



FULL LENGTH ARTICLE

SFRP5 inhibits melanin synthesis of melanocytes in vitiligo by suppressing the Wnt/ β -catenin signaling

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Received 7 February 2020; received in revised form 25 May 2020; accepted 5 June 2020

Available online 15 June 2020

KEYWORDS

Melanin synthesis;
Melanocytes;
MITF;
SFRP5;
Vitiligo;
Wnt signaling

Abstract Secreted frizzled-related protein 5 (SFRP5) plays a pivotal role in regulating the development of many tissues and organs, however, as an inhibitor of Wnt signaling, the role of SFRP5 in vitiligo remains unknown. Hence, we speculated that SFRP5 might be associated with melanogenesis in melanocytes by regulating Wnt signaling in vitiligo. In this study, we found that SFRP5 was overexpressed in the skin lesions of patients with vitiligo. Compared with that in normal epidermal melanocytes (PIG1), the expression of SFRP5 was increased in vitiligo melanocytes (PIG3V). To investigate the effect of SFRP5 on melanin synthesis, PIG1 cells were infected with recombinant SFRP5 adenovirus (AdSFRP5), and PIG3V cells were infected with recombinant siSFRP5 adenovirus (AdsiSFRP5). The results showed that SFRP5 overexpression inhibited melanin synthesis in PIG1 cells through downregulation of microphthalmia-associated transcription factor (MITF) and its target proteins via suppression of the Wnt/ β -catenin signaling pathway. Accordingly, SFRP5 silencing increased melanin synthesis and activated the Wnt signaling pathway in PIG3V cells. Moreover, SFRP5 overexpression also downregulated the transcriptional activity of T cell factor/lymphoid enhancer factor (TCF/LEF) in PIG1 cells. Furthermore, this inhibitory effect of SFRP5 on melanin synthesis was reversed by treatment with the β -catenin agonist, SKL2001. The inhibitory action of SFRP5 in pigmentation was further confirmed *in vivo* using a nude mouse model. Hence, our results indicate that SFRP5

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Peer review under responsibility of Chongqing Medical University.

can inhibit melanogenesis in melanocytes. Additionally, our findings showed that SFRP5 plays a vital role in the development of vitiligo, and thus may serve as a potential therapeutic target for vitiligo.

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Introduction

Vitiligo is an acquired chronic depigmentation disorder characterized by the destruction of melanocytes in the epidermis,^{1–3} which affects 0.5–1% individuals worldwide.^{4,5} Almost half of the patients have been shown to present symptoms before the age of 20 years.^{6,7} Moreover, vitiligo often affects the face and other visible areas of the body, resulting in a severe psychological burden and decreased quality of life in patients.^{8–10} Although several etiological theories have been proposed, which indicate that genetic predisposition,¹¹ immune responses,^{12,13} melanocytorrhagy¹⁴ and metabolic abnormalities¹⁵ might be involved in the pathogenesis of vitiligo, the exact mechanism of loss of melanocytes in depigmented lesions is still unclear.

Wnt signaling is known to play a crucial role in melanocyte stem cell differentiation.^{16,17} Wnt1 and Wnt3a promote the differentiation of neural crest cells into melanocytes.^{18,19} Xingyu Mei et al²⁰ showed that Wnt5a gene in the Wnt/ β -catenin classical pathway promoted the differentiation and proliferation of melanocytes. Moreover, it has been shown that Wnt/ β -catenin signaling plays a pivotal role in proliferation, migration, and differentiation in vitiligo pigmentation systems.²¹ Claire Regazzetti et al²² demonstrated that in lesional vitiligo, oxidative stress decreases Wnt expression/activation in melanocytes, which supports Wnt agonists to repigment vitiligo lesions. In addition, under certain conditions, the Wnt/ β -catenin pathway has been shown to participate in the activation of microphthalmia-associated transcription factor (MITF) and its downstream melanogenic enzymes in vitiligo.^{23,24} These data suggest that stimulation of Wnt signaling may serve as an adjunct to current therapies for vitiligo.

Secreted frizzled-related protein 5 (SFRP5) is a member of the SFRP family of secreted proteins.²⁵ SFRP5 is structurally very similar to the Fz receptor in the Wnt signaling pathway, and can inhibit the activity of Wnt signaling pathway by competitively inhibiting the Fz receptor.²⁶ Hence, it is well known as an inhibitor of the Wnt signaling pathway.^{25,27} SFRP5 plays a crucial role in regulating the development of other tissues and organs but its effect on vitiligo remains unclear.

Our interest in SFRP5 involves its upregulation in the depigmentation lesions of vitiligo. In our previous study, we found that the mRNA and protein expression of SFRP5 was significantly increased in vitiligo melanocytes (PIG3V) than the expression in normal epidermal melanocytes (PIG1). Hence, we hypothesized that SFRP5 plays a vital role in the onset of vitiligo. In this study, we aim to investigate the

effect of SFRP5 on melanogenesis in human melanocytes and elucidate the underlying mechanism *in vitro* and *in vivo*.

Materials and methods

Biopsy collection

Skin specimens for immunohistochemistry were taken from sixteen vitiligo patients and sixteen matched healthy volunteers. All the biopsy samples were obtained from patients diagnosed with vitiligo upon examination of their clinical and histologic manifestations at the Department of Dermatology, the First Affiliated Hospital of Chongqing Medical University. Skin biopsy samples were obtained from lesional and adjacent perilesional normal skin (usually within 1 cm from the lesion margin) for all patients. Written informed consent was obtained from each patient prior to skin biopsy. This study was approved by the Clinical Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Approval Number: 2020039) and conducted in accordance with the Declaration of Helsinki.

Immunohistochemistry

The biopsy samples were embedded in paraffin, sectioned (4 μ m thick sections), and incubated with primary antibody at 4 °C overnight after antigen retrieval. Next, all sections were subjected to immunohistochemical analysis using immunohistochemistry SP-9000 Kit (Zhongshan Golden Bridge, Beijing, China).

