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Epigenetic Downregulation of SFRP4 Contributes to Epidermal Hyperplasia in Psoriasis

Jing Bai,* Zhaoyuan Liu,* Zhenyao Xu,* Fang Ke,* Lingyun Zhang,* Huiyuan Zhu,* Fangzhou Lou,* Hong Wang,* Ye Fei, † Yu-Ling Shi,‡ and Honglin Wang*

Psoriasis is a chronic recurrent inflammatory skin disorder characterized by the dysregulated cross-talk between epidermal keratinocytes and immune cells, leading to keratinocyte hyperproliferation. Several studies demonstrated that Wnt pathway genes were differentially expressed in psoriatic plaques and likely were involved in the pathophysiology of disease. However, the molecular mechanisms underlying Wnt signaling regulation in epidermal hyperplasia in psoriasis remain largely unknown. We report that the expression of secreted frizzled-related protein (SFRP) 4, a negative regulator of the Wnt signaling pathway, was diminished in lesional skin of mouse models and patients with psoriasis. SFRP4 directly inhibited excessive keratinocyte proliferation evoked by proinflammatory cytokines in vitro. Pharmacological inhibition of Wnt signaling or intradermal injection of SFRP4 decreased the severity of the psoriasiform skin phenotype in vivo, including decreased acanthosis and reduced leukocyte infiltration. Mechanistically, we identified that aberrant promoter methylation resulted in epigenetic downregulation of SFRP4 in inflamed skin of patients with psoriasis and in the IL-23–induced mouse model. Our findings suggest that this epigenetic event is critically involved in the pathogenesis of psoriasis, and the downregulation of SFRP4 by CpG island methylation is one possible mechanism contributing to the hyperplasia of epidermis in the disease. The Journal of Immunology, 2015, 194: 000–000.

Psoriasis vulgaris is the most common chronic inflammatory skin disorder, with a prevalence of 0.1–6.5% depending on the ethnic and geographical characteristics of the studied population (1). The disease is characterized by keratinocyte hyperproliferation and massive leukocyte infiltration in inflamed skin, and the dysregulated cross-talk between epidermal keratinocytes and immune cells leads to epidermal hyperplasia in psoriasis (2).

The pathogenesis of psoriasis is complex and involves genetic, environmental, immunological, and even, neurologic, factors. Genetic-associated studies identified dozens of psoriasis-associated genes and the signaling pathways (3), such as the IL-23/Th17 axis (4), the NF-κB signaling pathway (5), and the epidermal differentiation complex (6, 7). Recently, several independent array-based studies revealed that Wnt signaling was altered in psoriatic skin compared with normal skin (8–10), suggesting that this pathway might be involved in psoriasis pathogenesis.

The Wnt proteins are highly conserved, lipid-modified, secreted molecules participating in multiple developmental processes during embryogenesis, and they have fundamental roles in controlling cell proliferation, cell fate determination, and differentiation during adult homeostasis (11, 12). Classically, Wnt signaling is categorized into two major pathways. The canonical Wnt pathway involves wnt ligands binding to frizzled receptors in conjunction with low-density lipoprotein receptor–related proteins 5 and 6 as coreceptors (11). This pathway activates nuclear translocation of β-catenin and consequently leads to regulation of gene transcription, which are usually linked to cell fate determination and stem cell maintenance (13). The noncanonical Wnt pathway is β-catenin independent and also participates in cell proliferation, adhesion, and differentiation (13). This pathway includes the planar cell polarity pathway typically regulating the cytoskeleton, which is responsible for the shape of the cell, and the Wnt/calcium pathway, which regulates calcium inside the cell (14). Wnt antagonists can be classified into three classes: secreted frizzled-related proteins (SFRPs), Dickkopf (Dkk) proteins, and Wnt inhibitory factors (WIFs) (11, 13). SFRPs were the first Wnt antagonists to be identified. Five mammalian SFRPs have been identified and named SFRP1–5 (13). These proteins bind Wnt ligands and prevent them from binding to the frizzled receptors, thus inhibiting Wnt signaling activity (15). SFRPs are expressed in a variety of embryonic and adult tissues, indicating that they may provide a common mechanism for inhibiting Wnt signaling in diverse tissues and cell types (16). Despite the intensive studies of Wnt proteins in controlling cell proliferation and differentiation, very little is known about the functions of such Wnt modulator molecules in epidermal hyperplasia in psoriasis (10).

Because Wnt signaling was first identified for its role in carcinogenesis, studies showed CpG island methylation of Wnt pathway genes in various cancers (17, 18). Considering the negative regulating function of SFRPs, we hypothesized that CpG

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The microarray data presented in this article have been submitted to the Gene Expression Omnibus database under accession number GSE60804.

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methylation also occurred in the promoter of SFRPs in psoriasis, which might cause the decreased expression of SFRPs. Interestingly, the discordance rate for psoriasis among monozygotic twins is 35–72%, which also strongly suggests the contribution of epigenetic or environmental factors in the pathogenesis of disease (19-21). DNA methylation, a critical epigenetic modification of the genome, is involved in the regulation of several developmental and cellular processes, including transcription, embryonic development, X-chromosome inactivation, chromosome stability, and genomic imprinting (22). Using methylated DNA immunoprecipitation sequencing, the whole-genome DNA methylation patterns in involved and uninvolved skin lesions from patients with psoriasis were characterized recently, suggesting that DNA methylation might act in an epigenetic dysregulation of biological pathways in psoriasis (23). The aim of the current study was to investigate whether the Wnt antagonist SFRP4 is functionally involved in the hyperproliferation of epidermis, as well as the upstream epigenetic mechanisms responsible for the downregulation of SFRP4 in psoriasis.

