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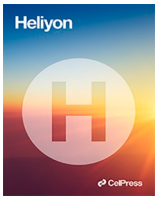

Research article

# Differentiation of cultured hair follicle neural crest stem cells into functional melanocytes

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ABSTRACT

Many autologous melanocytes are required for surgical treatment of depigmentation diseases such as vitiligo. However, primary cultured melanocytes have a limited number of in vitro passages. The production of functional epidermal melanocytes from stem cells provides an unprecedented source of cell therapy for vitiligo. This study explores the clinical application of melanocytes induced by hair follicle neural crest stem cells (HFNCSCs). This study established an in vitro differentiation model of HFNCSCs into melanocytes. Results demonstrate that most differentiated melanocytes expressed the proteins C-KIT, MITF, S–100B, TYRP1, TYRP2, and tyrosinase. The HFNCSC-derived melanocytes were successfully transplanted onto the dorsal skin of mice and survived in the local tissues, expressing marker protein of melanocytes. In conclusion, HFNCSCs in mice can be induced to differentiate into melanocytes under specific conditions. These induced melanocytes exhibit the potential to facilitate repigmentation in the lesion areas of vitiligo-affected mice, suggesting a promising avenue for therapeutic intervention.

## 1. Introduction

As a prevalent dermatosis, vitiligo is characterized by patches of depigmentation affecting approximately 0.1–2.0 % of the global population [1]. The pathogenesis of vitiligo remains elusive, making its treatment challenging. Various therapeutic options, such as topical and oral agents, phototherapy, laser procedures, and surgery, are available for managing vitiligo [2]. For patients in stable phases with localized manifestations or those unresponsive to alternative therapeutic approaches, surgical interventions become the primary approach. These interventions encompass tissue and cell transplantation [3,4]. Autologous non-cultured epidermal suspension is a well-established technique for vitiligo surgery, which boasts a remarkable repigmentation rate of 50 %–100 % and achieves satisfactory color matching [5]. In treating recalcitrant vitiligo, the use of cultured melanocytes has become a widely accepted and effective modality. A noteworthy finding from a previous study emphasizes the impact of different in vitro melanocyte culturing techniques on post-transplant growth and pigment recovery in vitiligo-affected regions [6]. Both methods involve harvesting skin from flat regions of the patient, often inflicting pain and potentially leading to insufficient donor site availability. Furthermore, primary cultured human melanocytes have a limited count of in vitro passage [7,8].

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Hair follicles serve as a reservoir for melanocytes in the interfollicular epidermis. In the repigmentation process in vitiligo, melanocyte precursors migrate upwards from the hair follicles to the interfollicular epidermis as needed [9]. In the context of vitiligo recovery, it is frequently observed that punctate pigmentation, concentrated around hair follicles, becomes evident. Pathological examinations reveal the migration of immature melanocytes, with or without pigmentation, from the hair follicles to the epidermis during this recovery phase. However, the challenge arises in areas with sparse or no hair, where repigmentation proves to be difficult. Furthermore, transplanting normal hair follicles to vitiligo-affected sites has emerged as a viable therapeutic approach. Within the bulge region of hair follicles, pluripotent neural stem cells exist, demonstrating the capacity for in vitro expansion and efficient differentiation [10]. Adult neural stem cells possess the remarkable capacity for directed differentiation into various cell types, including adipocytes, osteoblasts, chondrocytes, melanocytes, neurons, and Schwann cells [11]. Yu et al. [12]. induced differentiation of hair follicle neural crest stem cells isolated in vitro into neuronal cells. These cells, labeled with a fluorescent probe as a cell proliferation tracer, were subsequently transplanted into the brains of adult nude mice. The results showed that the neuronal cells not only exhibited normal biological functions in vivo but also retained their distinct neuronal phenotypes. Within the hair follicle eminence, neural crest stem cells are present, offering the potential for in vitro expansion and efficient differentiation [13]. The understanding that hair follicle neural crest stem cells (HFNCSCs) can differentiate into melanocytes holds significant implications for the treatment of disorders related to melanocyte dysfunction. Studies suggest that HFNCSCs can undergo differentiation into melanocyte precursors when induced by BMP4 and α-MSH [14]. NB-UVB has been shown to induce the differentiation of HFNCSCs into melanocytes and facilitate cell migration [15].

This study explores whether HFNCSC-derived melanocytes exhibit the function of normal melanocytes and tries to transplant them onto the dorsal skin of mice. This study provides a clinical exploration for the induction and transformation of HFNCSCs into melanocytes.