Cell culture

Human normal melanocyte cell line (PIG1) and vitiligo melanocyte cell line (PIG3V) were kindly donated by Dr. Chunying Li (Xijing Hospital, Fourth Military Medical University, Xi'an, China). Cells were cultured in Medium 254 (Gibco, Grand Island, NY, USA) supplemented with Human Melanocyte Growth Supplement (Gibco), 5% fetal bovine serum (Gibco), and 1% penicillin-streptomycin antibiotic mix (Invitrogen, CAL, USA) at 37 °C with 5% CO₂.²⁸ The B16 melanoma cell lines were cultured in Dulbecco's modified Eagle's medium (HyClone Laboratories, UT, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin antibiotic mix at 37 °C in a 5% CO₂ atmosphere.

Adenoviral transfection

Recombinant adenovirus SFRP5 (AdSFRP5) and recombinant adenovirus red fluorescent protein (AdRFP, as control) were kindly donated by Dr. T.C. He (Medical Center of University of Chicago, Chicago, IL, USA). Recombinant adenovirus siSFRP5 (AdsiSFRP5) and recombinant adenovirus green fluorescent protein (AdGFP, as control) were obtained from Hanheng Biotechnology Co., Ltd (Shanghai, China). They were transfected into PIG1 and PIG3V cells, respectively. After 8 h of cultivation, the medium was replaced with fresh medium.

Melanin content and tyrosinase activity assay

The melanin content of melanocytes was determined as previously described with slight modifications.^{29,30} Briefly, PIG1 or PIG3V cells were treated with different adenoviruses. When the fluorescence brightness reached 40–50%, the cells were digested, collected (1×10^7 cells), and lysed in 0.1 M phosphate buffer (pH 6.8). For melanin content determination, pellets were solubilized in 1 M NaOH. The absorbance at 490 nm was measured using a microplate reader. For the tyrosinase activity assay, each sample was incubated with 2 mM L-DOPA (Sigma–Aldrich, Shanghai, China) in 0.1 M phosphate buffer (pH 6.8) for 90 min at 37 °C. After incubation, the tyrosinase activity was measured at 490 nm. The melanin content and tyrosinase activity were normalized with that in the control group.

Immunocytochemical staining

Immunocytochemical staining was performed as previously described.³¹ After treatment with different processing factors, melanocytes were examined for α -melanocyte-stimulating hormone (α -MSH), SFRP5, and β -catenin expression. Rabbit polyclonal anti- α -MSH antibody (Bioss, Beijing, China), anti- β -catenin antibody (Cell Signaling Technology), and mouse monoclonal anti-SFRP5 antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used as the primary antibodies. The secondary antibodies included Alexa Fluor 594 goat anti-mouse IgG (Zhongshan Golden Bridge, Beijing, China) and FITC goat anti-rabbit IgG (Zhongshan Golden Bridge, Beijing, China). 4',6-Diamidino-2-phenylindole (DAPI; Beyotime, Beijing, China) was used as a nuclear counterstain. The fluorescence was detected and captured using a confocal microscope.

Transmission electron microscopy (TEM)

TEM was performed using a previously described method.^{2,32,33} In brief, after experimental treatments, cells were collected, washed twice with phosphate buffered saline (PBS), and fixed in ice-cold glutaraldehyde for 24 h at 4 °C. The cell pellet was then rinsed with Millonig's buffer and minced after fixing in 1.0% OsO₄. The pellet was stained with 2.0% aqueous uranyl acetate, followed by ethanol dehydration and embedding in Spurr's plasticresin. Ultrathin sections were visualized using the Tecnai™ G2 Spirit BioTWIN (FEI, Hillsboro, OR, USA).

Real-time polymerase chain reaction (RT-PCR)

After experimental treatments, total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) in accordance with the RNA extraction protocol. Total RNA (1.5 μ g) was used for cDNA synthesis by reverse transcriptase PCR. The cDNA was amplified on a RT-PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using SYBR-Green PCR Master Mix. β -actin was used as the endogenous control. The primers were designed and synthesized by Takara (Table 1). Each experiment was performed three times.

Western blotting

Cells were washed three times with PBS and lysed in RIPA lysis buffer (Beyotime, Beijing, China) supplemented with a protease inhibitor, phenylmethanesulfonyl fluoride (Sigma–Aldrich). Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The primary antibodies used were as follows: anti-SFRP5 (GTX114747) obtained from GeneTex (Irvine, CA, USA); anti-MITF (ab140606), anti-TYR (ab170905), anti-TRP1 (ab178676), and anti-TRP2 (ab221144) purchased from Abcam (Cambridge, MA, USA); anti- β -catenin (#8480), anti- β -catenin^{S675} (#4176), anti-GSK3 β (#12456), anti-GSK3 β ^{S9} (#9322), and anti- β -actin (#3700) purchased from Cell Signaling Technology (Danvers, MA, USA). The PVDF membranes were incubated with primary antibody overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody (Zhongshan Golden Bridge, Beijing, China) for 1 h at 37 °C. Finally, the protein bands were visualized with the SuperSignal West Pico Chemiluminescent Substrate Kit (EMD Millipore, Billerica, MA, USA).

Promoter analysis

Promoter analysis was performed as previously described.^{34,35} Melanocytes were seeded in T-25 flasks and transfected with 4 μ g of p-BGlu T cell factor/lymphoid enhancer factor (TCF/LEF) plasmids. After 24 h, the

Table 1 Primer sequences for RT-PCR.

Genes	Sequence of primers 5' to 3'
<i>β-actin</i>	F: CACCACACCTTCTACAATGAGC R: GTGATCTCCTTCTGCATCCTGT
<i>SFRP5</i>	F: TGCCCTTGCCACAGTTAGA R: GAGGGAACAGGGATAGGAGAACA
<i>MITF</i>	F: CAACAACCTCGGAAGTGGGACT R: TCCATGCTCATCTGCTCCTCC
<i>TRP1</i>	F: CTGTGCTGAGCCAGGCTAAGTG R: GCCATCACCCAGGAGACAA
<i>TRP2</i>	F: GGAGTGGTCCCTACATCCTACG R: GAATGGATGTTCTGCCGAATC
<i>TYR</i>	F: GACAAATCCAGAACCCCAAGG R: GAGGCATCCGCTATCCCACT

F, forward; R, reverse.

transfected melanocytes were seeded into 24-well plates and treated with AdSFRP5, AdRFP, AdGFP, and AdsiSFRP5 for 12 h. At the scheduled time points, cell supernatants were assayed using the luciferase assay kit, and the level of luciferase expression was detected by GloMax luminescence detector (Gairdner, Wuhan, China).

