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ORIGINAL RESEARCH

Role of Autophagy Induced by Pmel17 in the Pathogenesis of Vitiligo

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Background: Vitiligo is a prevalent depigmentation skin disorder with a complex etiology and incompletely understood pathogenesis. Previous studies have suggested a potential involvement of premelanosome protein 17 (Pmel17) in vitiligo, although the specific mechanism remains unclear. This study aimed to explore the association between Pmel17 and vitiligo development, as well as its mechanistic actions.

Methods: Initially, clinical samples from vitiligo patients and healthy individuals were collected to assess Pmel17 and tyrosinase (TYR) expression levels in tissues using immunohistochemistry and RT-PCR. Subsequently, the effect of Pmel17 on the vitiligo phenotype was validated in a mouse model. Finally, at the cellular level, Pmel17-siRNA was transfected into melanocytes to evaluate the effect and mechanism of Pmel17 on melanin synthesis.

Results: Compared with normal skin tissues, the expressions of TYR and Pmel17 in the lesions of patients with advanced vitiligo was significantly reduced. The results of animal experiments demonstrated that Pmel17-shRNA lentivirus infection induced depigmentation in mice and exacerbated the vitiligo phenotype in monobenzone model mice. At a cellular level, down-regulation of Pmel17 expression reduced melanin synthesis and induced autophagy in melanocytes, concomitant with inhibition of the PI3K-AKT-mTOR signaling pathway. Treatment with LY294002, a PI3K/AKT inhibitor, enhanced the suppressive effects of Pmel17 down-regulation on p-AKT and p-mTOR proteins. Conversely, upregulation of Pmel17 in melanocytes did not impact TYR expression or melanin content.

Conclusion: The findings demonstrate that downregulation of Pmel17 contributes to the pathogenesis of vitiligo by inducing autophagy and inhibiting melanin synthesis, through the suppression of the PI3K-AKT-mTOR signaling pathway. These results provide new insights into the molecular mechanism between Pmel17 dysfunction and depigmentation.

Keywords: vitiligo, Pmel17, melanin synthesis, autophagy

Introduction

Vitiligo is a depigmentation disease characterized by the loss of functional melanocytes in the epidermis, with a global prevalence of approximately 0.5%-2.0%. Its typical clinical manifestations are opalescent spots with clear boundaries, which tend to occur on the face, limbs and other exposed areas, seriously affecting the patients' mental health and quality of life.[1] At present, the etiology and pathogenesis of vitiligo are not fully understood. Studies have shown that its development results from the interaction of multiple factors, including genetic susceptibility, oxidative stress, autoimmune responses and intrinsic defects in melanocytes.[2] In particular, the disorder of melanosome synthesis due to melanocyte dysfunction is considered the final pathway leading to depigmentation, although the key molecular mechanisms regulating this process have not been fully elucidated.

Premelanosome Protein 17 (Pmel17, also known as gp100) plays an important role in the formation of signature fibers.[3] Studies have shown that mutations in the *Pmel17* gene can lead to structural abnormalities in melanosomes. For

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example, in mice, mutations in *Pmel17* lead to a silvery coat color, accompanied by disturbances in the structure of melanosome membranes.[4] However, the functional regulatory network of Pmel17 in human skin melanocytes and the association of this network with depigmentation diseases such as vitiligo have not been systematically investigated.

Several studies suggest that Pmel17 may play an important role in the pathogenesis of vitiligo. Yu et al found that *Pmel17* was significantly reduced in vitiligo skin lesions through gene chip analysis.[5] Through genome-wide association analysis, Tang et al identified an important vitiligo susceptibility locus 12q13.2 (rs10876864), which is located in the upstream promoter region of *Pmel17* gene. Patients carrying the risk allele at this locus exhibited an earlier age at onset and a more extensive lesion distribution.[6] Our previous studies found that knockdown of Pmel17 in melanocytes activated lysosomes and led to the degradation of melanosomes.[7] These findings suggest that functional deficiency of Pmel17 may contribute to melanocyte inactivation and the vitiligo phenotype by disrupting melanosome stability and melanin synthesis. In this study, we systematically analyzed the functional regulatory mechanisms of Pmel17 in vitiligo to provide a new perspective on the molecular basis of the disease.

Methods

Antibodies and Reagents

Rabbit anti-Pmel17 (cat. no. ab137078), mouse anti-TYR (cat. no. Ab170905) and mouse anti-β-actin (cat. no. ab8227) antibodies were obtained from Abcam. Rabbit anti-AKT (cat. no. 9272), rabbit anti-phosphorylated AKT (cat. no. 9271), rabbit anti-mTOR (cat. no. 2972), rabbit anti-phosphorylated mTOR (cat. no. 2971) and rabbit anti-P62 (cat. no. 5114) monoclonal antibodies were obtained from Cell Signaling Technology, Inc. Rabbit anti-LC3 antibody (cat. no. L7543) was obtained from Sigma. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (cat. no. HS201) and HRP-conjugated goat anti-rabbit IgG (cat. no. HS101) secondary antibodies were obtained from TransGen Biotech. CY3-labelled goat anti-rabbit secondary antibody (cat. no. A0516) was obtained from Beyotime Biotechnology. LY294002 (cat. no. HY-10108), 3-MA (cat. no. HY-19312) and L-DOPA (cat. no. HY-N0304) were obtained from MedChemExpress.

Clinical Samples

Skin tissues from patients who were diagnosed as advanced non-segmental vitiligo, including four males and four females, aged 19–52 years, were obtained from the Department of Dermatology of Shaanxi Provincial People's Hospital. The exclusion criteria were as follows: a history of other related skin or autoimmune diseases within the past 6 months or treatment with glucocorticoids or immunosuppressive drugs. Skin tissues from healthy individuals (four men and four women, aged 21–56 years) were obtained from the plastic surgery Department of Shaanxi Provincial People's Hospital. These individuals had no history of vitiligo, no systemic immune disease and no acute or chronic medical history. This study was performed in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Shaanxi Provincial People's Hospital, and all participants provided written informed consent.

Immunohistochemistry

Paraffin sections were dewaxed and incubated with 3% H_2O_2 for 20 minutes at room temperature to eliminate endogenous peroxidase. The sections were then washed three times with PBS for 5 minutes each time. After blocking with 10% normal goat serum for 20 minutes at room temperature, the sections were incubated with the primary antibody overnight at 4°C. The HRP-labelled secondary antibody was added and the sections were incubated at 37°C for 20 minutes. The sections were stained with hematoxylin for 3 minutes and observed under a BX41 fluorescence microscope (Olympus).

Melanin Staining

Melanin staining was performed using a Masson-Fontana melanin staining kit (Leagene Biotech. Co., ltd). The procedure was as follows: First, skin sections were gently washed in distilled water, then immersed in Fontana ammonia-silver solution and kept protected from light for 24 h. This was followed by several rinses with distilled water. The sections

were then treated with seaborne solution for 5 minutes and gently counterstained with neutral red dye solution for 5 minutes and rinsed again with distilled water. Finally, the sections were dehydrated using 95% ethanol and anhydrous ethanol, transparent with xylene, and sealed with neutral gum.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using an RNA extraction kit (TIANGEN BIOTECH Co.,Ltd). For quantitative RT-PCR, the SYBR™ Green PCR Master Mix Kit was used (Aucurate Biology). Primers were designed with the following sequences: Pmel17 forward: 5′– CCCCAGGAAACTGACGATGC–3′, Pmel17 reverse: 5′– AGCCACAGGAGGTGAGAGGAAT–3′, TYR forward: 5′–GGCCTCAATTTCCCTTCACA–3′, TYR reverse: 5′– CAGAGCACTGGCAGGTCCTAT–3′, β-actin forward: 5′–CTGGAACGGTGAAGGTGACA–3′, β- actin reverse: 5′–AAGGGACTTCCTGTAACAATGCA–3′. The PCR thermocycling conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 30 cycles at 95°C for 30 seconds, 54°C for 30 seconds and 72°C for 40 seconds. Changes in transcript abundance were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Culture

PIG1 cells, an immortalized human melanocyte cell line, were kindly gifted by Dr. Chunying Li (Xijing Hospital, Air Force Military Medical University). The cells were cultured in M254 medium (Gibco), supplemented with Human Melanocyte Growth Supplement-2 (HMGS-2, Gibco) and 5% fetal bovine serum (FBS, Gibco). Cultures were maintained in an incubator at 37°C with 5% CO_2.