Materials and Methods

Mice

C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were kept under specific-pathogen-free conditions, in compliance with the National Institutes of Health Guide 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. To ameliorate any suffering of mice observed throughout these experimental studies, they were euthanized by CO2 inhalation.

Imiquimod and IL-23–induced mouse model of psoriasis

In the IL-23–induced mouse model, mice were injected i.p. (24) with 300 μl CG-3 (MedShine) or 300 μl control (PBS) or 1 or 2.5 mg/kg (body weight) 5-aza-2’dC (MedShine). A total of 25 6–8 wk of age) were sacrificed, and the ear samples were collected. The fresh tissue samples were snap-frozen in liquid nitrogen and stored at −80°C. The paraffin sections for immunohistochemistry and methylation analysis were provided by Y.-L.S. and Y.F., and patient information is included in Supplemental Table I. All individuals provided informed consent. The study was performed in accordance with the Declaration of Helsinki Principles and was approved by the Research Ethics Board of Shanghai Ten People’s Hospital, Tongji University School of Medicine, and Shanghai Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine.

RNA extraction, reverse transcription, and qPCR

Total RNA was extracted from skin biopsies using TRizol reagent (Invitrogen; catalog no. 15596-026). For epidermis RNA extraction, skin samples were incubated overnight at 4°C in Dispe II (2.5 U/ml; BD Biosciences; catalog no. 354235) to completely separate the epidermis from the dermis. CDNA was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen; catalog no. 1209902). qPCR was carried out with FastStart Universal SYBR Green Master (Roche; catalog no. 0413914001) in a 7500 Real-Time PCR system (ABI). The relative expression of target genes was confirmed using quantity of target gene/quantity of GAPDH.

The primer sequences used were:

- β-actin, forward primer, 5’-TTGTCCTCCCGAGAAGC-3’; reverse primer, 5’-AGATACCCCTCAACAACAGG-3’.
- GAPDH, forward primer, 5’-GGAGGGCCGTTGCTGG-3’; reverse primer, 5’-GACATGGAGTGTAGTGGCA-3’.
- SFRP4, forward primer, 5’-CTGACGGACATGCACTGTC-3’; reverse primer, 5’-CTTTTGCGCTCTGTTCAAG-3’.
- CO2, forward primer, 5’-GGACGACCCAGGCCGTC-3’; reverse primer, 5’-TAAAGACGGTACCTGACAAG-3’.

Microarray experiments

Gene-expression profiles were analyzed with the Whole Mouse Genome Microarray (Agilent; catalog no. 014868). Total RNA was extracted using TRizol reagent (Invitrogen; catalog no. 15596-026). The following experiments and data normalization were performed by Shanghai Biotechnology. Microarray data have been submitted to the Gene Expression Omnibus database under accession number GSE60804 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60804).

Human subjects

Twelve patients with mild to severe plaque psoriasis and 10 healthy controls (6 males/4 females for patients and 2 males/8 females for controls; mean age ± SD 32.1 ± 10.6 and 37.3 ± 9.4, respectively) were enrolled for sampling, quantitative real-time PCR (qPCR) analysis, and Western blotting of SFRP4. The Psoriasis Area and Severity Index for patients was 13.8 ± 4.2 (mean ± SD). Psoriatic skin samples (n = 12) were obtained by punch biopsy under local lidocaine anesthesia. Nonlesional skin was also taken from three patients and used for Western blotting. The locations of harvest sites were usually leg, arm, back, waist, and chest. The studied subjects did not use any systemic antipsoriatic biological medicine. Some patients who used topicals stopped the treatment 2 wk prior to biopsy. Normal adult human skin specimens (n = 10) were taken from healthy adults undergoing plastic surgery. The fresh tissue samples were snap-frozen in liquid nitrogen and stored at −80°C. The paraffin sections for immunohistochemistry and methylation analysis were provided by Y.-L.S. and Y.F., and patient information is included in Supplemental Table I. All individuals provided informed consent. The study was performed in accordance with the Declaration of Helsinki Principles and was approved by the Research Ethics Board of Shanghai Ten People’s Hospital, Tongji University School of Medicine, and Shanghai Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine.