Xenograft mouse experiment

The *in vivo* experiments were approved by the Animal Experiment Administration Committee of Chongqing Medical University, Laboratory Animal Research. Approximately 5×10^6 AdRFP-, AdSFRP5-, AdGFP-, or AdsiSFRP5-infected B16 cells were briefly suspended in 100 μ L of serum-free media and injected subcutaneously into nude mice. The changes in skin color of nude mice were observed every other day. The mice were sacrificed by cervical vertebra dislocation after 2 weeks, and the skin tissues around the injection site were collected and embedded in paraffin for immunohistochemical staining. All procedures related to animal handling, care, and treatment were performed in strict accordance with the recommendations in the guidelines for the management of experimental animals. Additionally, the experimental protocol was approved by the Animal Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Approval Number: 2020039).

Statistical analysis

All statistical analyses were performed with SPSS software (version 20.0; SPSS, Inc., Chicago, IL, USA). Student's *t*-test was used to evaluate differences between two groups. Data represent mean $\pm$ standard deviation (SD) for at least 3 independent experiments. A value of $P < 0.05$ was considered statistically significant.

Results

SFRP5 is upregulated in vitiligo lesions and vitiligo melanocytes

Immunohistochemical staining was performed to detect endogenous SFRP5 expression in the skin tissues of healthy control volunteers as well as lesional and perilesional normal skin of vitiligo patients. The results revealed that the protein expression of SFRP5 was higher in vitiligo lesions compared with that in perilesional and healthy control skin (Fig. 1A). The levels of SFRP5 expression in PIG1 and PIG3V cells were analyzed by RT-PCR and Western blotting. The results indicated that the mRNA and protein expression of SFRP5 was significantly increased in PIG3V cells compared with that in PIG1 cells (Fig. 1B and C).

SFRP5 inhibits pigmentation of melanocytes

To investigate the role of SFRP5 in the regulation of melanogenesis, PIG1 cells were infected with AdSFRP5 and AdRFP (as control), separately. Western blot analysis revealed that AdSFRP5 was successfully transfected into PIG1 cells (Fig. 2A, left panel). SFRP5 overexpression did

not affect PIG1 cell proliferation (Fig. S1 online); moreover, flow cytometry revealed that apoptosis and cell cycle were not altered by SFRP5 overexpression (Fig. S2, S3 online). However, SFRP5 overexpression significantly decreased the level of tyrosinase activity (Fig. 2B, left panel). Consistent with the changes in tyrosinase activity, there was a significant decrease in melanin content (Fig. 2B, right panel) and α -MSH expression (Fig. 2D). In addition, the number of dendrites was also detected by scanning electron microscopy, and the results indicated that SFRP5 overexpression remarkably decreased the number of dendrites, as well as the number of melanosomes, as assayed by TEM (Fig. 2E). Furthermore, the effect of SFRP5 downregulation on melanogenesis was also examined. As shown in Fig. 2A (right panel), siSFRP5 adenovirus transfection markedly reduced the expression of SFRP5 in PIG3V cells compared with the AdGFP group. In addition, SFRP5 knockdown significantly upregulated the tyrosinase activity (left panel) and melanin content (right panel) of PIG3V cells (Fig. 2C). Consistently, the expression of α -MSH was also remarkably increased (Fig. 2F). The numbers of dendrites and melanosomes were also increased by SFRP5 downregulation (Fig. 2G). Taken together, these results indicate that SFRP5 has an inhibitory effect on melanin synthesis.

SFRP5 inhibits the expression levels of melanogenesis-associated proteins

To investigate whether SFRP5 affects the expression of melanogenesis-related proteins, we further examined the mRNA and protein expression of the melanogenesis-associated proteins, MITF, tyrosinase (TYR), tyrosine-related protein 1 (TRP1), and tyrosine-related protein 2 (TRP2) in PIG1 cells treated with AdSFRP5 and AdRFP. Results showed that the mRNA and protein levels of MITF, TYR, TRP1, and TRP2 were significantly decreased in AdSFRP5-treated cells compared with those in AdRFP-treated cells (Fig. 3A and B). Similarly, the effect of SFRP5 downregulation on PIG3V cells was examined. As shown in Fig. 3C and D, silencing of SFRP5 significantly increased the mRNA and protein levels of MITF, TYR, TRP1, and TRP2.

SFRP5 inhibits Wnt/ β -catenin signaling in melanocytes

SFRP5 is a known antagonist of the Wnt signaling pathway. While Wnt signaling plays a significant role in regulating melanocyte function, it is unclear whether SFRP5 inhibits melanogenesis in melanocytes through this pathway. Hence, we examined the effect of SFRP5 on the Wnt/ β -catenin signaling pathway in melanocytes. Results showed that SFRP5 downregulation decreased the phosphorylation level of glycogen synthase kinase 3 β (GSK3 β) at Ser9, resulting in a decrease in the phosphorylation of β -catenin at Ser675 and total β -catenin level (Fig. 4A). Consistently, SFRP5 knockdown significantly increased the phosphorylation of GSK3 β at Ser9 and of β -catenin at Ser675 (Fig. 4B). Next, we performed TCF/LEF luciferase reporter assay to investigate whether SFRP5 inactivates β -catenin signaling.