Mouse Model Construction

Four-week-old SPF grade female C57BL/6 mice, weighing 15–18 g, were purchased from Xi'an Jiaotong University Medical Science Center. All animal experiments were approved by the Ethics Committee of Xi'an Jiaotong University. Furthermore, the experiments adhered to the guidelines on animal protection, Laboratory animal - Guideline for ethical review of animal welfare (GB/T 35892–2018, China). Vitiligo was induced in the mice by shaving a 2×2 cm area on the abdomen and applying 40% monobenzone cream (4-benzyloxy phenol, Sigma) until fully absorbed. Application of the cream was discontinued on day 50 and observation continued until day 65. The mice were randomly divided into four groups: control group injected with Lv-sh-NC (Control group), control group injected with Lv-sh-Pmel17 (Pmel17-shRNA group), monobenzone group injected with Lv-sh-NC (Monobenzone group) and monobenzone group injected with Lv-sh-Pmel17 (Monobenzone+shRNA group). For lentivirus injection, 20 μL of 10^8 TU lentivirus was intradermally injected into the shaved back skin of each mouse every 3 days for 5 times. The mice were maintained at a constant temperature of 20±2°C, 50±10% relative humidity, and a 12 h light/dark cycle.

HE Staining

Tissues were fixed in 4% paraformaldehyde for 24 hours, then dehydrated and embedded in paraffin. Then, tissue sections 4 µm thickness were stained with hematoxylin and eosin, respectively. Finally, the images were observed and captured using a BX41 fluorescence microscope (Olympus).

Western Blotting

Total proteins were extracted from cell and tissue samples. The proteins were separated by SDS-PAGE gels, transferred to NC membranes, and blocked with 5% skim milk at room temperature for 2 h. The membranes were then incubated with primary antibodies overnight at 4°C. The primary antibodies used were anti-Pmel17 (1:300), anti-TYR (1:200), anti-β-actin (1:1000), anti-LC3 (1:1000), anti-P62 (1:500), anti-AKT (1:500), anti-phosphorylated AKT (1:500), anti-mTOR (1:400) and phosphorylated mTOR (1:400). After washing three times with PBST, the membranes were incubated with HRP-conjugated secondary antibodies for 40 min at room temperature. Finally, the blots were visualized using a gel imaging system (Alpha Innotech).

Transmission Electron Microscopy (TEM)

Cells were initially fixed with 2.5% glutaraldehyde, rinsed and then fixed with 1% osmium tetroxide. Following fixation, the cells were fractionally dehydrated using acetone, embedded in epoxy resin and sectioned into ultrathin slices. The sections were stained with lead citrate and uranyl acetate, then observed using a transmission electron microscope (JEM-1200EX, JEOL).

Transfection of siRNAs and Overexpressed Vectors

For the targeted knockdown of Pmel17, a pair of Pmel17-siRNA were designed and synthesized by Invitrogen (Thermo Fisher Scientific, Inc). The nucleotide sequences of the siRNAs were as follows: Pmel17-homo-829, sense 5′-GGACUUUGGAGACAGUAGUTT-3′, antisense 5′- ACUACUGUCUCCAAAGUCCTT-3′; negative control (NC)-siRNAs with the following sequences were also used: sense 5′-UUCUCCGAACGUGUCACGUTT-3′ and antisense 5′- ACGUGACGUUCGCAGAATT-3′.

For the overexpression of Pmel17, a pair of primers was designed and synthesized by Invitrogen. The nucleotide sequences of the primers were as follows: sense 5′-CTAGCGTTTAAACTTAAGCTTGCCACCATGGATCTGGTGCTAAAAAGATGCCTTCTT-3′, antisense 5′-CCACACTGGACTAGTGGATCCTCAGACCTGCTGCCCACTGAGGAGGGGGCTGTTC −3′. In total, 5×10^4 melanocytes were seeded in six-well plates and cultured for 24 hours to reach 80% cell fusion. Pmel17-siRNA or pcDNA3.1-Pmel17 was mixed with the transfection reagent Lipofectamine 2000 (Invitrogen) and then added to serum-free medium. After 4–6 hours, the medium was replaced with complete culture medium and the cells were cultured for a further 48 or 72 hours to assess the expression of related genes.

Cell Viability Assay

To assess cell viability, cells were collected into a suspension, and 2×10^3 cells per well were seeded into 96- well plates. After 48 hours of treatment, 30 µL of MTT solution was added to each well, and the cells were incubated at 37°C for 4 hours. The supernatant was then removed, and 100 µL of DMSO was added to lyse the cells. Absorbance was measured at 490 nm.

Tyrosinase Activity Assay

Cells were washed twice with PBS and then lysed using lysis buffer to obtain the supernatant. A 50 µL aliquot of the supernatant was mixed with 100 µL of PBS (0.1 M, pH 6.8) and 50 µL of 0.1% L-DOPA, then incubated at 37°C for 15 minutes. Dopachrome was measured by absorbance at 475 nm using an ELISA microplate reader.

Determination of Melanin Content

Melanin content was determined using the NaOH dissolution method. Melanocytes were collected, and dissolved in 1 mL of 1 M NaOH, then incubated at 80°C for 30 minutes. Absorbance was measured at 405 nm using an ELISA microplate reader.

Immunofluorescence

Cultured melanocytes were seeded onto coverslips overnight then fixed with 4% paraformaldehyde for 30 minutes. After the cells were washed with PBS three times, they were permeated with 0.5% Triton X-100 at room temperature for 15 minutes and then washed with PBS three times. Next, the cells were blocked with 5% normal goat serum for 30 minutes at room temperature and incubated with rabbit anti-LC3B monoclonal antibody overnight at 4°C, followed by CY3-labelled goat anti-rabbit secondary antibody for 2 hours at room temperature. The cells were counterstained with 1 mg/mL DAPI for 5 minutes to visualize the nuclei. Images were captured using an Olympus Fluoview FV1000 confocal laser scanning microscope and analysed with Olympus FV1000 software.

Statistical Analysis

Statistical analysis was performed with SPSS 19.0 statistical software. Significance was determined by one- way ANOVA and post hoc Tukey's test. Data are presented as mean ± SEM. A significance level of $P < 0.05$ was considered statistically significant.

Results

Expression of Pmel17 and TYR Was Decreased in Vitiligo Tissues

The expression of TYR and Pmel17 in skin tissues from eight patients with advanced vitiligo and eight healthy individuals was examined by immunohistochemistry. The results showed that TYR and Pmel17 were primarily expressed in melanocytes located in the basal layer of the skin. Compared with normal skin tissues, the expressions of TYR and Pmel17 in the lesions of patients with advanced vitiligo was significantly reduced (Figure 1a and b). Staining for Pmel17 and TYR was scored separately, and Pearson correlation analysis was performed. The results showed a positive correlation between Pmel17 and TYR expression (Figure 1c and d). The RT-PCR results were consistent with the immunohistochemistry findings, indicating the abnormal expression of Pmel17 in the skin lesions of patients with vitiligo, this may suggest a potential role of Pmel17 in vitiligo (Figure 1e). Melanin staining results revealed higher melanin content in normal skin tissues, while almost no obvious melanosomes were observed in the skin tissue of patients with advanced vitiligo. This confirms that pigmentation was significantly reduced in vitiligo lesions (Figure 1f).

Pmel17 Knockdown Induced Vitiligo Phenotype in Mice

The mice were randomly assigned to four groups: control group injected with Lv-sh-NC (Control group), control group injected with Lv-sh-Pmel17 (Pmel17-shRNA group), monobenzone group injected with Lv-sh-NC (Monobenzone group) and monobenzone group injected with Lv-sh-Pmel17 (Monobenzone+shRNA group). The results showed that mice in both the Monobenzone group and the Pmel17-shRNA group developed white skin spots compared with the Control group, indicating that Pmel17-shRNA can induce depigmentation phenotypes in mice. Moreover, the white skin spots in the Monobenzone+shRNA group were more pronounced than those in either the Monobenzone or Pmel17-shRNA group, suggesting that Pmel17-shRNA enhances the vitiligo phenotype induced by monobenzone (Figure 2a).

After 65 days of observation, the mice were euthanized by cervical dislocation, and the skin tissues were collected for fixation and staining. The results of HE staining and melanin staining showed that the hair follicles in the skin of normal mice contained abundant melanin. By contrast, the other three groups (the Monobenzone group, the Pmel17-shRNA group, and the Monobenzone+shRNA group) showed significantly reduced melanin in the skin hair follicles (Figures 2b and c).

Melanosomes and autophagosomes in the mouse skin tissues were observed by TEM. The results revealed a higher number of melanosomes in the Control group, whereas the Pmel17-shRNA, Monobenzone, and Monobenzone+shRNA groups all showed a marked reduction in melanosomes. Additionally, the Monobenzone+shRNA group showed significant autophagosome formation (Figures 2d and e).