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FIGURE 1. Diminished SFRP4 expression in IMQ- and IL-23–induced psoriasiform skin disease. (A) Total RNAs prepared from lesional skin of three mice treated with vehicle or IMQ were subjected to a microarray analysis. Transcripts of Wnt genes are listed. (B) qPCR analysis of SFRP4 expression in lesional skin of mice treated with vehicle or IMQ (n = 6–10) and in ear skin of mice treated with PBS or rmIL-23 (n = 10). (C) Immunofluorescence staining of SFRP4 in skin of mice treated with IMQ or vehicle. IgG was used as the negative control. (D) Immunofluorescence staining of SFRP4 in ear skin of mice treated with rmIL-23 or PBS. IgG was used as the negative control. (E) Western blotting analysis of SFRP4 in epidermis of mice treated with vehicle or IMQ (n = 5). (F) Western blotting analysis of SFRP4 in ear skin of mice treated with PBS or rmIL-23 (n = 5). Densitometry of (Figure legend continues)
**FIGURE 2.** Decreased SFRP4 expression in psoriatic epidermis of patients. (A) qPCR analysis of SFRP4 expression in epidermis of healthy skin and lesional skin from patients with psoriasis (n = 8–12). (B) Western blotting analysis of SFRP4 in epidermis of skin from healthy controls (NN), as well as in paired nonlesional (PN) and lesional (PP) skin from patients with psoriasis (n = 3). Densitometry of Western blots is shown (right panel). (C) Immunohistochemical staining of SFRP4 in epidermis of healthy skin and lesional skin from patients with psoriasis. Data are from one experiment representative of two independent experiments (mean ± SEM) (A) or from one experiment representative of three (C). The black dashed lines indicate the border between the epidermis and dermis. Scale bars, 100 µm. *p < 0.05, **p < 0.01, two-tailed Student t test. ns, not significant.

Western blotting

After incubating at 4°C in Dispase II (2.5 U/ml; BD Biosciences, catalog no. 354235) overnight, epidermis was completely separated from the dermis. The epidermis or the whole skin was lysed in radioimmunoprecipitation assay buffer (Cell Signaling Technology; catalog no. 9806S) supplemented with protease and phosphatase inhibitor cocktails (Thermo Scientific; catalog no. 78440). Mouse anti-actin Ab (1:3000; Cell Signaling Technology; catalog no. 3700S), rabbit anti-SFRP4 Ab (1:2000; Abcam; catalog no. ab154167), HRP-labeled Goat Anti-Mouse IgG (H+L) (1:1000; Beyotime; A0216), and HRP-labeled Goat Anti-rabbit IgG (H+L) (1:1000; Beyotime; A0208) were used.

**Immunohistochemistry**

For immunohistochemistry, paraflin-embedded tissue sections (5 µm) from normal and lesional psoriatic skin were cut from routine blocks, deparaffinized with xylene, rehydrated, and subjected to heat-induced epitope retrieval methods before incubation with the appropriate Abs. Sections were immersed in 1 mM EDTA (pH 8) and subsequently heated in a pressure cooker for 10 min. After rinsing in PBS, the sections were incubated in 3% H2O2 for 25 min at room temperature. After rinsing in PBS, the sections were blocked with a rabbit monoclonal anti-SFRP4 Ab (1:1000; Abcam; catalog no. ab154167), HRP-labeled Goat Anti-Mouse IgG (H+L) (1:1000; Beyotime; A0216), and HRP-labeled Goat Anti-rabbit IgG (H+L) (1:1000; Beyotime; A0208) were used.

**Immunofluorescence staining**

The method used for immunofluorescence staining was described previously (25). The primary Abs were rabbit anti-SFRP4 Ab (1:100; Abcam; catalog no. ab154167), mouse anti-actin Ab (1:3000; CELL Signaling Technology; catalog no. A7030), rabbit anti-SFRP4 Ab (1:2000; Abcam; catalog no. ab154167), HRP-labeled Goat Anti-Mouse IgG (H+L) (1:1000; Beyotime; A0216), and HRP-labeled Goat Anti-rabbit IgG (H+L) (1:1000; Beyotime; A0208) were used.

**Pharmacological inhibition of tankyrases**

XAV-939 (R&D Systems; catalog no. 3748), a selective inhibitor of tankyrase (TNKS)-1 and TNKS-2, was injected i.p., at a dose of 1 mg/ml, once a day for seven consecutive days of IMQ treatment (injection volume...
Control mice were injected with 100 μl 10% DMSO/90% 0.9% NaCl, the solvent for XAV-939 (26).

**In vivo administration of SFRP4**

rmSFRP4 (Sino Biological; catalog no. 50053-M08H), 15 ng/ml, was injected intracutaneously every other day during IMQ treatment (injection volume 75 μl) (27, 28). Control mice were injected with 75 μl PBS, the solvent of recombinant protein.

**Histological analysis of skin inflammation**

After treatment with IMQ, the mouse back skin was fixed in formalin and embedded in paraffin. Sections (6 μm) were stained with H&E. Epidermal hyperplasia (acanthosis) and the number of dermal-infiltrating cells were assessed as histological features. Briefly, for measuring acanthosis, the epidermal area was outlined, and its pixel size was measured using the lasso tool in Adobe Photoshop CS4. For counting dermal-infiltrating cells, nine areas (1.5 in² each) in three sections of each sample were taken randomly, and the number of infiltrating cells was calculated.

**BrdU incorporation**

Normal human epidermal keratinocytes (NHEKs; catalog no. FC-0007) were cultured with DermaLife basal medium supplemented with a DermaLife K LifeFactors Kit (catalog no. LL-0007), and 0.05% trypsin/0.02% EDTA and Trypsin Neutralizing Solution (TrypKit; catalog no. LL-0013; all from Lifeline Cell Technology) were used to subculture NHEKs. To monitor the effect of SFRP4 on cell proliferation in vitro, NHEKs were seeded in a six-well plate and starved for 24 h with basal medium. The medium was changed back to complete medium. Subsequently, 50 ng/ml recombinant human (rh)IL-6 and 0, 25, or 50 ng/ml rhSFRP4 (R&D Systems; catalog no. 1827-SF-025/CF) were added to the medium. After 24 h of incubation, cells were incubated with 10 μM BrdU for 1 h at 37°C before being harvested. BrdU staining was performed using an APC BrdU flow kit (BD Biosciences; catalog no. 552598). Cells were analyzed by flow cytometry (BD Biosciences; FACSCanto II), and the numbers of cells in the S phase were counted as proliferative cells.