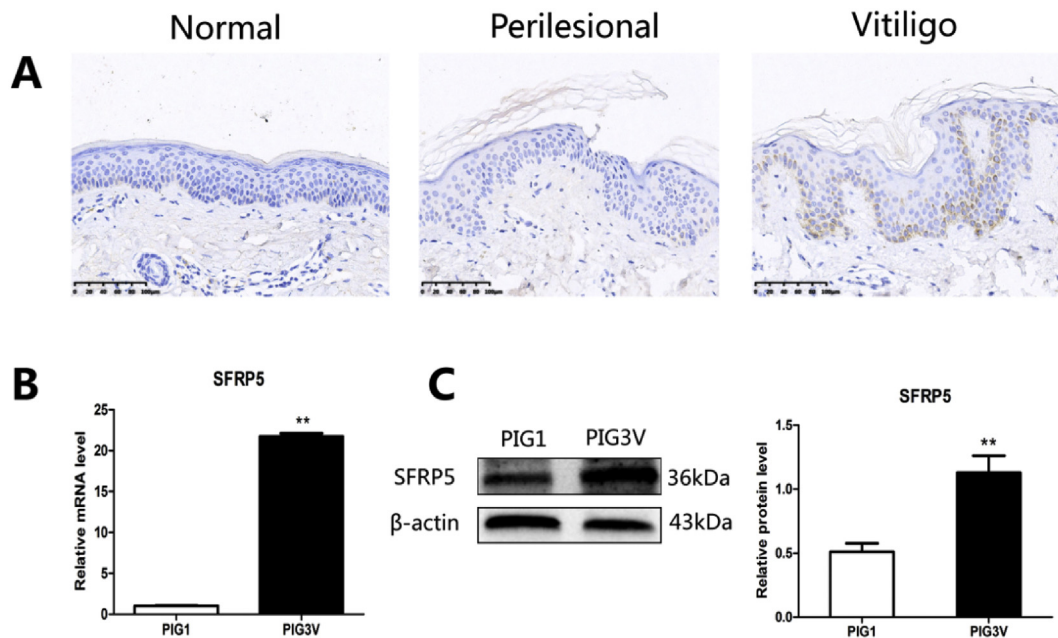


Figure 1 Relative expression of SFRP5 in vitiligo and normal skin. **(A)** Immunohistochemical staining of SFRP5 in vitiligo lesional ($n = 16$), perilesional normal skin ($n = 16$) and normal healthy control skin ($n = 16$). Scale bar = 100 μm (magnification: 400 $\times$). **(B)** The mRNA levels of SFRP5 in normal melanocytes (PIG1) and vitiligo melanocytes (PIG3V). **(C)** The protein levels of SFRP5 in PIG1 and PIG3V. Error bars are the means $\pm$ SD, $n = 3$. ** $P < 0.01$ vs. control.

Treatment with AdSFRP5 inhibited luciferase activity and green fluorescence protein expression in PIG1 cells, while treatment with AdsiSFRP5 increased luciferase activity compared with control treatment (Fig. 4C). Finally, to further confirm the effect of β -catenin signaling on melanogenesis, double immunocytochemical staining was performed. Confocal microscopic examination showed a significantly reduced proportion of nuclear β -catenin in PIG1 cells overexpressing SFRP5 (Fig. 4D), but a significantly increased proportion of nuclear β -catenin in SFRP5-silenced cells (Fig. 4E).

The inhibitory effect of SFRP5 on melanogenesis can be reversed by the β -catenin agonist SKL2001

To further confirm the role of Wnt/ β -catenin signaling pathway in the inhibitory effects of SFRP5 on melanogenesis in PIG1 cells, SKL2001 was used to activate the Wnt/ β -catenin signaling pathway. The results of concentration screening showed that SKL2001 had an optimal activation effect on Wnt signaling pathway at a concentration of 10 μM (Fig. 5A). Hence, this concentration was selected for subsequent experiments. As shown in Fig. 5B, after treatment with SKL2001, the expression levels of the melanogenesis-associated proteins, MITF, TYR, TRP1, and TRP2, were significantly increased. In addition, β -catenin activation markedly abrogated the SFRP5-induced downregulation of melanogenesis-associated proteins (Fig. 5B). Consistently, the effect of SFRP5 on the level of tyrosinase activity (Fig. 5C) and melanin content (Fig. 5D) was also reversed by treatment with SKL2001.

SFRP5 suppresses melanogenesis of B16 cells *in vivo*

Since PIG1 cells are normal human-derived immortalized melanocytes,³⁶ pigmentation changes are difficult to observe after transplanting them into nude mouse. Although B16 cells are mouse-derived melanoma cells, they were widely used for the research of melanin synthesis.^{37–40} Hence, we chose B16 cells to establish a xenograft model in nude mice. The results showed that the skin was slightly lighter in the AdSFRP5 group than in the control group. Similarly, the AdsiSFRP5 group showed skin hyperpigmentation compared with the AdGFP group (Fig. 6A). Subsequently, *ex vivo* mouse skin was used for immunohistochemical staining, and the results showed that α -MSH expression was decreased in the AdSFRP5 group, but increased in the AdsiSFRP5 group (Fig. 6B).

Discussion

In the present study, we first revealed the overexpression of SFRP5 in vitiligo lesions and vitiligo melanocytes. In addition, we proved that SFRP5 overexpression suppressed melanogenesis by inhibiting the Wnt/ β -catenin signaling pathway in normal melanocytes. Subsequently, we showed that treatment with the β -catenin agonist reversed the inhibitory effect of SFRP5 on melanogenesis in normal melanocytes. Our data further demonstrated that SFRP5 silencing contributed to repigmentation in vitiligo melanocytes through the Wnt/ β -catenin signaling. Finally, we confirmed the inhibitory effects of SFRP5 on melanogenesis