The expression of related proteins in mouse skin tissues was analyzed by RT-PCR. The results showed that the gene expression levels of Pmel17 and TYR were significantly decreased in the Monobenzone group, the Pmel17-shRNA group, and the Monobenzone+shRNA group compared with the Control group. These findings indicate that Pmel17 knockdown reduces the expression of genes involved in melanin synthesis (Figure 3a and b).

Western blotting was used to detect the expression of melanin synthesis and autophagy-related proteins in the skin tissues of mice from each group. The results showed that compared with the Control group, the protein expression levels of Pmel17 and TYR were significantly decreased in the Monobenzone group, the Pmel17-shRNA group, and the Monobenzone+shRNA group. Conversely, expression of the autophagy protein LC3 was elevated, suggesting that Pmel17 knockdown reduces melanin synthesis and induces autophagy (Figure 3c and d).

Pmel17 Knockdown Reduced Pigment Synthesis and Activated Autophagy

Pmel17-siRNA was transfected into immortalized human melanocytes PIG1, and Western blotting results showed that Pmel17-siRNA transfection significantly downregulated Pmel17 protein levels (Figure 4a). At the same time, Pmel17-

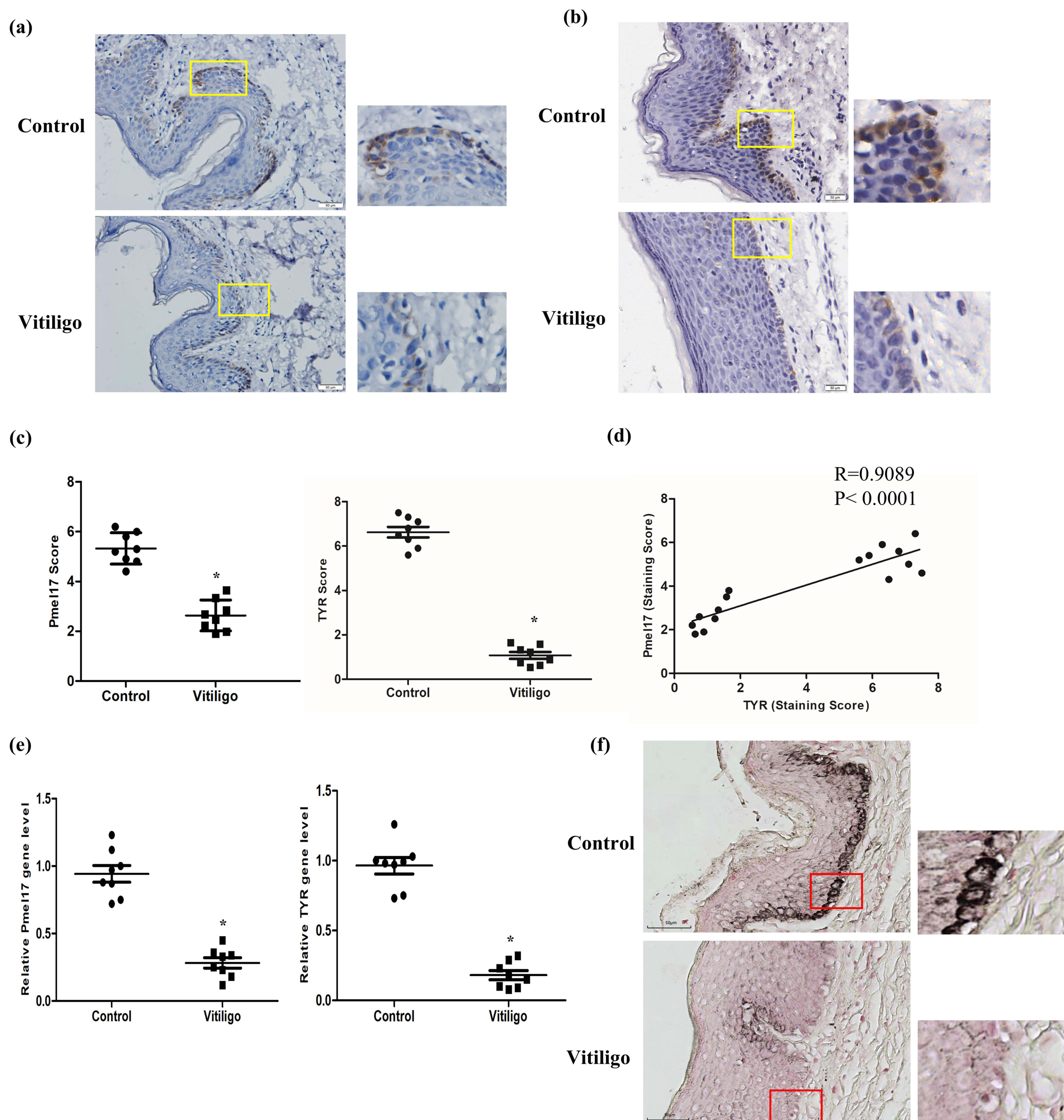

Figure 1 The expression of Pmel17 and TYR in vitiligo tissues were decreased. (**a**) Immunohistochemical staining of Pmel17 in skin tissues from patients with advanced vitiligo and healthy individuals at 400x magnification. The yellow boxes indicate the enlarged parts of the figure. (**b**) Immunohistochemical staining of TYR in skin tissues from patients with advanced vitiligo and healthy individuals at 400x magnification. The yellow boxes indicate the enlarged parts of the figure. (**c**) Statistical analysis of Pmel17 and TYR expression in skin tissues from patients with advanced vitiligo and healthy individuals, n=8, *P<0.05. (**d**) Correlation analysis of Pmel17 expression with TYR expression. (**e**) The expression levels of Pmel17 and TYR in skin tissues from patients with advanced vitiligo and healthy individuals were detected by RT-PCR. (**f**) Melanin staining of skin tissues from patients with advanced vitiligo and healthy individuals. The red boxes indicate the enlarged parts of the figure.

siRNA transfection had no significant effect on cell viability (Figure 4b). Tyrosinase is a key enzyme in melanin synthesis and plays a critical role in determining the rate of pigment production. We examined changes in tyrosinase activity and melanin content in melanocytes after transfection with Pmel17-siRNA. The results showed that Pmel17-siRNA transfection significantly reduced both tyrosinase activity and melanin content (Figure 4c and d), suggesting that downregulation of Pmel17 expression can reduce pigment synthesis.