## 2. Methods details

### 2.1. Isolation and cultivation of HFNCSCs

HFNCSCs were obtained from the vibrissae of C57BL/6 mice at six weeks of age. C57BL/6J mice were humanely euthanized via cervical dislocation. The vibrissae were excised from the mice using sterile scissors. The tissue blocks were then placed into Dispase II and left overnight in a refrigerator at 4 °C (approximately 12 h). Subsequently, the tissue blocks were retrieved, and intact hair follicles with surrounding connective tissue sheaths were dissected. The connective tissue sheaths were carefully opened with sterile microsurgical scissors, and the outer root sheaths of the hair follicles were gently extracted with microsurgical forceps and placed into a culture dish. The culture dish was supplemented with DMEM/F12 medium (Gibco, USA) containing 10 % FBS, 2 % B-27 supplement, 1 % penicillin-streptomycin, 20 ng/ml EGF, and 40 ng/ml bFGF. The dish was then placed in a 37 °C, 5 % $CO_2$ incubator and cultured, with media changes performed every three days [16].

### 2.2. Characterization of cultured HFNCSCs

Cultured HFNCSCs were characterized for the presence of SOX10 and nestin, which are markers of neural crest cells. Upon achieving approximately 80 % cell detachment from the hair follicles, the cells were seeded into a culture dish and washed thrice with PBS, 3 min each time. Fixation was carried out with 4 % paraformaldehyde in ice-cold for 15 min, followed by three PBS washes. Permeabilization was achieved using 1 % Triton X-100 (prepared in PBS) at room temperature for 20 min. After three PBS washes, the cells were incubated with normal goat serum at room temperature for 1 h. Subsequently, the blocking solution was discarded, and the primary antibody working solutions for SOX10 (1:100) (Bioss, China) and Nestin (1:100) (Bioss, China) were added, ensuring complete coverage of the cells. The dish was then placed in a humid chamber and incubated overnight at 4 °C. Following this, the cells were incubated with a fluorescent secondary antibody (Goat Anti-rabbit IgG; 1:200) in a humid chamber at 37 °C for 1 h. After three PBS washes (each lasting 3 min), nuclear counterstaining was performed by adding DAPI and incubating for 5 min in the dark. Excess DAPI was washed away with PBS, and 1 mL PBS was added for the specimens to be observed under the laser confocal microscope.

### 2.3. Differentiation of HFNCSCs into melanocytes

HFNCSCs in a robust growth state were seeded into culture dishes at a density of 5 × 10^3 cells/dish. Differentiation was induced with an induction medium developed in our lab composed of advanced DMEM/F-12 supplemented with 2 % FBS, 4 mM L-glutamine (Gibco, USA), 100 units/mL penicillin,100 μg/ml streptomycin,20 μg/ml IBMX (MedChemExpress, China), 200 ng/ml α-MSH (MedChemExpress, China), 20 ng/ml b-FGF (PeproTech, USA) [16]. Cultivation took place at 37 °C in a 5 % $CO_2$ incubator, with medium changes every two days and regular monitoring of cell quantity and morphology.

### 2.4. l-DOPA (3, 4- dihydroxyphenylalanine) assay

Upon reaching a confluence of approximately 70–80 %, the cells were harvested and carefully deposited onto glass slides previously positioned in culture dishes. The slides were then placed in a 37 °C, 5 % $CO_2$ incubator for 24 h. Following incubation, the glass slides were retrieved, and the cells were rinsed twice with ice-cold PBS. Fixation was performed using 5 % formaldehyde for 30 min, followed by two additional PBS washes. Subsequently, 0.1 % L-DOPA(Solarbio, China) phosphate-buffered solution (pH 7.4) was applied, and

the cells were incubated at 37 °C for 4 h. The surplus DOPA solution was discarded, and the extent of cellular pigmentation was assessed.

### 2.5. Immunostaining of differentiated melanocytes

After a 14-day differentiation assay, the melanocytes were checked for the markers C-KIT, MITF, S-100, TYR, TYRP1, and tyrosinase by immunostaining. The cells were then seeded into a 6-well plate at a density of 5 × 10^3 cells per well. 2 mL of melanocyte culture medium was added, and the cells were incubated overnight at 37 °C in a 5 % $CO_2$ incubator. The primary antibody working solutions for C-KIT (1:100) (Bioss, China), MITF (1:100) (Bioss, China), S-100 (1:100) (Bioss, China), TYR (1:100) (Bioss, China), TYR1 (1:100) (Bioss, China), and tyrosinase (1:100) (Bioss, China) were appropriately diluted. Cellular morphology was observed using a fluorescence microscope, and images were captured. The fluorescence was quantified using ImageJ software.