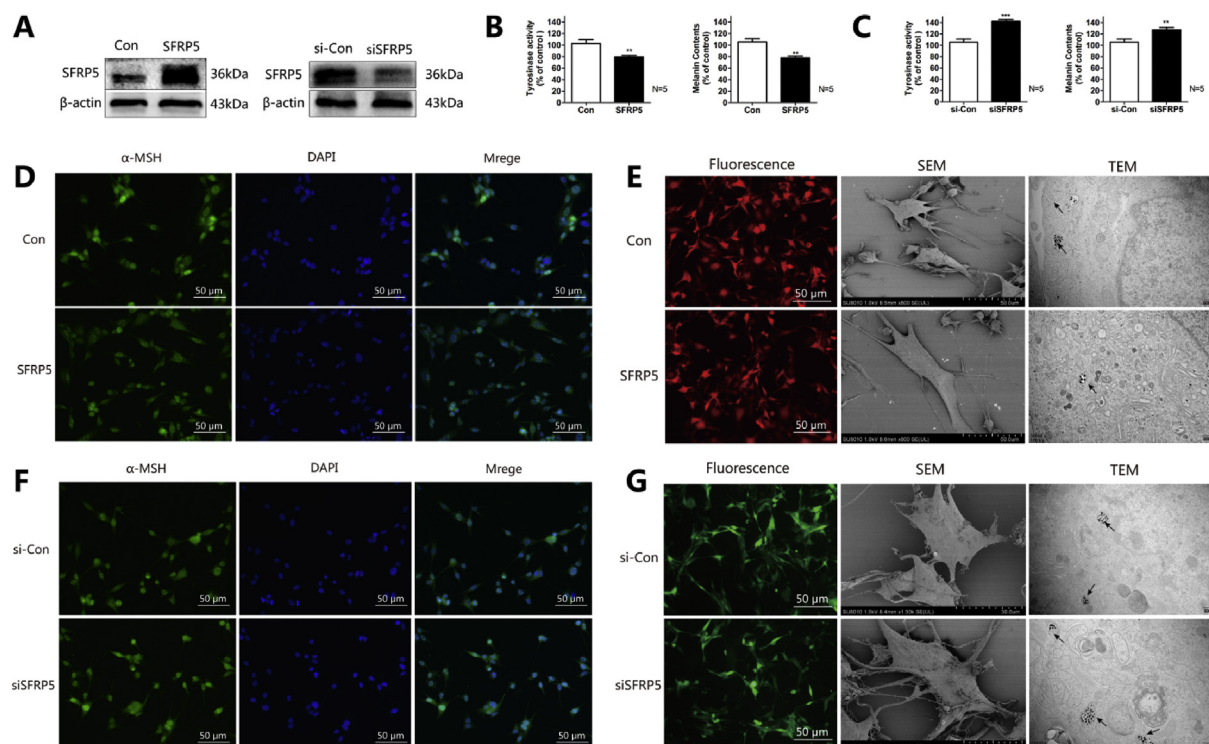


Figure 2 SFRP5 inhibits melanogenesis in normal human melanocytes. (A) Western blot analysis of the expression levels of SFRP5 in AdSFRP5-infected PIG1 cells (left panel) and AdsiSFRP5-infected PIG3V cells (right panel). (B) SFRP5 inhibits tyrosinase activity (left panel) and melanin content in PIG1 cells (right panel). (C) Tyrosinase activity (left panel) and melanin content were measured in SFRP5 knockdown PIG3V cells (right panel). (D) Immunocytochemical analysis the α -MSH expression in the PIG1 cells infected with AdSFRP5 or AdRFP. Nuclei were counterstained with DAPI (blue). (E) SFRP5 decreases the numbers of dendrites and melanosomes. (F) Immunocytochemical analysis the α -MSH expression in the PIG1 cells infected with AdsiSFRP5 or AdGFP. Nuclei were counterstained with DAPI (blue). (G) The numbers of dendrites and melanosomes in SFRP5 knockdown PIG1 cells. Error bars are the means $\pm$ SD, $n = 3$. *** $P < 0.001$ or ** $P < 0.01$ vs. control.

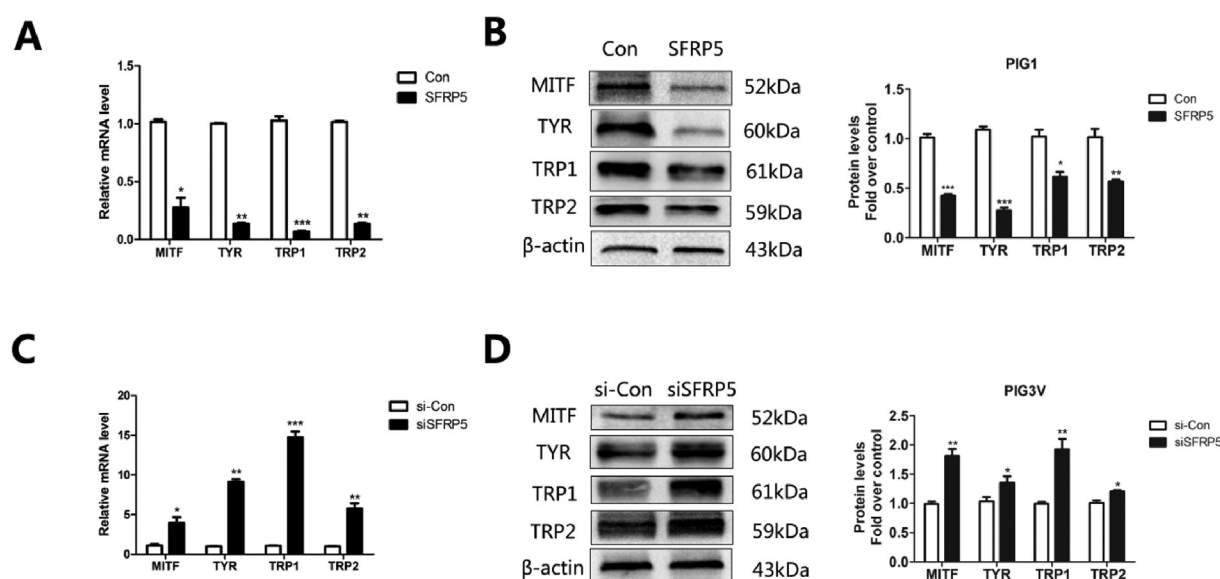


Figure 3 SFRP5 down-regulates the melanogenesis-associated proteins. (A) The expressions of MITF, TYR, TRP1 and TRP2 were examined by means of RT-PCR in AdSFRP5-infected PIG1 cells. (B) Western blot analysis of the protein expression levels of MITF, TYR, TRP1 and TRP2 in AdSFRP5-infected PIG1 cells. (C) RT-PCR analyzed the MITF, TYR, TRP1 and TRP2 mRNA expression levels in AdsiSFRP5-infected PIG3V cells. (D) Western blot analysis of the protein expression levels of MITF, TYR, TRP1 and TRP2 in AdsiSFRP5-infected PIG3V cells. Error bars are the means $\pm$ SD, $n = 3$. *** $P < 0.001$, ** $P < 0.01$ or * $P < 0.05$ vs. control.