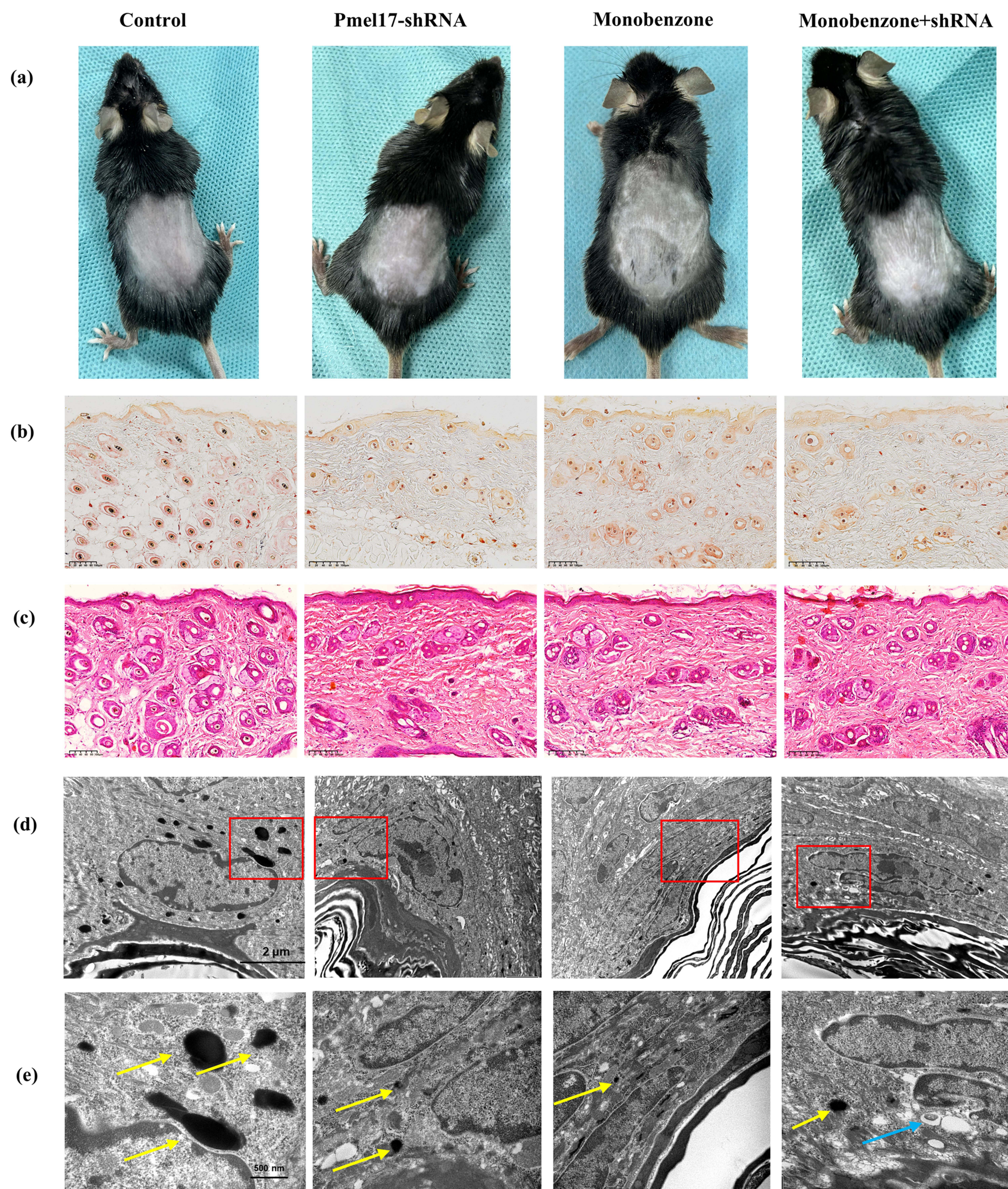

Figure 2 Pmel17 knockdown induced vitiligo phenotype in mice. The mice were randomly assigned to four groups: Control group, Pmel17-shRNA group, Monobenzone group and Monobenzone+shRNA group. (**a**) Photographs of skin decolorization in mice. (**b**) Masson-Fontana staining of melanosomes at 200x magnification. (**c**) HE staining of melanosomes at 200x magnification. (**d**) The melanosomes and autophagosomes were observed by electron microscope with magnification of 10000×. The red boxes indicate the enlarged parts of the figure. (**e**) The melanosomes and autophagosomes were observed by electron microscope with magnification of 30000×. Yellow arrows indicate melanosomes and blue arrows indicate autophagosomes.

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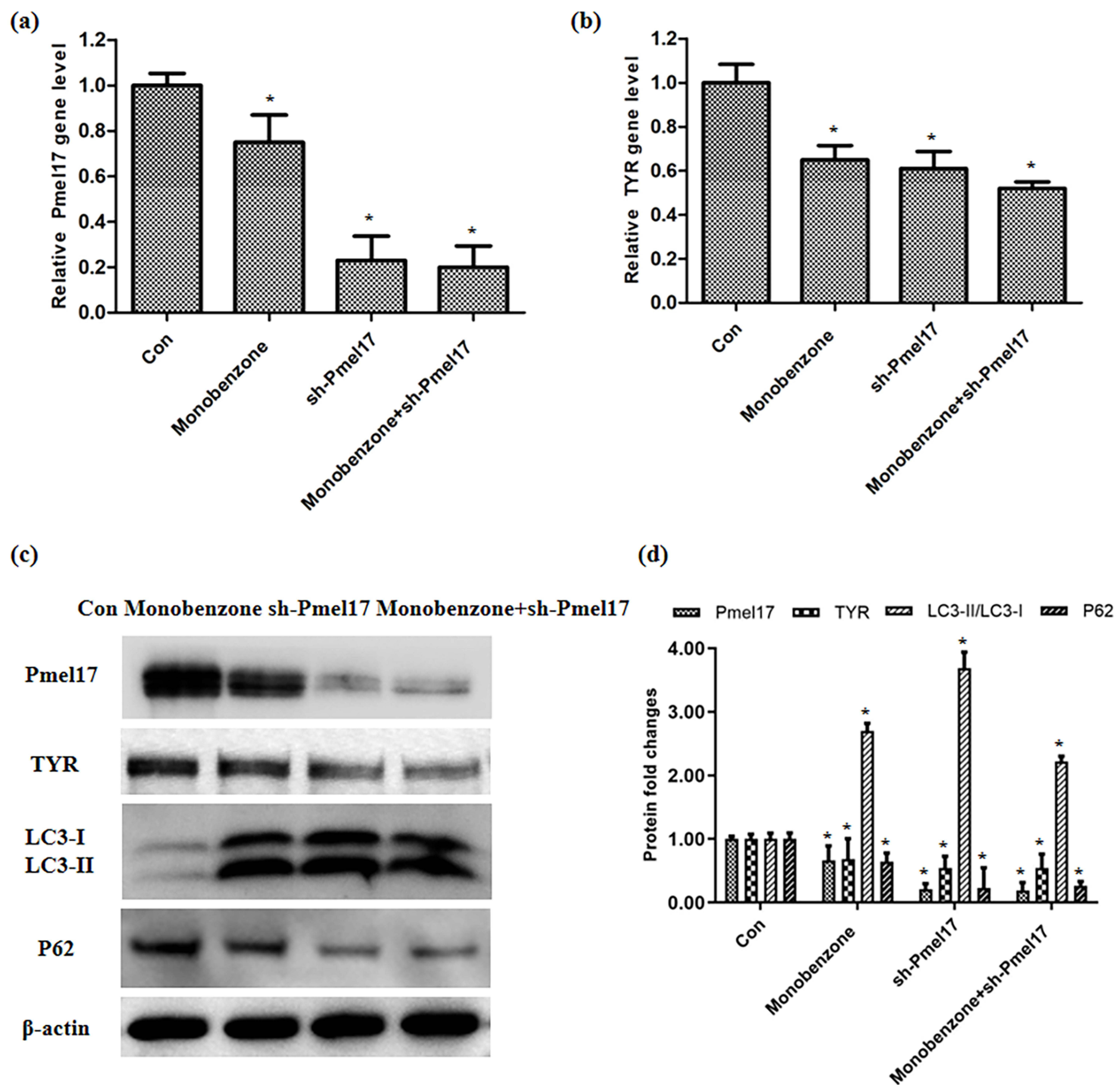

Figure 3 The expression of melanosome proteins and autophagy proteins in mouse tissues were detected by RT-PCR and Western blotting. (**a**) The expression of Pmel17 in mouse skin tissues was detected by RT-PCR. (**b**)The expression of TYR in mouse skin tissues was detected by RT-PCR. (**c**) The expression of related proteins in mouse skin tissues was detected by Western blotting. (**d**) Statistical analysis of related proteins expression. *P<0.05 indicates a significant difference compared with the control group.

Since autophagy was activated in the mouse model of vitiligo, we further examined the effect of Pmel17 on autophagy at the cellular level. Western blotting results showed that downregulation of Pmel17 led to an increase in the LC3-II/LC3-I ratio and a decrease in P62 protein expression, indicating that autophagy was activated in melanocytes (Figure 5a). Next, immunofluorescence was used to examine the cellular localization of the autophagy protein LC3. As shown in Figure 5b, compared with the control group, LC3 puncta were significantly increased following Pmel17 knockdown, further supporting the activation of autophagy in melanocytes. Observation of autophagosomes by TEM provides the most direct evidence of autophagy. To further verify that melanin synthesis is regulated by autophagy, we used TEM to examine both autophagosomes and melanosomes. TEM analysis showed that Pmel17 knockdown resulted in a decrease in melanosomes and an increase in autophagosomes (Figure 5c). Taken together, these results confirmed that downregulation of Pmel17 expression induces autophagy in melanocytes.

