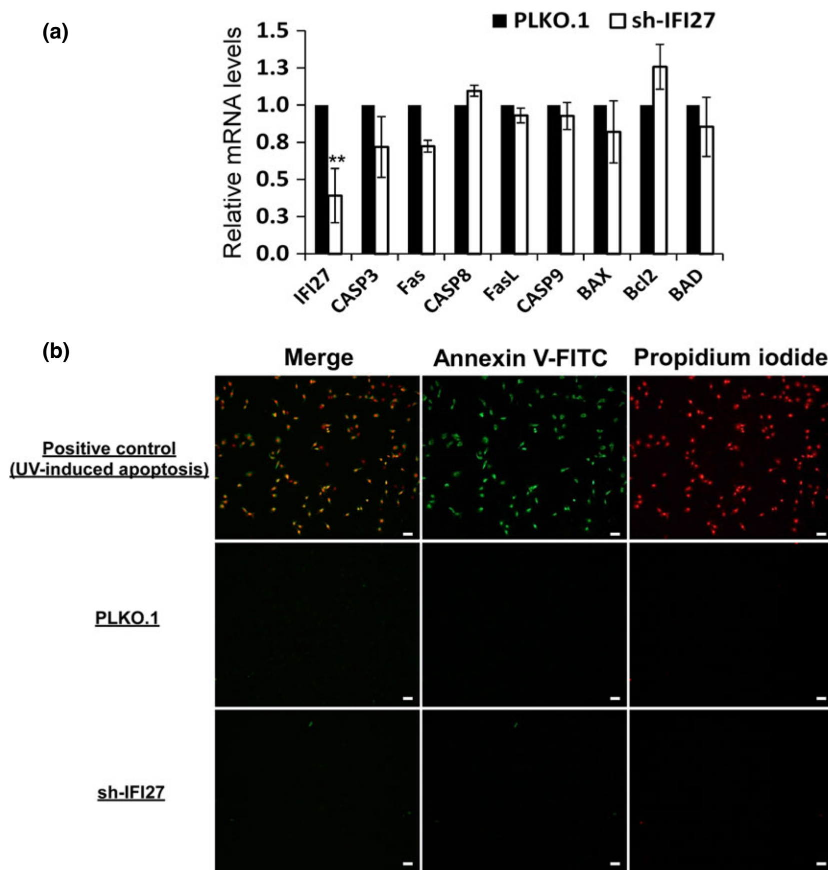

Figure 3. Knockdown of IFI27 expression did not trigger apoptosis of HaCaT cells. (a) mRNA expressions of apoptosis-related genes were analysed by RT/real-time PCR. Experiments were performed in triplicate. Data are presented as mean ± SD; $^{**}P < 0.01$. (b) Representative photos in 100-fold magnification show results of keratinocytes stained with FITC-conjugated annexin-V (green) and PI (red). Positive control was prepared by treating keratinocytes with UV for 6 h to induce apoptosis (scale bar = 50 μm).

patterns for apoptosis were observed (Fig. 3b); similar results obtained from IFI27 knockdown keratinocytes by the use of sh-IFI27-2 are shown in Fig. S4.

IFI27 knockdown altered expressions of cyclins

To further understand the cause of S-phase arrest in IFI27 knockdowned keratinocytes, we measured expression of cyclins A, B, D and E, by RT/real-time PCR and western blot analysis. Results revealed down-regulation of cyclin B and up-regulation of cyclin A in IFI27 knockdowned keratinocytes at both mRNA and protein levels (Fig. 4a, b). Cyclin A is a stable protein in S and G_2 phases, but is destabilized when cells enter mitosis and is almost completely degraded before the metaphase to anaphase transition (26,27). Our results ruled out the possibility that cyclin A in IFI27 knockdown keratinocytes might acquire a longer half-life. Half-life of cyclin A was then measured and compared in control keratinocytes and IFI27 knockdown ones. However, half-life of cyclin A after addition of cycloheximide did not differ between control and IFI27 knockdown keratinocytes (Fig. 4c), indicating that S-phase arrest in IFI27 knockdown cells was not caused by delayed degradation of cyclin A protein. Similar results obtained from IFI27 knockdown keratinocytes by use of sh-IFI27-2 are shown in Fig. S5.