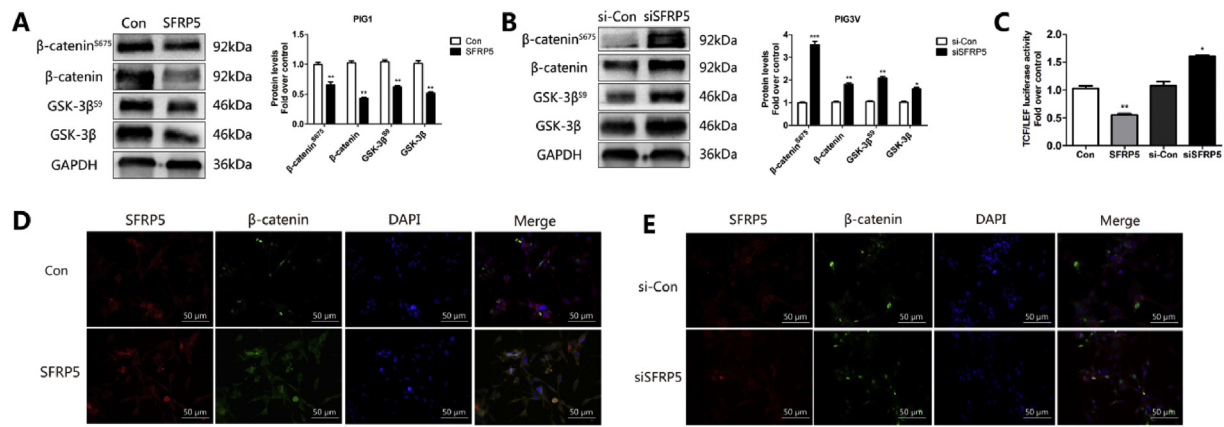


Figure 4 SFRP5 inhibits β -catenin signaling in melanocytes. (A) Western blot analysis of the protein expression levels of β -catenin^{S675}, total β -catenin, GSK-3 β ^{S9} and GSK-3 β in AdSFRP5-infected PIG1 cells. (B) Western blot analysis of the protein expression levels of β -catenin^{S675}, total β -catenin, GSK-3 β ^{S9} and GSK-3 β in AdsiSFRP5-infected PIG3V cells. (C) A TCF/LEF reporter luciferase assay was performed using PIG1 cells infected with AdSFRP5, AdRFP, AdsiSFRP5 or AdGFP. (D) The localization of β -catenin was analyzed using a confocal microscope in PIG1 cells that overexpress SFRP5. Nuclei were counterstained with DAPI (blue). (E) The localization of β -catenin was analyzed using a confocal microscope in PIG3V that knockdown SFRP5. Nuclei were counterstained with DAPI (blue). Error bars are the means $\pm$ SD, $n = 3$. *** $P < 0.001$, ** $P < 0.01$ or * $P < 0.05$ vs. control.

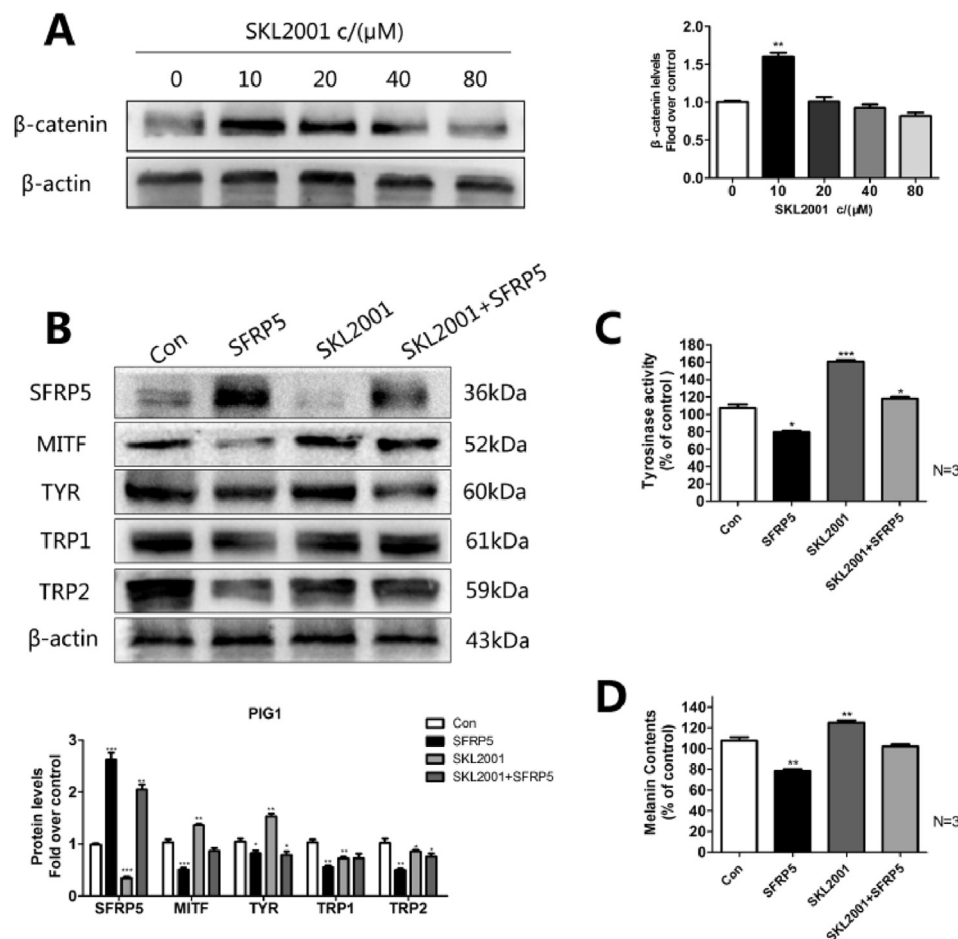


Figure 5 SKL2001 reverses the inhibitory effect of SFRP5 on melanogenesis. (A) The optimal concentration of SKL2001 was detected by Western blot. (B) Western blot analysis of protein expression levels of SFRP5, MITF, TYR, TRP1 and TRP2 in PIG1 cells. (C) Results of tyrosinase activity measurement. (D) Results of melanin content measurement. Error bars are the means $\pm$ SD, $n = 3$. *** $P < 0.001$, ** $P < 0.01$ or * $P < 0.05$ vs. control.