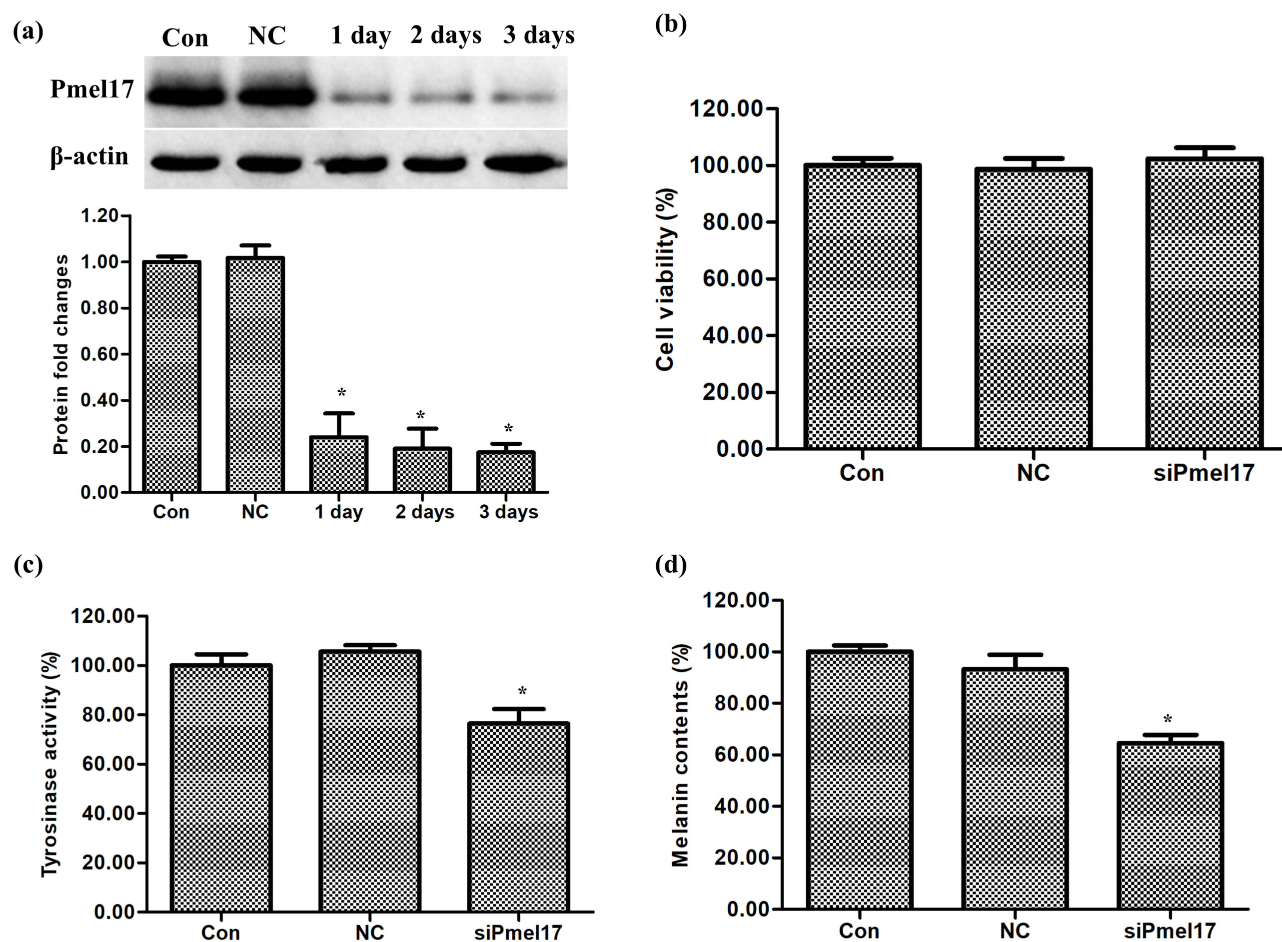

Figure 4 Pmel17 knockdown reduces pigment synthesis in melanocytes. Pmel17-siRNA was transfected into epidermal melanocytes, (**a**) The expression of Pmel17 was detected by Western blotting. (**b**) The cell viability was detected by MTT method. (**c**) The tyrosinase activity was detected as described in the Materials and Methods section. (**d**) The melanin content was determined as described in the Materials and Methods section. *P<0.05 indicates a significant difference compared with the control group.

To verify whether Pmel17 knockdown reduces pigment synthesis through autophagy, we treated human epidermal melanocytes transfected with Pmel17-siRNA using the autophagy inhibitor 3-MA. Western blotting results showed that tyrosinase expression decreased following Pmel17 knockdown, while 3-MA treatment restored tyrosinase expression (Figure 6a). However, melanin content assays revealed that 3-MA did not reverse the reduction in melanin synthesis caused by Pmel17 knockdown (Figure 6b). These findings suggest that Pmel17 knockdown reduces tyrosinase expression via autophagy. Nevertheless, the restoration of tyrosinase expression alone was insufficient to recover melanin synthesis, possibly due to the absence of Pmel17.

Down-Regulation of Pmel17 Inhibited the PI3K-AKT-mTOR Signaling Pathway

The PI3K-AKT-mTOR signaling pathway plays a key role in the regulation of autophagy. The PI3K/AKT pathway also contributes to melanin synthesis by upregulating MITF and melanin-synthesis enzymes. Inhibition of the PI3K pathway leads to reduced melanin production. mTOR, a component of the PI3K/AKT pathway, suppresses autophagy by phosphorylating the ULK1 complex and autophagy-associated protein 13. We therefore further investigated whether the PI3K-AKT-mTOR signaling pathway is involved in the effect of Pmel17 on pigment synthesis.

After transfection with Pmel17-siRNA for 48 hours, melanocytes were treated with the PI3K/AKT inhibitor LY294002 for 24 hours, and the protein expression levels of AKT, p-AKT, mTOR, and p-mTOR were assessed by Western blotting. The results showed that Pmel17-siRNA treatment significantly reduced the expression of p-AKT and

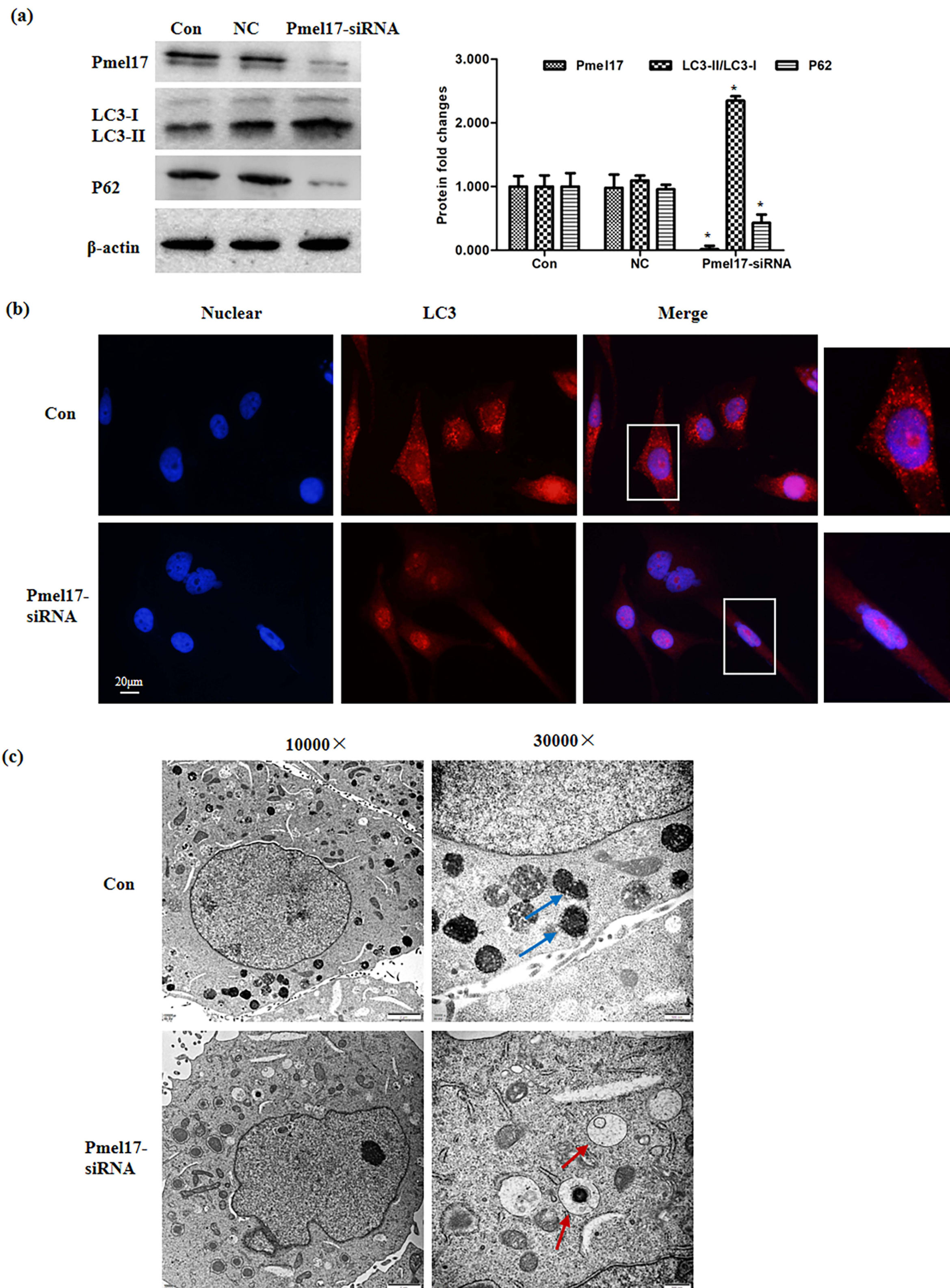

Figure 5 Pmel17 knockdown activated autophagy in melanocytes. Pmel17-siRNA was transfected into epidermal melanocytes, (**a**) The expression of autophagy proteins was detected by Western blotting. (**b**) The localization of LC3 in cells was detected by immunofluorescence. The white boxes indicate the enlarged parts of the figure. (**c**) The melanosomes and autophagosomes were observed by electron microscope. Blue arrows indicate melanosomes and red arrows indicate autophagosomes. *P<0.05 indicates a significant difference compared with the control group.