**Methylation analysis**

Bisulfite conversion of genomic DNA of formalin-fixed, paraffin-embedded skin tissues from either 10 psoriasis patients or 10 normal individuals or mouse models of psoriasis and normal mice were processed with an EpiTect Fast Bisulfite Kit (QIAGEN). For human samples, a 220-bp sequence from +180 to +440 of SFRP4 was amplified with forward primer, 5'-GTGTTTTGTGTGTTAGA-3' and reverse primer, 5'-CCACTAAAA- TAAAAAACAATCAACA-3' (17). For mouse samples, a 256-bp sequence from -184 to +72 of mouse SFRP4 was amplified with forward primer, 5'-GGGGAAATGTAGAATTATGGTTTTT-3' and reverse primer, 5'-CAACACTCAACCTCCAAAACA-3'. Barcodes were added for the

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**FIGURE 3.** Inhibitory function of SFRP4 for keratinocyte hyperproliferation in vitro. **(A)** BrdU staining of cultured NHEKs with various concentrations of rhSFRP4 or XAV-939 in the presence or absence of rhIL-6 (50 ng/ml). Numbers in the FACS plots indicate the frequencies of gated cells. **(B)** BrdU staining of cultured NHEKs with various concentrations of rhSFRP4 in the absence of rhIL-6 (50 ng/ml). Data are from one experiment representative of two independent experiments.
FIGURE 4. Wnt signaling inhibitor XA V-939 ameliorated the psoriasiform skin disease induced by IMQ. H&E staining of the back skin injected with VC (A) or Wnt-specific inhibitor XA V-939 (B) from mice administered IMQ. Dotted line indicates the border between the epidermis and dermis. Acanthosis (C) and dermal cellular infiltrates (D) of skin injected with PBS or Wnt-specific inhibitor XA V-939 from mice administered IMQ. Cryosections from skin of normal mice (E), IMQ-induced mouse model treated with VC (F), and IMQ-induced mouse model treated with XA V-939 (G) were double stained for mouse macrophage marker F4/80 (green) and T cell marker CD3 (red). DAPI was used to stain nuclei. (H) Quantification of F4/80+ macrophages and CD3+ T cells in normal skin, as well as in skin from IMQ-induced mouse model treated with VC and XA V-939 (n = 5). The data are presented as the numbers of positive cells in the entire fields of view of multiple images. (I) Representative H&E staining shows reduced neutrophilic infiltrates in (Figure legend continues)
amplification products obtained from each sample, and they were pooled together based on similar amounts of DNA. The concentration of each sample was measured with an ND-2000 spectrometer (Nanodrop Technologies). The pools were purified using a QIAquick PCR Purification Kit (QIAGEN; catalog no. 28106). The mixed sample was used for library construction and sequenced on an Illumina MiSeq System using the MiSeq Reagent Kit v3 (2 x 300 cycle; Illumina, San Diego, CA). Barcode-separated pairs of FASTQ files were obtained from the Illumina MiSeq Reporter. The FASTQ files were transformed into FASTA format before methylation analysis at http://jquma.cdb.riken.jp/.

Statistical analysis
Data are mean ± SEM and were analyzed with GraphPad Prism 5, using the Student t test when two conditions were compared. Probability values < 0.05 were considered significant, and the tests performed were two-tailed.

Results
Expression of SFRP4 is diminished in IMQ- and IL-23–induced skin inflammation in mice
We performed a microarray analysis to examine the global gene expression in lesional skin in an IMQ-induced psoriasis mouse model that closely resembles the human disease phenotype (29, 30). The gene-expression analysis showed significant changes in several members of the Wnt signaling molecules in lesional skin treated with IMQ compared with vehicle-treated normal skin (Fig. 1A). Of the Wnt signaling molecules, we further found that SFRP4 expression was most strongly decreased in lesional skin induced by IMQ compared with controls (7.5-fold decrease, p < 0.001) (Fig. 1B, upper panel, Supplemental Fig. 1). In another mouse model of psoriasis induced by rmIL-23, SFRP4 also was found to be significantly decreased compared with the control group (Fig. 1B lower panel). Furthermore, in contrast to vehicle-treated controls, protein levels of SFRP4 were diminished in IMQ- and IL-23–induced psoriasiform lesions, as detected by both immunofluorescence staining (Fig. 1C, 1D) and Western blotting (Fig. 1E, 1F). Together, these data revealed that SFRP4 expression was decreased in lesional skin, particularly in epidermis, in two established mouse models of psoriasis.