IFI27 knockdown reduced formation of cyclin A/CDK1 complex, and increased expression of p21

As formation of cyclin A/CDK1 complex is required for entry into G_2 phase (26), we next examined whether IFI27 knockdown altered interaction of cyclin A and CDK1, by a co-immunoprecipitation (Co-IP) method. Results clearly showed that, although protein level of cyclin A was increased in IFI27 knockdowned keratinocytes, amount of complex formed with CDK1 was reduced (Fig. 5a); IgG was used as a negative control in the co-IP reaction. In addition, CDC25B was found to be reduced, and phosphorylation of CDK1 on Tyr15 was significantly higher in IFI27 knockdown keratinocytes (Fig. 5b), indicating greater inhibition of CDK1 activity in IFI27 knockdown keratinocytes. In our studies, formation of cyclin A-CDK2 complex, and activation of CDK2 showed no difference between control and IFI27 knockdowned keratinocytes (data not shown). As CDK1 activity can also be antagonized by cyclin-dependent kinase (CDK) inhibitors, we further analysed

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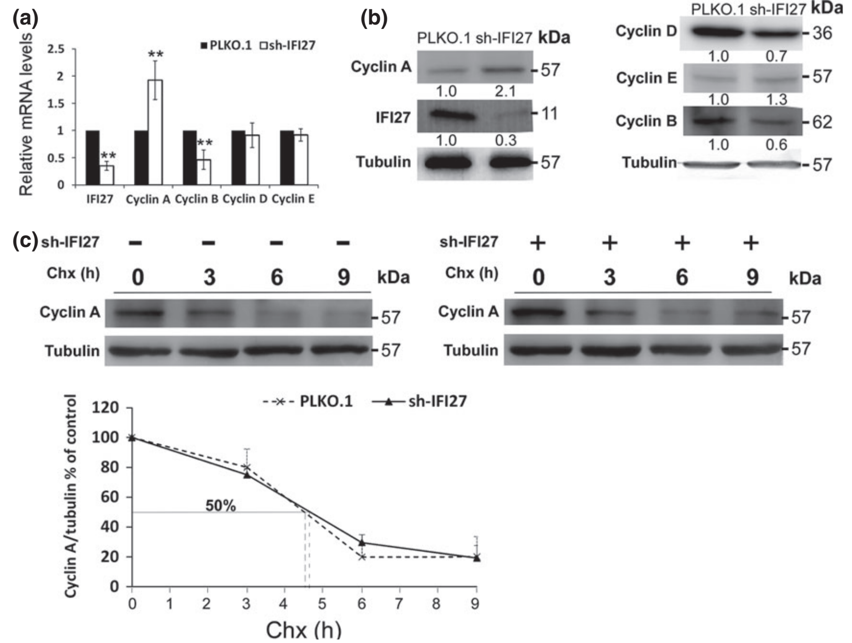

Figure 4. Knockdown of IFI27 in HaCaT cells resulted in up-regulation of cyclin A expression without affecting its stability. Expression levels of cyclins in HaCaT cells transfected with PLKO.1 or sh-IFI27 were analysed by (a) RT/real-time PCR and (b) western blotting. Results are presented as mean ± SD from three independent experiments (**$P < 0.01$). (c) Keratinocytes transfected with PLKO.1 or sh-IFI27 were treated with 50 μg/ml cycloheximide (CHX), and the remaining cyclin A protein level was determined by western blotting. Upper panel shows representative results of immunoblotting from three independent experiments. Data quantified by densitometry analysis are shown as mean ± SD in lower panel.