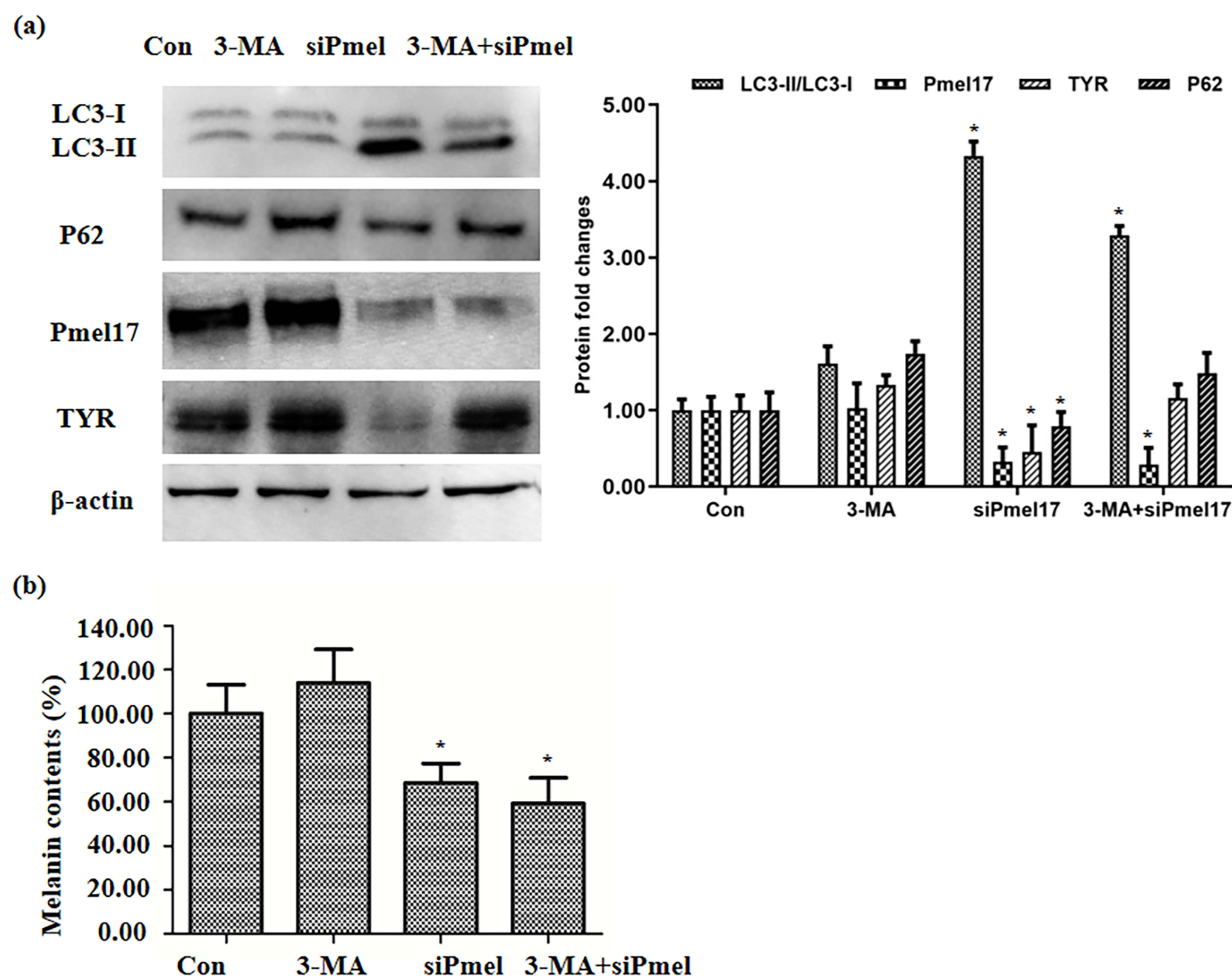

Figure 6 Effects of inhibition of autophagy on protein expression and melanin content. After a 6 h pretreatment with 3- MA, melanocytes were transfected with Pmel17-siRNA for 48 h. (**a**) Detection of protein expression levels of LC3, Pmel17 and TYR by Western blotting. (**b**) Determination of melanin content by the NaOH dissolution method described above. *P<0.05 indicates a significant difference compared with the control group.

p-mTOR proteins, while no significant changes were observed in AKT and mTOR proteins. LY294002 alone also decreased the expression of p-AKT and p-mTOR. Furthermore, LY294002 enhanced the inhibitory effect of Pmel17-siRNA on p-AKT and p-mTOR expression (Figure 7a). These findings indicated that downregulation of Pmel17 inhibits the PI3K-AKT-mTOR signaling pathway.

We further examined the expression of TYR and LC3 proteins in each group. Following treatment with either Pmel17-siRNA or LY294002, TYR expression was significantly reduced, while LC3 expression was increased. Moreover, LY294002 enhanced the inhibitory effect of Pmel17-siRNA on TYR expression (Figure 7b). These data suggest that Pmel17-siRNA induces autophagy by inhibiting the PI3K-AKT-mTOR signaling pathway in melanocytes.

Effects of Overexpression of Pmel17 on Protein Expression and Melanin Content

To further investigate the role of Pmel17, an overexpression vector (pcDNA3.1-Pmel17) was constructed and transfected into epidermal melanocytes. Western blotting results showed that overexpression of Pmel17 did not affect the protein expression levels of TYR or LC3 (Figure 8a).

We also examined the effects of Pmel17 overexpression on cell viability and melanin content. The results indicated that overexpression of Pmel17 had no significant effect on cell viability and melanin content (Figure 8b and c).