Expression of SFRP4 is decreased in psoriatic epidermis in humans
We next investigated SFRP4 expression levels in lesional skin of patients with psoriasis. qPCR analysis demonstrated a significant decrease in SFRP4 mRNA levels in psoriatic epidermis compared with epidermis from healthy subjects (Fig. 2A). In contrast to epidermis derived from normal skin of healthy subjects or nonlesional skin of psoriasis patients, SFRP4 protein levels were significantly downregulated in epidermis of lesional skin derived from psoriasis patients, as revealed by Western blotting (Fig. 2B). However, there was no significant difference in SFRP4 expression between normal skin from healthy subjects and nonlesional skin from psoriasis patients (Fig. 2B). Consistently, in contrast to a clear staining of SFRP4 in basal keratinocytes in normal human skin, SFRP4 was almost undetectable in hyperproliferative keratinocytes in human psoriatic lesions (Fig. 2C). Together, these data indicate that the Wnt antagonist SFRP4 is diminished in psoriatic epidermis in humans, and SFRP4 may be functionally important in keratinocyte hyperproliferation in psoriasis.

SFRP4 inhibits keratinocyte hyperproliferation in vitro
Hyperproliferation of epidermal basal keratinocytes is one of the histopathological hallmark features for psoriasis (31). The mean cell cycle duration of keratinocytes is reduced from 13 d to 36 h in psoriatic lesions (32). To test whether SFRP4 is capable of inhibiting keratinocyte hyperproliferation, we cultured NHEKs with 50 ng/ml rhIL-6 in the absence or presence of rhSFRP4. We found that rhSFRP4, at concentrations of 25 and 50 ng/ml, sufficiently suppressed keratinocyte hyperproliferation, as indicated by reduced cell numbers in the S phase (Fig. 3A, upper panel). XAV-939, a small molecule that selectively inhibits TNKS-1 and TNKS-2, was reported to inhibit the canonical Wnt signaling pathway (33). In this study, we used it as a positive control for inhibiting the proliferation of NHEKs because it was reported to suppress the proliferation of human leiomyoma cells (34) and the colon carcinoma cell line DLD-1 (33). XAV-939 substantially inhibited the hyperproliferation of NHEKs (Fig. 3A, lower panel). Interestingly, we found that rhSFRP4 did not affect the proliferation of NHEKs in the absence of rhIL-6 (Fig. 3B). Together, these findings suggest that SFRP4 is capable of inhibiting keratinocyte hyperproliferation in vitro.

Wnt signaling is critical for mouse epidermal hyperplasia induced by IMQ
We investigated the in vivo functionality of Wnt signaling by injecting Wnt signaling inhibitor XAV-939 in an IMQ-induced mouse model of psoriasis. In contrast to vehicle control (VC), administration of XAV-939 resulted in a significant decrease in the IMQ-induced epidermal hyperplasia (indicated by acanthosis) and dermal inflammatory infiltrates in mice (Fig. 4A–D). Immunofluorescence staining revealed that XAV-939 administration remarkably decreased the infiltration of F4/80+ macrophages and CD3+ T cells in inflamed skin lesions induced by IMQ (Fig. 4E–H). Furthermore, reduced neutrophil infiltrates in microabscesses were observed in the lesional skin treated with XAV-939 compared with VC (indicated by arrows) (Fig. 4I). Moreover, XAV-939 significantly suppressed the expression of proinflammatory cytokines, such as IL-17 and IL-22, with a trend toward decreased expression of IL-1α, IL-6, TNF-α, and IFN-γ in the IMQ-induced mouse model (Fig. 4J–O). These cellular players and proinflammatory cytokines are well known to be pivotal in the pathophysiology of psoriasis. Thus, our data indicate that Wnt signaling is critical for the epidermal hyperplasia mediated by IMQ.

SFRP4 alleviates skin inflammation in the IMQ-induced mouse model
Because SFRP4 was found to be diminished in the epidermis of lesional skin in mouse models and patients with psoriasis, we investigated whether the Wnt gatekeeper SFRP4 harbored a therapeutic potential for the treatment of psoriasis. The intradermal injection of rmSFRP4 substantially improved the psoriasiform phenotype in the IMQ-induced mouse model (Fig. 5A). The epidermal thickness and dermal cellular infiltrates were reduced significantly in mice treated with rmSFRP4 compared with PBS-treated controls (Fig. 5B), suggesting that the Wnt negative regulator rmSFRP4 was able to decrease epidermal hyperplasia in vivo. Furthermore, in contrast to un-
FIGURE 5. rmSFRP4 treatment improved the psoriasiform phenotype in the IMQ-induced mouse model. (A) Representative phenotype of IMQ-induced mouse model of psoriasis treated with PBS or rmSFRP4. Acanthosis (B) and dermal cellular infiltrates (C) in skin injected with PBS or rmSFRP4 in mice administered IMQ. Cryosections from skin of normal mice (D), as well as from IMQ-induced mouse model treated with PBS (E) and rmSFRP4 (F), were double stained for mouse macrophage marker F4/80 (green) and T cell marker CD3 (red). IgG control for immunofluorescence. (Figure legend continues)
treated control mice, rmSFRP4 significantly inhibited the infiltration of F4/80+ macrophages and CD3+ T cells (Fig. 5D–H), as well as the expression levels of proinflammatory cytokines, such as IL-6, IL-17, IL-22, and TNF-α, in inflamed skin lesions in the IMQ-induced mouse model (Fig. 5I–N). Together, these findings suggested that SFRP4 was capable of alleviating skin inflammation by decreasing keratinocyte hyperproliferation, cellular infiltration, and proinflammatory cytokine production in vivo.