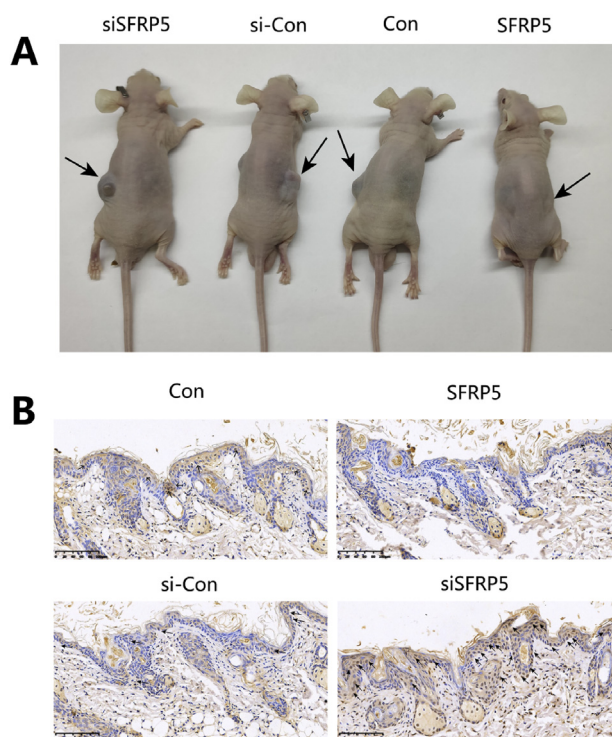


Figure 6 SFRP5 inhibits the melanogenesis of B16 cells *in vivo*. **(A)** The pigmentation on the back skin of nude mice. **(B)** Immunohistochemical staining for the expression of α -MSH. Scale bar = 100 μ m (magnification: 400 $\times$).

in a nude mouse model. Collectively, our study demonstrates that SFRP5 inhibits melanin synthesis in vitiligo melanocytes via the Wnt/ β -catenin signaling pathway, and more importantly, specific knockdown of SFRP5 in melanocytes may be a promising therapeutic approach for vitiligo treatment (Fig. 7).

Vitiligo is an acquired disfiguring skin disorder, mainly characterized by progressive depigmentation due to the destruction of epidermal melanocytes.^{41,42} Although multiple mechanisms are involved in the pathogenesis of vitiligo, the most accepted hypothesis is that both genetic and non-genetic factors affect the function and survival of melanocytes, eventually leading to their immune-mediated destruction.^{42–44} As one of the SFRP family members, SFRP5 has been reported to be associated with several immune-related disorders, such as rheumatoid arthritis,⁴⁵ psoriasis,⁴⁶ and type 2 diabetes mellitus,²⁷ which are close to vitiligo.⁷ In addition, Wnt signaling pathway activation is closely related to repigmentation in vitiligo.^{47–50} However, as an antagonist of the Wnt signaling pathway, the link between SFRP5 and vitiligo remains indistinct. In our previous study, we found that SFRP5 had a lower expression in melanoma tissues than in normal skin tissues, which inhibited the migration and invasion of melanoma cells.⁵¹ In this study, we first demonstrated that the expression of SFRP5 in vitiligo skin lesions was increased. Furthermore, the mRNA and protein expression of SFRP5 was significantly increased in PIG3V cells compared with that in PIG1 cells. Emerging evidences highlight that vitiligo has an inverse relationship with malignant melanoma,^{52–56} which is consistent with our results.

The formation and maturation of melanosomes and the ability to synthesize melanin are key to vitiligo repigmentation.^{57,58} On the basis of its overexpression in vitiligo skin *in vivo*, we hypothesized that SFRP5 inhibits melanogenesis. Overexpression or silencing of SFRP5 expression was then achieved by recombinant or control adenoviruses, respectively, and the direct biological effects of SFRP5 on melanocytes were investigated. The results showed that SFRP5 overexpression reduced melanin content and tyrosinase activity and inhibited α -MSH immunoreactivity in PIG1 cells. Interestingly, SFRP5 overexpression was found to be associated with reduced numbers of dendrites and melanosomes. It is well known that melanin synthesis is an extremely complex process, which mainly involves three enzymes of the tyrosine gene family, namely, TYR, TRP1, and TRP2, of which TYR is the key enzyme for this process.⁵⁹ MITF can regulate the expression of TYR, TRP1, and TRP2, thereby participating in the regulation of the whole melanin synthesis process.⁶⁰ As the ability to synthesize melanin is the key to vitiligo repigmentation, we further investigated the effect of SFRP5 on the levels of melanogenesis-associated proteins. The results indicated that SFRP5 inhibited the mRNA and protein expression of the melanogenesis-associated proteins. These data revealed that SFRP5 can inhibit the biological function of melanocytes.

Next, we also provided evidence that SFRP5 behaves as a negative regulator of the canonical Wnt pathway in human melanocytes. Increasing evidence has highlighted the negative effect of SFRP5 as an antagonist of β -catenin.^{61,62} Moreover, previous findings have also revealed that activation of Wnt/ β -catenin signaling results in accumulation of β -catenin, which forms a complex with LEF-1 to upregulate the gene expression of MITF.⁶³ In addition, β -catenin directly interacts with the MITF protein and activates the MITF-specific target genes.^{50,64,65} In our study, SFRP5 overexpression in PIG1 cells resulted in decreased levels of the active form of β -catenin. Moreover, we found that SFRP5 overexpression induced the downregulation of GSK3 β ⁵⁹, β -catenin^{56,75}, and total β -catenin. Furthermore, SFRP5 overexpression significantly decreased the expression of MITF and LEF-1, which are known downstream target genes of β -catenin. More importantly, the inhibitory effect of SFRP5 on melanogenesis in melanocytes was reversed by treatment with the β -catenin agonist.

Finally, we assessed the efficacy of the SFRP5 overexpression on B16 cells *in vivo*. The results indicated that SFRP5 overexpression lightened the back skin of nude mice, while knockdown of SFRP5 promoted repigmentation of the back skin. Moreover, the most suitable animal model can be generated by using ultraviolet B (UVB) to induce pigmentation in brown guinea pigs^{66–68} or C57BL/6J mouse,^{69,70} and then performing various treatments and observing the color changes. However, because of our limited experimental conditions, UVB induction could not be performed. Moreover, the above-mentioned method is applicable to processing factors that can inhibit pigmentation in mouse skin, but our processing factors both increased and decreased pigmentation. Furthermore, there are several recent studies using nude mice for pigmentation research.^{71–73} Hence, we selected a normal nude mouse model.