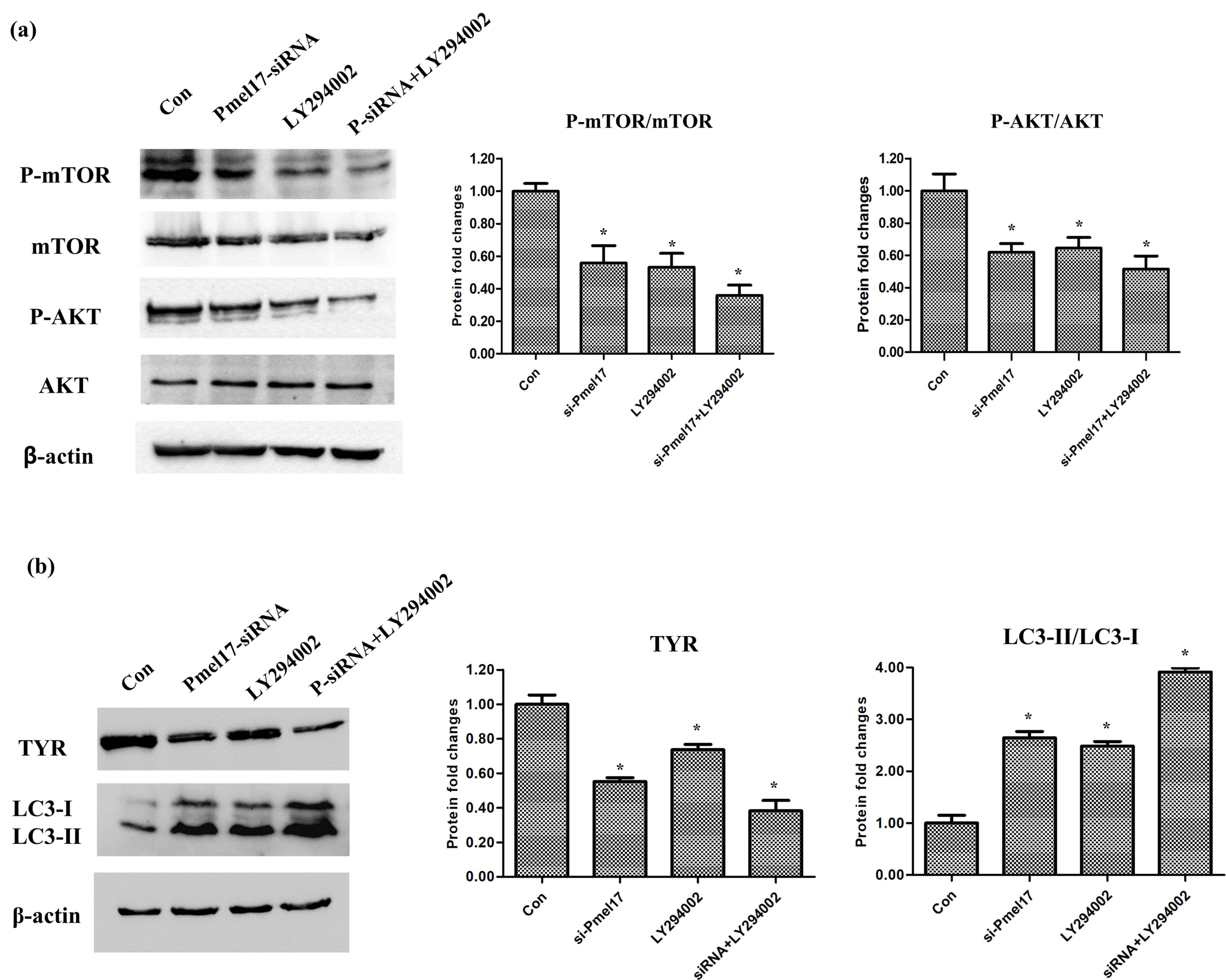

Figure 7 Down-regulation of Pmel17 inhibited the PI3K-AKT-mTOR signaling pathway. After transfection with Pmel17-siRNA for 48 hours, melanocytes were treated with the PI3K/AKT inhibitor LY294002 for 24 hours, (**a**) Detection of protein expression levels of p-AKT, p-mTOR, AKT and mTOR by Western blotting. (**b**) Detection of protein expression levels of LC3 and TYR by Western blotting. *P<0.05 indicates a significant difference compared with the control group.

Therefore, we speculate that overexpression of Pmel17 alone is insufficient to influence melanin synthesis and that additional factors may be involved.

Discussion

Pmel17, a type I membrane protein, is a key structural component required for the transformation of melanosomes from Phase I to Phase II. During melanosome development, Pmel17 is cleaved into several fragments following the phase I stage, forming the fibrillar matrix of the melanosome.[8] Defects or mutations in the Pmel17 gene can lead to albinism of the skin, hair, or eyes in animals; however, there is still some debate regarding the precise mechanisms by which this protein functions.[9,10] While previous studies suggest that Pmel17 may be involved in the pathogenesis of vitiligo, the exact relationship between Pmel17 and vitiligo remains unclear.

In this study, we collected skin tissues from patients with vitiligo and healthy individuals for immunohistochemical and RT-PCR analysis. The results showed that the expression of Pmel17 and TYR was simultaneously decreased in vitiligo skin lesions, and the expression levels of Pmel17 and TYR were significantly positively correlated. This suggests that Pmel17 may play a role in the pathogenesis of vitiligo and the reduced expression of Pmel17 may contribute to pigment loss by inhibiting TYR activity.

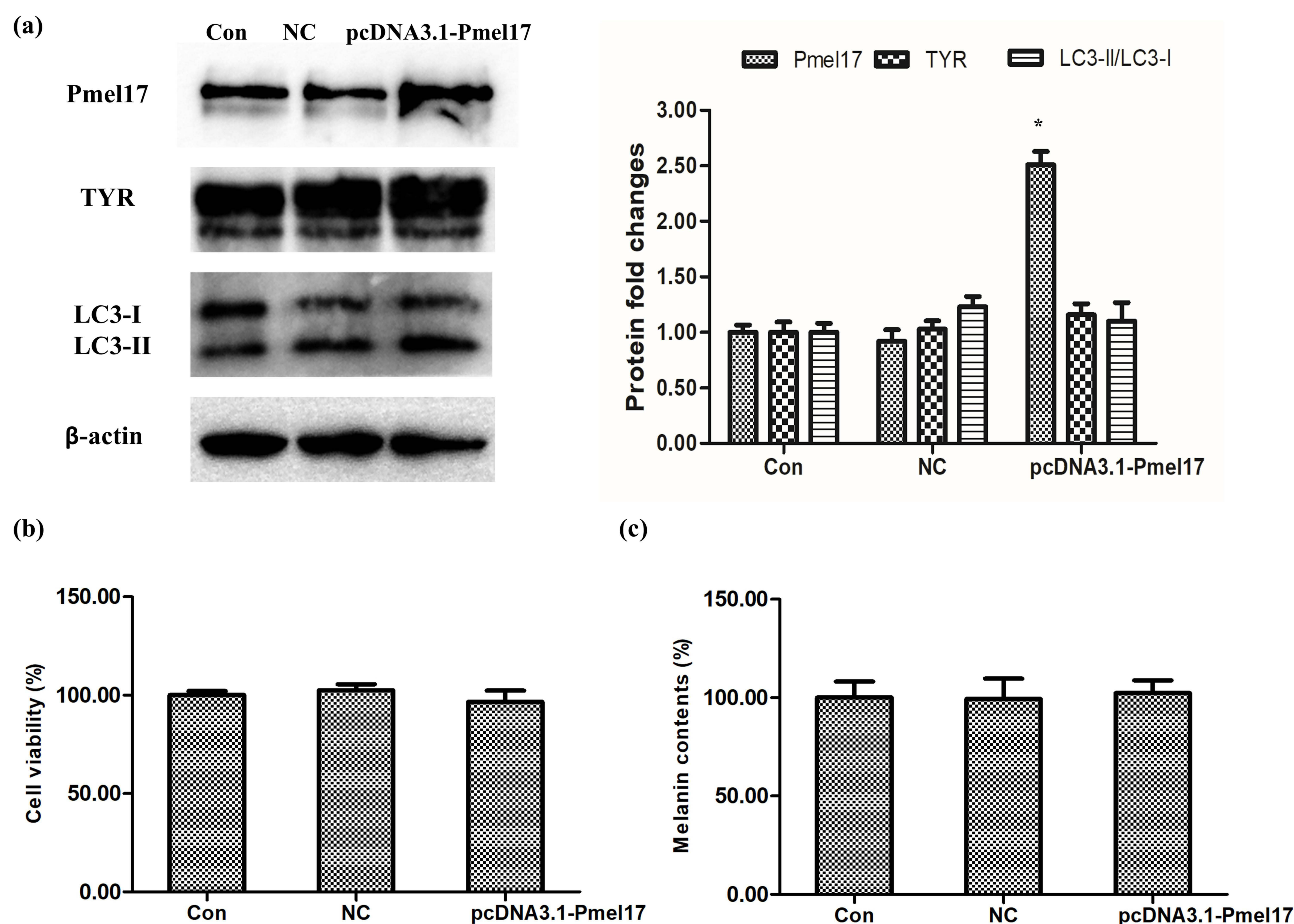

Figure 8 Effects of overexpression of Pmel17 on protein expression and melanin content. pcDNA3.1-Pmel17 was transfected into epidermal melanocytes, (**a**) Detection of protein expression levels of LC3 and TYR by Western blotting. (**b**) The cell viability was detected by MTT method. (**c**) The melanin content was determined as described in the Materials and Methods section. *P<0.05 indicates a significant difference compared with the control group.

To investigate the function of Pmel17, a mouse model of vitiligo was established using monobenzone. In parallel, mice were infected with Pmel17-shRNA lentivirus to explore the effects of Pmel17 on the mouse phenotype and its influence on depigmentation in the monobenzone-induced model. Monobenzone is a skin-depigmenting agent. As early as 1939, it was observed that depigmentation caused by monobenzone was similar to vitiligo resulting from occupational exposure. With prolonged exposure, monobenzone-induced depigmentation may even spread to unexposed areas of the skin.[11] Studies have shown that monobenzone promotes the presentation of melanocyte differentiation antigens by triggering melanosome autophagy and tyrosinase ubiquitination, inducing $CD4^+$ and $CD8^+$ T-cell responses that destroy melanocytes and lead to skin depigmentation.[12] Our results demonstrated that both the Monobenzone group and the Pmel17-shRNA group exhibited skin depigmentation. Moreover, combined treatment with monobenzone and Pmel17-shRNA significantly accelerated the development of white spots, suggesting that Pmel17-shRNA can enhance the onset and progression of depigmentation in mice with vitiligo.