SFRP4 promoter is hypermethylated in psoriatic skin in patients

Several studies suggested that DNA methylation may be involved in the pathogenesis of psoriasis (23, 35). To investigate whether aberrant CpG island methylation of SFRP4 exists in lesional skin of patients with psoriasis, we used bisulfite genomic next-generation sequencing on DNA samples derived from normal and psoriatic skin. Bisulfite conversion of genomic DNA and PCR-based MiSeq analysis were performed. By using primers of SFRP4 for Bisulfite Sequencing PCR (BSP), we obtained BSP fragments (the PCR products) that were 220 bp in length and contained 18 CpG dinucleotide sites (Supplemental Table I, upper panel). We then used next-generation sequencing to determine the sequence of BSP fragments; the sequencing depth ranged from 283 to 3181 for all of the PCR products prepared from 10 normal individuals and 10 psoriasis patients (Supplemental Table I, lower panel). By comparing sequencing results with the original SFRP4 sequence, we were able to calculate the percentage of methylation of 18 CpG sites of all of the bisulfite sequences. Methylation at CpG islands in normal skin was at low levels, whereas dense methylation was detected at CpG islands in psoriatic lesions (Fig. 6A). In contrast to normal skin, the percentage of methylation in the SFRP4 promoter region was increased significantly in lesional skin from patients with psoriasis, and half of the tested lesional skin samples (5 of 10 patients) showed >50% methylation (Fig. 6B). These data strongly suggested that the SFRP4 promoter was hypermethylated in psoriatic lesions, and the epigenetic modification was responsible for its downregulation in psoriatic skin in patients.

Methylation of SFRP4 in lesional skin in the IL-23–mediated mouse model

We next evaluated DNA methylation of SFRP4 in the mouse model of psoriasis. Bisulfite next-generation sequencing revealed that the CpG dinucleotides in the S′ region of SFRP4 were significantly methylated in lesional skin in the IL-23–induced mouse model (Fig. 7A, 7B, top panel). However, little aberrant methylation in the S′ region of SFRP4 was found in inflamed skin in the IMQ-induced model (data not shown). Notably, the mouse SFRP4 promoter was more frequently hypermethylated in several CpG sites after rmIL-23 treatment (Fig. 7A). The mean percentage of methylation of all 19 CpG sites in lesional ear skin induced by rmIL-23 was increased significantly compared with controls (Fig. 7B, top panel). However, the mean percentage of methylation of four CpG sites (CpG sites 2, 4, 13, and 19 of the BSP fragments) after rmIL-23 treatment was 8%, which was 6.3-fold higher than that of PBS-treated controls (Fig. 7B, middle panel), and the mean percentage of methylation of CpG site 19 was 9.3%, which was 13-fold higher than that of PBS-treated controls (Fig. 7B, bottom panel). To determine whether SFRP4 expression is regulated by DNA methylation, we investigated its expression in the IL-23–induced mouse model treated with 5-aza-dc. 5-aza-dc is a nucleotide analog of cytosine that is integrated into DNA where it covalently binds DNA methyltransferases to inhibit their catalytic activities and lead to demethylation (36). qPCR analysis showed that 5-aza-dc treatment restored SFRP4 expression in lesional skin induced by rmIL-23 (Fig. 7C). Correspondingly, the acanthosis, ear thickness, and dermal infiltration were reduced significantly after 5-aza-dc administration compared with untreated controls. These data suggested that, in the IL-23–induced mouse model, DNA methylation was involved either directly or indirectly in the downregulated expression of SFRP4.

Discussion

The Wnt pathways are evolutionarily conserved signaling pathways that participate in myriad biological phenomena throughout the development and adult life of human beings and all other animals (37). Abnormal Wnt signaling has been associated with various human diseases, ranging from cancer to degenerative disorders (37). Actually, Wnt signaling has been implicated in almost all types of biological events relevant to skin (13), including cancer (38), inflammation (10), wound healing (39), stem cell biology (40), and aging (41). Psoriasis is the most common chronic skin disorder, which is characterized by relapsing and remitting disease courses, red and scaly patches, papules, and plaques (2). Although it is immune mediated, psoriasis is a complex disease involving inflammation, as well as aberrant wound healing, disturbed stem cell biology, and hyperproliferation of keratinocytes that partially resembles the excessive proliferation of cancer cells (2).