### 2.6. Establishing a mouse model of vitiligo-like skin lesions and microscopic analysis

6-week-old C57BL/6 male mice were utilized in this study. The animal facility maintained a clean and quiet environment, with a room temperature of (22 ± 3)°C and a relative humidity of 40 %–60 %. The lighting conditions provided 8 h of light daily. Clean-grade bedding was used, and the mice had unrestricted access to food and water throughout the study period. All mice were in good health, and none exhibited any signs of skin diseases. 24 C57BL/6J mice were randomly assigned to three groups: Control, Model, and Transplantation, with eight mice in each group. An area of 2 cm × 2 cm on the dorsal skin was shaved. In the Model and Transplantation groups, mice underwent depilation followed by daily application of 5 % $H_2O_2$ [17], while the Control group received daily application of physiological saline. This regimen continued for 36 days, during which visible depigmentation at the local site was

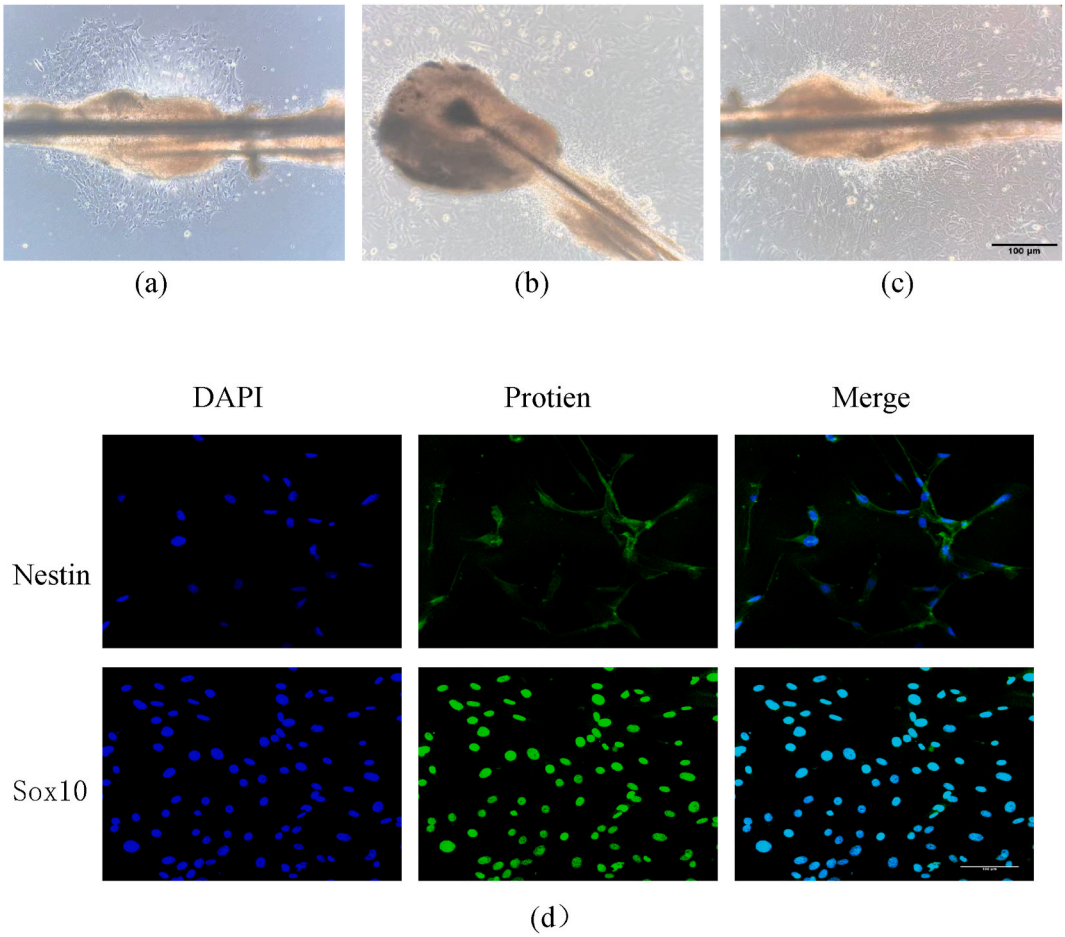


**Fig. 1.** Primary culture HFNCSCs and immunofluorescence results of HFNCSCs (scale bar = 100 μm). (a) A small population of HFNCSCs emerging from tissue blocks. (b) After 12–14 days, HFNCSC achieved a confluence of 60 %–70 %; (c) Approximately 20 days later, HFNCSC reached a confluence of 80 %–90 %, mostly exhibiting spindle-shaped and triangular morphologies. (d) The HFNCSCs showed positive staining for nestin and SOX10.