Studies have shown that autophagy is involved in skin depigmentation. For instance, autophagic activity is decreased in hyperpigmented skin such as senile freckles, and melanocytes in Caucasian skin exhibit higher autophagic activity than do those in African American skin.[13,14] However, the role of autophagy in vitiligo remains unclear. Some evidence suggests that impairment of the Nrf2/p62 pathway in vitiligo melanocytes may lead to autophagy defects, rendering the cells more susceptible to oxidative damage.[15] Autophagy induction in vitiligo has also been reported. For example, IL-17-induced melanocyte death in vitiligo has been shown to require autophagic

processes,[16] and deficiency of the glutamate/cysteine reverse transporter leads to cell death via activation of endoplasmic reticulum stress and autophagy in melanocytes.[17] Additionally, keratinocytes and the few remaining melanocytes in vitiligo lesions display significantly more autophagic vacuoles than do those in control and non-lesional skin.[18] In this study, our results showed that the expression of the autophagy marker protein LC3 was elevated in the mouse model of vitiligo, initially indicating that autophagy may be involved in the progression of vitiligo.

Autophagy plays a key role in melanin synthesis.[19] Tranexamic acid, an ingredient commonly used in whitening cosmetics, has been shown to reduce melanin synthesis while increasing the expression of autophagy-related proteins such as LC3-II, Beclin-1, and ATG12.[20] ARP101 inhibits melanin production by activating autophagy in melanoma cells, and its effect on melanin synthesis is diminished when autophagy is suppressed using ATG5-siRNA.[21] Similarly, resveratrol and saponin induce autophagy in melanocytes and reduce the levels of TYR and TRP-1, demonstrating anti-melanogenic activity.[22–24] These findings suggest that activation of autophagy can inhibit melanin synthesis. Autophagy also plays a role in the clearance of defective melanosomes; for example, Devillers et al[25] observed that dysfunctional melanosomes in melanocytes from depigmented areas were phagocytosed by keratinocyte autophagosomes. In our study, Pmel17 knockdown at the cellular level led to a reduction in tyrosinase expression and inhibition of melanin synthesis, while simultaneously activating lysosome-mediated autophagy. These findings reveal a novel mechanism by which Pmel17 dysfunction induces autophagy to regulate melanin synthesis. Currently, studies on drugs that induce autophagy-related skin depigmentation are mainly focused on inhibiting pigment loss by knocking down autophagy-related genes or using autophagy inhibitors. Going forward, we can delve deeper into the targets and mechanisms, which can provide new therapeutic insights for the treatment of vitiligo.

The PI3K/Akt/mTOR axis plays a key role in regulating several physiological processes, including cell proliferation, apoptosis, autophagy, migration, invasion, metabolism, and angiogenesis.[26–28] Dysregulation of the PI3K/Akt/mTOR pathway has been linked to the pathophysiology of skin cancers and immune-mediated skin diseases such as acne, psoriasis, vitiligo, and scleroderma.[29–31] The present study confirmed that Pmel17 knockdown activates autophagy by inhibiting the PI3K-Akt-mTOR signaling pathway, thereby promoting lysosome-mediated TYR degradation. These findings suggest that Pmel17 may influence autophagy through regulation of the PI3K-Akt-mTOR axis and could represent a potential therapeutic target. Further studies are required to confirm the direct interactions between Pmel17 and components of this pathway using immunoprecipitation and other methods, as well as to elucidate the structural basis of these interactions.

In our study, Pmel17 overexpression had no significant effect on melanocyte function, suggesting that Pmel17 dysfunction rather than overexpression is the key driver in the pathogenesis of vitiligo. The inability of Pmel17 overexpression to restore melanin synthesis may be attributed to the cooperative nature of melanosome assembly. Specifically, Pmel17 needs to cooperate with proteins such as MART-1 and TYRP1 to form functional complexes, and its overexpression is insufficient to reconstruct the matrix network.[32] Additionally, excessive deposition of amyloid glycoproteins in the human body can have pathological consequences, as seen in diseases such as Alzheimer's. Pmel17, as a functional amyloid glycoprotein, is an important participant in melanogenesis and storage and under normal conditions does not induce pathology.[3,33] However, our data indicate that the overexpression of Pmel17 under these experimental conditions does not reach cytotoxic levels and does not disrupt normal melanocyte function.

In conclusion, this study systematically revealed the key role of Pmel17 and its regulatory network in the pathogenesis of vitiligo through clinical sample analysis, animal model construction, and molecular mechanism investigation (Figure 9). In the future, it will be important to further clarify how upstream regulatory factors—such as methylation and miRNAs—affect Pmel17 expression and to analyze the dynamic role of Pmel17 across different stages of vitiligo using approaches such as single-cell sequencing and organoid models.

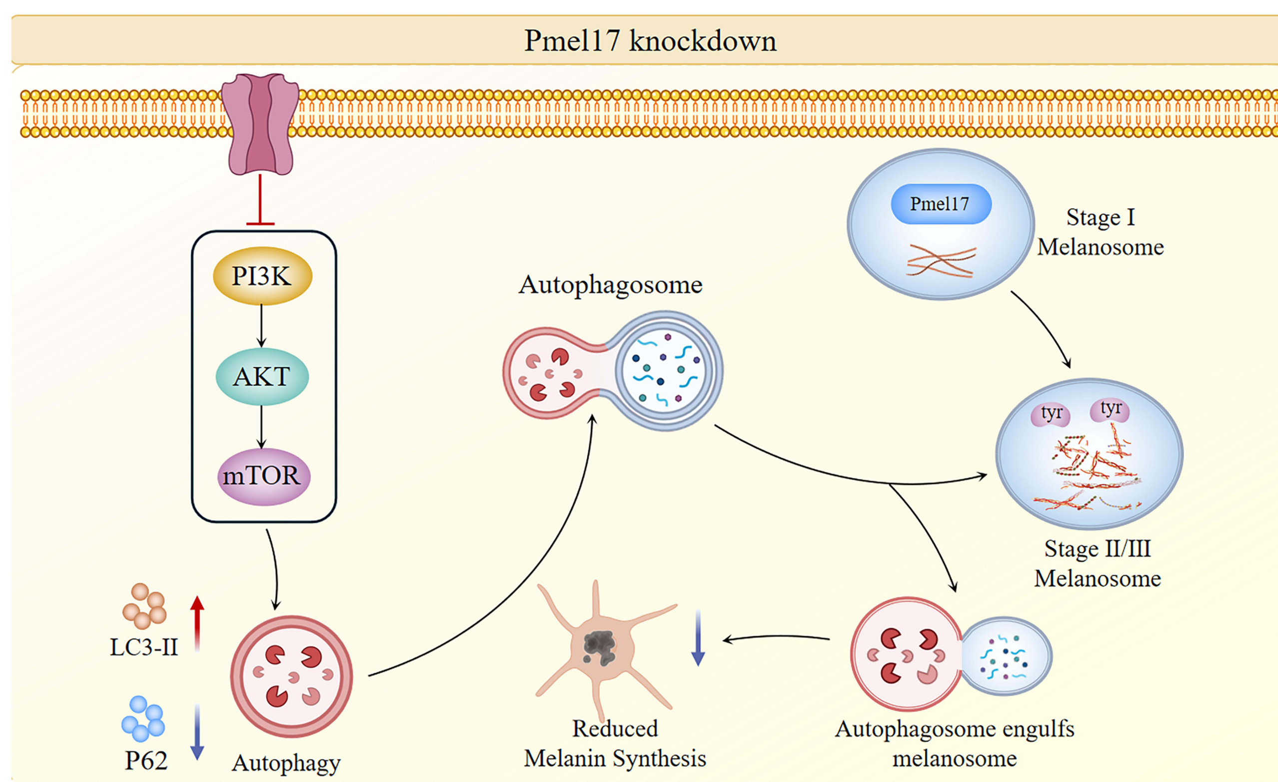

Figure 9 Diagram of melanin synthesis mechanism in Pmel17 knockdown melanocytes. After Pmel17 was knocked down, there were few fibrils in stage I melanosomes. In the stage II and stage III melanosomes, there was a disorder of the fibrillary matrix, and TYR cannot attach normally either. Meanwhile, the PI3K-AKT-mTOR pathway in the cytoplasm was inhibited, thereby activating autophagy and inducing the formation of autophagosomes. The melanosomes with structural abnormalities in stage II and stage III were phagocytosed and degraded by autophagosomes, eventually leading to a reduced melanin synthesis in melanocytes.

Abbreviations

Pmel17, premelanosome protein 17; TYR, tyrosinase; HRP, horseradish peroxidase; RT-PCR, reverse transcription polymerase chain reaction; ATG, autophagy-related gene; LC3, microtubule-associated protein 1 light chain 3; HMGS-2, Human Melanocyte Growth Supplement-2; FBS, fetal bovine serum; MART-1, melanoma-associated antigen recognized by T cells; TYRP1, tyrosinase related protein 1.

Data Sharing Statement

The data generated in the current study are available from the corresponding author Chunyan Guo on reasonable request.

Ethics Statement

The study was approved by the Ethics Committee of Shaanxi Provincial People's Hospital. All participants signed informed consent.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in financial or any other sphere.

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