Several studies reported that the global Wnt signaling pathway or expression of some specific Wnt proteins was altered in psoriasis patients (8, 10, 42, 43). In fact, a gene-expression study of 58 patients with psoriasis and 64 normal healthy controls by microarray showed that WIF1 was the most strongly downregulated gene in psoriatic skin versus normal skin (44). More microarrays demonstrated that Wnt5a was increased in psoriatic plaques of patients, indicating that the noncanonical Wnt signaling pathway might be activated in psoriasis (8, 43). Despite the controversial findings of whether the canonical or noncanonical Wnt pathway was activated in psoriasis (9, 10, 42), all of the studies pointed out that the expression of inhibitory factors related to Wnt signaling, such as SFRP, Dkk, and WIF, were reduced to some extent in psoriasis (8, 10). In the current study, we found that the expression of SFRP4, a negative modulator of Wnt signaling (45), was decreased in lesional skin of psoriasis mouse models and patients. SFRP4 is a protein that was identified in 1996; it contains an N-terminal cysteine-rich domain homologous to the putative Wnt-binding site of frizzled proteins and, thus, functions as a modulator of Wnt signaling (15, 46). In our study, we identified that SFRP4 expression was predominantly restricted to the basal and suprabasal cell layers of the epidermis in normal skin, whereas it was diminished in lesional skin from mouse models and patients. Interestingly, another Wnt antagonist, Dkk-1, also was prominently expressed in the epidermal basal cell layer in nonlesional psoriatic skin.
FIGURE 6. Methylation status of specific SFRP4 CpG sites in lesional skin from patients with psoriasis. (A) Schematic representation of the location of the CpG island in the transcriptional region of the human SFRP4 gene. CpG dinucleotides (CpG sites) are indicated by vertical lines, and the gray box shows the CpG island. The black horizontal line under the gray box indicates the region examined by bisulfite next-generation sequencing. Methylation status of specific SFRP4 CpG sites in healthy skin and psoriatic lesions are represented by circles, and the mean percentage of methylation at each CpG site is represented by the fraction of dark shading inside. (B) Statistical analysis of the mean percentage of methylation of SFRP4 CpG sites for normal and psoriatic skin (n = 10). Data are mean percentage (± SEM) of methylation of the 18 CpG sites. ***p < 0.001, two-tailed Student t test. TSS, transcriptional start site.
FIGURE 7. Methylation of SFRP4 in the IL-23–induced mouse model of psoriasis and treatment with 5-aza-dc. (A) Schematic representation of the location of the CpG island in the transcriptional region of mouse SFRP4. CpG sites are indicated by vertical lines, and the gray box shows the CpG island. The black line under the gray box represents the region examined by bisulfite next-generation sequencing. Methylation status of... (Figure legend continues)
skin but was decreased greatly in psoriatic lesions (47). Thus, the dysfunctional Wnt signaling in psoriatic epidermis, likely as a consequence of the diminished expression of Wnt antagonists, such as Dkk-1 and SFRP4, may contribute to the epidermal hyperplasia that occurs in psoriasis.

Exogenous SFRP4 was reported to reduce the proliferation and promote the apoptosis of HaCaT cells (48). Nevertheless, more evidence should be found to draw the conclusion that SFRP4 could inhibit epidermal keratinocyte proliferation. We used NHEKs to test whether SFRP4 was capable of inhibiting keratinocyte hyperproliferation. Using a BrdU-incorporation assay, we found that SFRP4 only decreased the number of NHEKs in the S phase in the presence of IL-6. Without IL-6, even a high dose of SFRP4 failed to reduce the cell number in the S phase. Our data suggested a potential benefit of using SFRP4 as a therapeutic agent for psoriasis treatment because it did not agitate the normal growth of keratinocytes in vitro but efficiently suppressed the keratinocyte hyperproliferation stimulated by IL-6.

The effects of SFRPs’ gain or loss of function also were evaluated in other biological systems, usually in different cancer models. For example, re-expression of SFRP4 in β-catenin–deficient mesothelioma cell lines induced apoptosis and suppressed cell growth (49). The restoration of SFRPs’ expression in colon cancer cell lines was able to attenuate Wnt signaling and elevate apoptosis responses (18). In a model of renal injury, i.p. administration of recombinant SFRP4 protein decreased β-catenin activation, as well as reduced the amount of myofibroblasts and suppressed renal fibrosis (27). Overexpression of SFRPs in mice appears to have a protective effect following induced myocardial infarction, suggesting that these proteins might be useful in conditions involving acute tissue injury (50, 51). With regard to other classes of antagonists of Wnt signaling, Dkk-1 was reported to be required for the development of ischemic neuronal death, and antisense-induced knockdown of Dkk-1 showed a substantial neuroprotective activity in both in vitro and in vivo models (52).

These studies suggested that it might be feasible to improve different diseases by manipulating the expression of these secreted Wnt modulators. To our knowledge, no functional analysis of soluble Wnt inhibitors has been performed in animal models of psoriasis. In the current study, we showed that the IMQ-induced psoriasiform skin disease in mice was improved substantially by injecting rmSFRP4 protein. Our data indicate that modulating Wnt signaling by administering SFRP4 may provide a basis for studies that lead to potential therapies for psoriasis.

Interesting transgenic studies also were conducted to build a genetic link between the pathogenesis of psoriasis and components of the Wnt signaling pathway. Increased Wnt5a expression was confirmed in psoriatic plaques (8, 43). However, transgenic mice with epidermal overexpression of Wnt5a under the control of the K14 promoter failed to show a psoriasis-like phenotype (53). Instead, these transgenic mice displayed impaired hair follicle development. In another study, the expression of Evi, which encodes the Wnt cargo receptor and the knockout of which would cause deficient Wnt secretion, was found to be downregulated in human psoriatic skin biopsies (54). In the same study, the epidermal conditional knockout of this gene led to the development of an inflammatory skin phenotype and loss of hair. However, the phenotype failed to reflect a psoriasis phenocopy due to the lack of αβ T cells or IL-23/Th17 cytokine axis involvement, which was the limitation of mouse models and indicated a neutrophil-driven disease model. Although major changes in skin biology could be induced by manipulating the expression of Wnt pathway components, these genetic studies seemed to implicate that the aberrant expression of these components could not necessarily be interpreted as pathogenesis of psoriasis. In our research, we found that SFRP4 was downregulated in psoriasis patients and mouse models of psoriasis, and we were able to partially rescue the IMQ-induced psoriasiform phenotype by injecting recombinant SFRP4 protein. Although we were far from concluding that ablation of SFRP4 alone was adequate to induce psoriasis, we were able to demonstrate that SFRP4 might be involved in the pathogenesis of psoriasis by agitating skin homeostasis, possibly by working in synergy with other mediators.