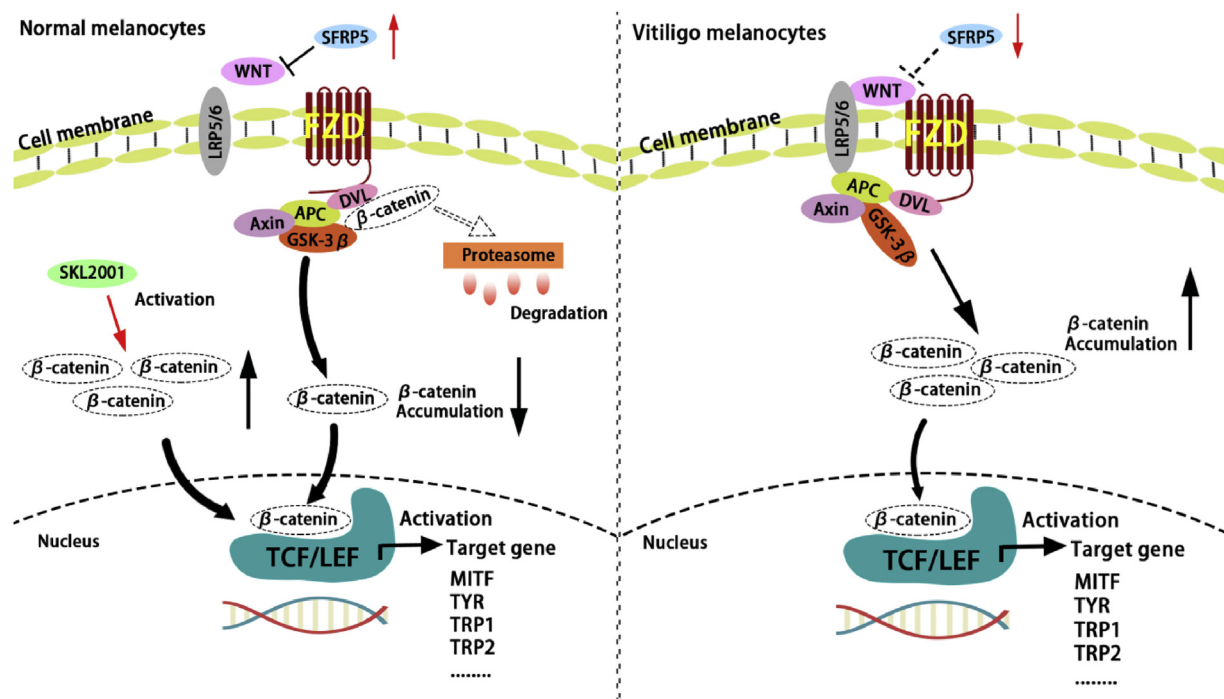


Figure 7 Wnt/ β -catenin signaling pathway regulates melanogenesis of melanocytes. The expression of SFRP5 is increased in vitiligo melanocytes compared with normal melanocytes. In normal melanocytes, overexpression of SFRP5 inhibits the Wnts and then reduces the accumulation of β -catenin in the cytoplasm, subsequently reduces β -catenin entering the nucleus. We use SKL2001 to activate β -catenin, increase its entry into the nucleus, and then proceed nuclear translocation to start the transcription and translation of downstream target genes. In vitiligo melanocytes, We knock down the SFRP5, then the Wnt signaling pathway is activated, β -catenin accumulates in the cytoplasm, subsequently enters the nucleus, nuclear translocation occurs, and transcription and translation of downstream target genes are initiated.

In conclusion, our results showed that SFRP5 inhibits melanin synthesis in melanocytes through the Wnt/ β -catenin signaling pathway *in vitro* and exhibits melanin-inhibiting activity *in vivo*. As for the possible mechanism of SFRP5 upregulation in vitiligo melanocytes and vitiligo skin lesions, we speculate that it may be regulated by epigenetics,⁷⁴ inflammation,⁷⁵ and immune-mediated factors.⁷⁶ In recent years, epigenetic changes in SFRP5, such as SFRP5 gene methylation have been closely related to the occurrence and development of various tumors. Moreover, SFRP5 has been reported to act as a tumor suppressor gene in various cancers,⁷⁷ and it is well known that transcriptional suppression of tumor suppressor genes leads to tumor development. For example, epigenetic inactivation of the SFRP5 gene in human breast cancer is associated with poor prognosis,⁷⁸ hypermethylation of SFRP5 in multiple myeloma is associated with advanced disease stage,⁷⁹ epigenetic silencing of SFRP5 promotes the metastasis and invasion of chondrosarcoma,⁸⁰ and SFRP5 methylation is closely related to the incidence of colorectal cancer and poor prognosis of gastric cancer.^{81,82} Therefore, we speculate that in vitiligo skin lesions, SFRP5 expression may increase due to its demethylation. However, this needs to be confirmed by further experiments. An in-depth investigation of the impact of SFRP5

epigenetic changes on the biological function of melanocytes may contribute to the clinical development of targeted vitiligo drugs.

Availability of data and materials

Data sets used or analyzed during the current study can be obtained reasonably request of the corresponding authors.

Data statement

All data generated or analyzed during this study are authentic. Besides, the datasets during the current study are available from the corresponding author on reasonable request.

Authors contribution

JC and TWG planned the experiments; YMC and DPZ performed the experiments, AI and PTKN prepared the figures and analyzed the data; XHY and LZZ helped to perform experiments; YJZ collected the samples; DPZ composed the

manuscript. All authors read and approved the final manuscript.

Conflict of Interests

The authors declare no conflict of interests.

Funding

This work was supported by the National Natural Science Foundation of China [grant number 81773307]; and a research grant from Chongqing Science and Technology Commission [grant number cstc2018jcyjAX0195].

Acknowledgements

We thank Dr. T.C. He (Medical Center of University of Chicago, Chicago IL, USA) for his kind provision of the recombinant adenoviruses, plasmid and Dr. Chunying Li (Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China) for her kind provision of the human melanocyte cell lines PIG1 and PIG3V. We also thank the vitiligo patients and healthy donors for their participation in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jgendis.2020.06.003>.

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