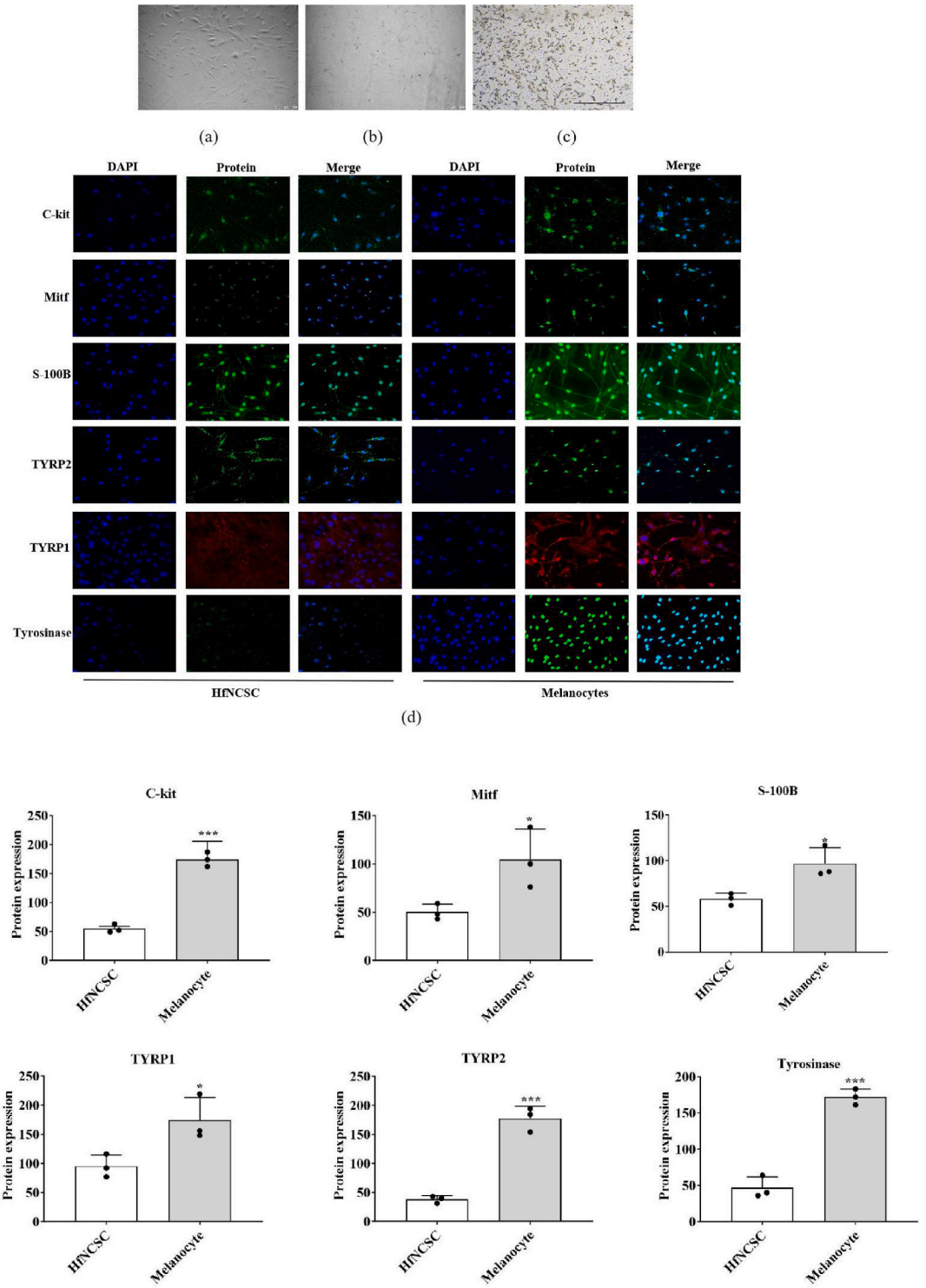


**Fig. 2.** Characterization of HFNCSCs-derived melanocytes(scale bar = 100 μm). (a)Microscopy images of HFNCSCs. (b) Microscopy images of melanocytic differentiation from HFNCSC. (c) L-DOPA staining: The cytoplasm and dendrites of the cells appeared in black or dark, indicating a positive reaction. (d) Immunofluorescence staining of C-KIT, MITf, S–100B, TYRP1, TYRP2, and tyrosinase in melanocytes (scale bar = 100 μm). (e) The fluorescence expression of C-KIT, TYRP2, tyrosinase, MITF, S-100, and TYRP1 in melanocytes (n = 3),*$P < 0.05$, ***$P < 0.001$.

monitored. On the 37th day of modeling, the Transplantation group received subcutaneous injections of melanocytes. After puncturing the blisters and draining the fluid, a cell suspension was injected, with an approximate cell count of 5~6 × 10^4 cells/cm$^2$. Injections were administered weekly for two consecutive weeks. Postoperative changes in the lesion site of the mice were observed, and photographs documenting the status of depigmentation were taken every ten days. Further assessments were conducted 52 days after the initial procedure.