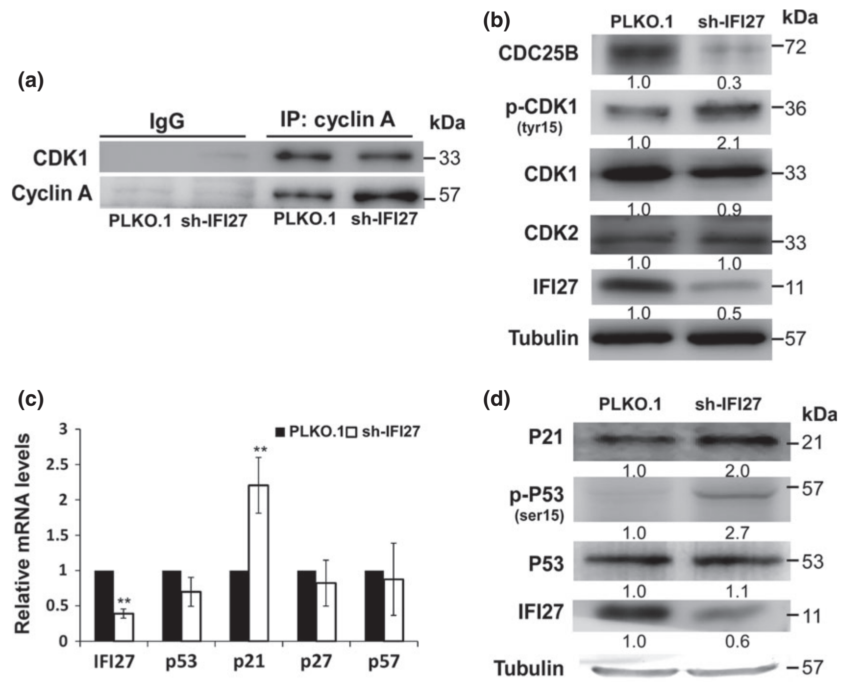

Figure 5. IFI27 knockdown reduced formation of cyclin A/CDK1 complex and increased expression of p21 in HaCaT cells. (a) Non-denatured cell extracts of HaCaT cells transfected with PLKO.1 or sh-IFI27 were immunoprecipitated with anti-Cyclin A or negative control IgG antibody. The immunoprecipitated protein complex was then analysed by western blotting using antibodies against CDK1 or Cyclin A. (b) Protein expression levels of IFI27, CDK1 (p-tyr15), CDC25B, CDK1 and CDK2 were analysed. Expressions of p53, p21, p27 and p57 were determined by (c) RT/real-time PCR and (d) western blot analysis. Data in (c) are presented as mean ± SD of three different experiments (**$P < 0.01$).

mRNA expressions of p53, p21, p27 and p57 by RT/real-time PCR. Results demonstrated marked increase in p21 mRNA expression level in IFI27 knockdown keratinocytes, but not of p53, p57 or p27 (Fig. 5c).

To further investigate p53 activation involved in cell cycle arrest after IFI27 knockdown in HaCaT cells, we examined post-translational modification of p53 by western blot analysis, using specific antibodies. As shown in Fig. 5d, although total level of p53 protein had no difference, its phosphorylation at Ser15 in IFI27 knockdowned keratinocytes significantly increased (Fig. 5d). Together, our data indicated that IFI27 played a role in progression of the cell cycle in keratinocytes and its function might be involved in transition from S to G2 phases. Similar results obtained from IFI27 knockdown keratinocytes by the use of sh-IFI27-2 are shown in Fig. S6.

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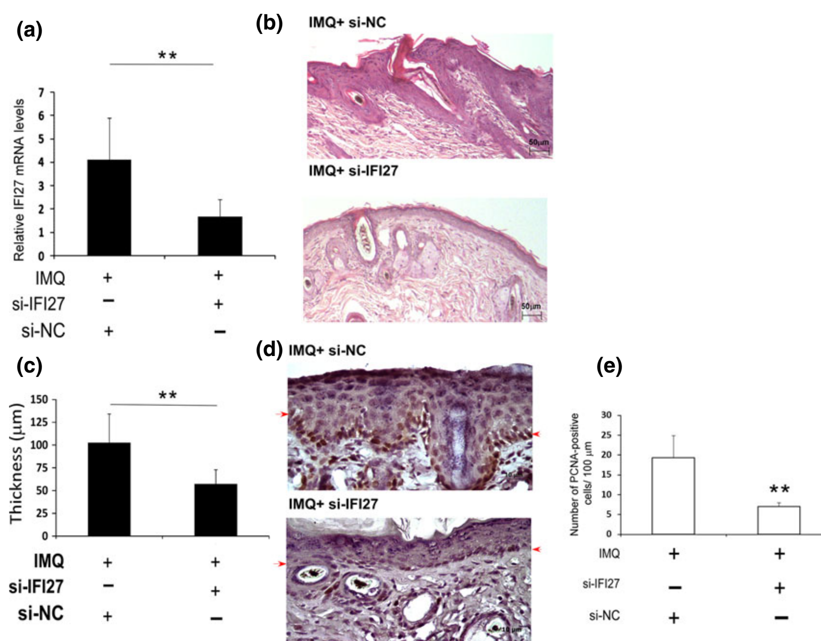

Figure 6. Reduction in epidermal thickness in imiquimod (IMQ)-induced psoriasis-like skin by IFI27 siRNA. IMQ-induced psoriasis mouse model was treated as described in the Materials and methods section. (a) IFI27 mRNA expression was determined after IFI27 siRNA treatment. (b) Histological analyses of H&E-stained back skins on day 10 are shown. Magnification 100× (scale bar = 50 μm). One representative picture is shown for each group. (c) Epidermal hyperplasia was quantified by measuring thickness in more than six fields, at intervals of 100 μm in each section and the mean was calculated. Data were calculated from at least five independent experiments and presented as means ± SD (**$P < 0.01$). (d) Representative results of immunohistochemical analysis using anti-PCNA antibody is shown. Red arrows indicate basal layer of the epidermis. Magnification 400× (scale bar = 10 μm). (e) PCNA-positive cells were counted in three different areas per skin and the mean was calculated. $n = 3$ each group, **$P < 0.01$.