In this study, we found that SFRP4 was significantly downregulated in human psoriatic plaques at both the mRNA and protein levels. Bisulfite next-generation sequencing demonstrated that the SFRP4 promoter region was densely methylated in lesional skin samples, whereas there was little methylation in normal human skin. In addition, significant, but moderate, methylation was further identified in psoriasiform lesions in the IL-23–induced mouse model of psoriasis. The difference in methylation status between psoriasis patients and the IL-23–induced model might be explained by the fact that DNA hypermethylation is a time-dependent process (55). Unlike the chronic inflammatory condition in patients with psoriasis, IL-23 induces the psoriasiform skin disease usually within 1 mo, which was sufficient to increase the aberrant DNA methylation in the promoter region of SFRP4, but the extent of methylation might be limited. However, the DNA methylation of SFRP4 in the IL-23–induced mouse model seemed to be heterogeneous, with frequent hypermethylation in some CpG sites. The relatively high level of methylation at several CpG sites might efficiently cause the downregulation of SFRP4 in the IL-23–induced mouse model. To provide more direct evidence, we treated the IL-23–induced mouse model with the demethylation agent 5-aza-dc, and found that it restored the expression of SFRP4 mRNA in vivo. Our results indicated that downregulation of SFRP4 in the IL-23–induced mouse model and psoriasis patients correlated with CpG methylation of the SFRP4 promoter. Unexpectedly, although the expression of SFRP4 also was significantly diminished in the IMQ-induced mouse model of psoriasis, the methylation level was low.

The discrepancy in CpG methylation of the SFRP4 promoter in these two mouse models may be due to the fact that induction of psoriasiform skin inflammation using mIL-23 takes longer than IMQ; however DNA hypermethylation was suggested to be a time-dependent process (55). Also, other epigenetic mechanisms might be involved in the regulation of SFRP4 expression in the IMQ-induced mouse model.

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each of the 19 SFRP4 CpG sites of normal skin and skin treated with IL-23 are represented by circles, and the mean percentage of methylation at each CpG site is represented by the fraction of dark shading inside. (B) Statistical analysis of the mean percentage of methylation of all of the 19 SFRP4 CpG sites (top panel), of 4 CpG sites in the dashed boxes in (A) (CpG sites 2, 4, 13, and 19; middle panel), and of CpG site 19 (bottom panel) for normal and inflammatory skin (n = 10). Data are mean percentage (± SEM) of methylation. (C) Expression levels of SFRP4 quantified using qPCR in control skin of normal mice, as well as from lesional skin of IL-23–treated mice and skin of mice treated with IL-23 and 5-aza-dc at different concentrations. Acanthosis (D), thickness of whole ear (E), and dermal cellular infiltrates (F) in skin from control normal mice, IL-23–treated mice, and mice treated with both IL-23 and 5-aza-dc at different dosages. Data are from one experiment representative of at least two independent experiments (mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.001, two-tailed Student t test. TSS, transcriptional start site.
In summary, we report that SFRP4, a WNT gatekeeper, was predominantly expressed in the epidermal basal layer consisting of keratinocyte stem cells in healthy skin; however it was diminished in the epidermis of lesional skin in psoriasis and its related mouse models. SFRP4 was functionally important for inhibiting the hyperproliferation of keratinocytes in vitro and hyperplasia of epidermis in vivo. The hypermethylated of the SFRP4 promoter region was responsible for its diminished expression levels in psoriatic skin of patients. Furthermore, aberrant DNA methylation of SFRP4 was found in inflamed skin in the IL-23–induced mouse model and administration of 5-aza-dc rescued its expression, reflecting a correlation between CpG island methylation and decreased SFRP4 expression. Hence, our study suggests that SFRP4 functions as a suppressor in psoriasis and that restoration of its expression may offer a new therapeutic approach for the treatment of psoriasis and possibly other autoimmune diseases.

Disclosures

The authors have no financial conflicts of interest.

References


### Supplemental Table 1

**Summary for promoter region of SFRP4.**

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Information of normal individuals and psoriasis patients whose samples were used for methylation analysis.

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**Gender:** F: Female, M: Male. **Age (years):** N1-N10: Normal healthy skin samples. **P1-P10:** Human psoriatic skin samples. **Disease duration (years):** Regular. **PASI:** Psoriasis area and severity index. **BSA:** Body surface area. **R:** Regular.
Supplemental Figure 1. mRNA levels of the Wnt genes. Mice treated with vehicle or IMQ for 7 days were used. qPCR analysis of the expression of Wnt genes in epidermis of mice treated with vehicle or IMQ (n=5-10). *p<0.05, **p<0.01, two-tailed Student’s t-test. Data are from one experiment representative of at least two independent experiments (mean ± SEM). Ctr, vehicle treated control mouse. IMQ, imiquimod treated mouse.