Reduction in epidermal thickness in IMQ-induced psoriasis- like skin by IFI27 siRNA

Given the functional role of IFI27 in cell proliferation, we hypothesized that topically applied IFI27 siRNA might slow down proliferation of epidermal keratinocytes and suppress progression of psoriasis. Effects of anti-IFI27 siRNA in the psoriasis-like mouse model with daily topical application of IMQ on the skin, was investigated. IMQ-treated mice were treated as described in the Materials and methods section. Knockdown of IFI27 mRNA expression was confirmed on day 10 (Figs 6a, S7a). Analysis of haematoxylin and eosin- stained back skin sections revealed IFI27 siRNA treatment reduced IMQ-induced epidermal thickness and formation of rete ridges (Figs 6b,S7b). Epidermal thickness was measured in more than six fields at intervals of 100 μm in each section, and the mean was calculated (Figs 6c,S7c). Moreover, number of PCNA-positive cells in epidermal basal layers, as indicated by red arrows, was significantly reduced in IFI27-siRNA-treated lesions compared to negative control siRNA- treated lesions (Fig. 6d,e). Reduction of psoriatic epidermal hyperplasia by IFI27 siRNA further confirmed the role of IFI27 in cell proliferation in this *in vivo* condition.

Discussion

IFI27, one of the interferon-α highly inducible proteins, was first reported to be up-regulated in psoriatic skins in a gene array study (9). Subsequently, *in situ* hybridization analysis showed that mRNA expression of IFI27 was abundantly distributed in epidermis of psoriatic lesions (10). Thus, it is interesting to be able to understand regulation of IFI27 in keratinocytes during development of psoriasis. A number of reports have indicated that the EGF receptor (EGFR) and its endogenous ligands are over-expressed in psoriatic lesions (25,28). In addition, multiplex cytokine assay also demonstrates 10-fold increase in serum EGF in psoriatic patients compared to normal controls (12). High expression of EGFR and its ligands in lesional skin, together with high expression of EGF in sera, suggest that this pathway contributes to pathophysiology of the disease. The present study, for the first time, demonstrates that IFI27 protein stability was prolonged by EGF treatment in keratinocytes. By the mechanism of stabilizing proteins to enhance their activities, two other growth-related proteins, NGFI-B and EGR1 have also been found to be increased and stabilized by EGF (29,30). It is still not clear how EGF increase protein stability; however, our

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data provide evidence to support the role of IFI27 in pathogenesis of psoriasis, specially in hyperproliferation of keratinocytes.

It has been speculated since 2004 (10) that IFI27 might be a novel marker of epithelial proliferation and neoplastic development, however, no study had been performed to investigate whether the function of IFI27 was indeed involved in cell proliferation. In this study, we modulated IFI27 expression in keratinocytes, and the results demonstrated that IFI27 function was required for proliferation and cell cycling of the cells. Knockdown of IFI27 resulted in cell cycle arrest in the S phase. Studies in recent years have shown that keratinocytes under treatment with Tanshinone IIA, glycolic acid or UV irradiation arrest in S phase and then undergo apoptosis (31–34). However, IFI27 knockdown did not trigger apoptosis, indicating a different mechanism involved. In addition by western blot analysis, no significant changes in involucrin or keratin-10 were found in IFI27 knockdown keratinocytes (data not shown). Results from cDNA microarray analysis comparing gene expression profiles between control and IFI27 knockdown keratinocytes did not identify changes in genes associated with skin differentiation either (data not shown). Together these show that the function of IFI27 is unlikely to be involved in the process of cell differentiation.

IFI27 knockdown unexpectedly altered expression profiles of cyclins, and expression level of cyclin A significantly increased, that is associated with S-phase arrest. Cyclin A is a stable protein in S and G_2 phases, but is destabilized when cells enter mitosis and is almost completely degraded before the metaphase to anaphase transition (26,27). Cyclin A/CDK1 is thought to be involved in activation and stabilization of cyclin B/CDK1 complex (35). Once cyclin B is activated, cyclin A is no longer needed and is subsequently degraded through the ubiquitin pathway (26,36). Degradation of cyclin A/CDK1 induces mitotic exit (26). Our results ruled out the possibility that cyclin A in IFI27 knockdown keratinocytes might acquire longer half-life. However, formation of cyclin A/CDK1 was shown to be reduced. Except for lower expression level of CDK1 caused by IFI27 knockdown, CDK1 activity was apparently inhibited due to higher level of phosphorylation at the Tyr15 residue and reduced CDC25B (a phosphatase responsible for removal of the phosphate group at Tyr15) and activated CDK1 (37,38).

Regulation of cyclin/CDK complexes occurs at multiple levels, including assembly of cyclin and CDK, phosphorylation/dephosphorylation events, and association of cyclin/CDK complexes with CDK inhibitors (CKIs). CDK1 activity is antagonized by CKIs such as p21, p27 and p57 (39–41). Among these, up-regulation of p21 expression was confirmed in IFI27 knockdown keratinocytes. Increase in p53 transcriptional activity is a molecular signature for cell cycle arrest, and its higher activity is driven by changes in p53 phosphorylation status (42). Phosphorylation of p53 at Ser15 inhibits interaction between Mdm2 and p53, and causes nuclear accumulation of p53 (43). However, HaCaT cells contain two mutant alleles of p53, which are unable to activate transcription from the p21 promoter when over-expressed (44,45). Up-regulation of *p21* gene expression in IFI27 knockdown HaCaT cells is likely to be due to a p53-independent pathway. In the absence of p53 function, up-regulation of p21 has been observed following destabilization of the actin cytoskeleton, mainly by reduced ubiquitination and delayed degradation of p21 protein (46). On the other hand, increased degradation of p21 has been found in HaCaT cells after UVB irradiation, suggesting a p53-independent regulation of p21 expression (47). Whether a similar mechanism is involved in IFI27 knockdowned HaCaT cells needs further investigation. However, a recent study has reported that mutp53 in HaCaT cells, though not binding to the *p21* gene promoter, has been shown to act both by tethering growth-controlling TFs and high-jacking p63 to discrete DNA elements in various promoters, and still participate in regulation of cell cycle. As analysed by microarray methods, removal of mutp53 has resulted in up-regulation of genes of the cell cycle process (48). Thus, higher phosphorylated level of p53 at Ser15 in IFI27 knockdowned HaCaT cells suggests enhanced control of cell cycle progression that deserves future study in more detail.

In recent studies, topical treatment with IMQ has been reported to be a novel mouse model for psoriasis-like skin inflammation, inducing acanthosis, parakeratosis and mixed inflammatory infiltrate (13). In our study, after IMQ treatment, C57BL/6 mice started to develop erythema, skin scaling with increased hardness, and thickening of the skin on days 2–4, maximal change being reached by days 8–10 (data not shown). Furthermore, attenuated epidermal hyperproliferation has been shown in the back skin of IMQ-treated mice by IFI27 siRNA treatment, represented by reduced epidermal thickness as well as by significant reduction in number of PCNA-positive cells in the epidermis.

In addition to the function of participating in regulation of proliferation and cell cycle as demonstrated in this study, IFI27 has been shown to be closely associated with some inflammatory conditions including virus infection, Sjogren's syndrome, lupus erythematosus and inflammatory bowel disease (49–52). It has also been shown that 3–17-fold up-regulation of IFI27 mRNA expression was stimulated by IFN-γ, TNF-α or TGF-β1 (10). Together,

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the function of IFI27 is expected to be involved in certain inflammatory mechanisms which are worth further investigation in the future.

In conclusion, the present study demonstrates that IFI27 was required for cell proliferation *in vitro* and *in vivo*, and its function was involved in transition of cells from S to G2 phases in the cell cycle. Inhibition of p53 phosphorylation and p53-independent p21 expression were shown. These results suggest that IFI27 could be a novel potential therapeutic target for treatment of psoriasis that deserves further investigation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. IFI27 was highly expressed in HaCaT keratinocytes.

Fig. S2. Effects of IFI27 downregulation in primary human keratinocytes are similar to HaCaT keratinocytes.

Fig. S3. IFI27 regulated the cell proliferation and cell cycling of keratinocytes.

Fig. S4. The knockdown of IFI27 expression did not trigger the cell apoptosis of HaCaT cells.

Fig. S5. The knockdown of IFI27 in HaCaT cells resulted in the up-regulation of cyclin A expression.

Fig. S6. Down-regulation of IFI27 increased the phosphorylation of CDK1 and expression of p21 in HaCaT cells.

Fig. S7. The reduction of epidermal thickness in imiquimod (IMQ)-induced psoriasis-like skin by IFI27 siRNA-2.

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