### 2.7. Immunohistochemistry of melanocyte grafted in mouse skin

The lesion tissues from the dorsal area were fixed in a 4 % formaldehyde solution, embedded in paraffin, and subsequently sectioned. Following hematoxylin staining, differentiation, counterstaining with blue, eosin staining, and dehydration, the tissue sections underwent two rounds of immersion in xylene for 4 min each. After drying, the sections were mounted with neutral gum, and photomicrographs were captured using an upright microscope at 200 × magnification. The sections underwent processes for immunofluorescence staining of skin tissues, including baking, tissue rehydration, endogenous enzyme removal, and antigen retrieval. The steps for primary and secondary antibody incubation were similar to those outlined in Section 4. A single drop of neutral gum was applied, and a coverslip was used for sealing. The fluorescence was quantified using ImageJ.

### 2.8. Statistical analysis

Data analysis and graphical representation were conducted via GraphPad Prism 10.0. Descriptive statistics were expressed as mean ± standard deviation (x ± s) for quantitative data. Single-factor analysis of variance (ANOVA) was applied for data comparisons, with Tukey's test used for pairwise multiple comparisons. A significance level of $P < 0.05$ was considered indicative of statistical significance.

## 3. Results

### 3.1. Primary culture and characterization of HFNCSCs

HFNCSCs were cultured from whisker hair cells of C57BL/6 mice. Hair follicles of mice were cultured adherently for 7–10 days, during which a small number of HFNCSCs migrated out from the tissue blocks (Fig. 1a). Subsequently, the cell proliferation rate increased, and after 12–14 days, the confluence of HFNCSCs reached 60 %–70 % (Fig. 1b). Around 20 days, the confluence of HFNCSCs reached 80 %–90 %, at which point the cells were passaged. The majority of the cells displayed spindle-shaped and triangular morphologies (Fig. 1c). Immunofluorescence identification of the cells at the second and third passages revealed that nestin was localized

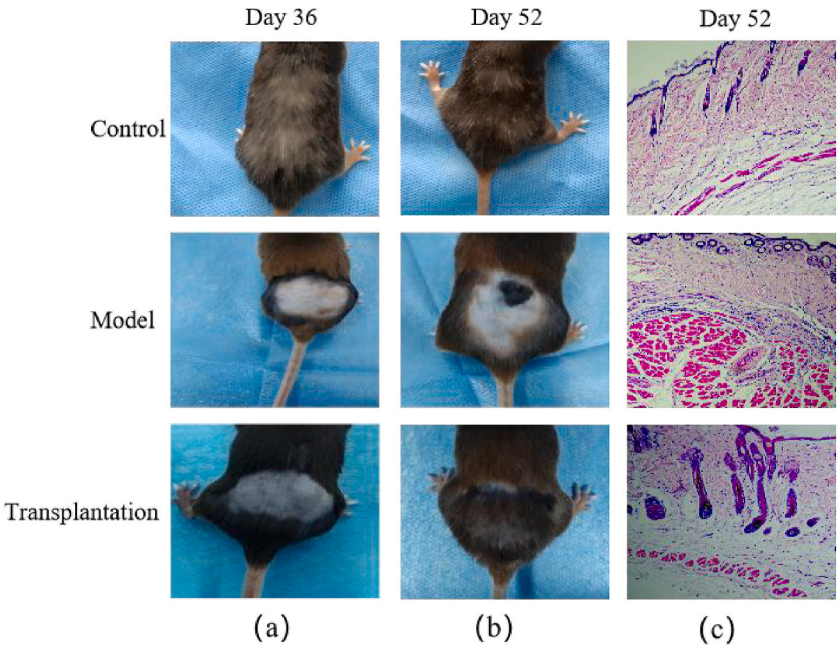


**Fig. 3.** Simultaneous grafting of melanocytes in mice. (a) On the 36th day, evident depigmentation was observed in the modeling area of mice in the Model group and Transplantation group. (b) By the 52nd day, significant pigmentation was noticeable in the Transplantation group. (c) In comparison to the Model group, HE staining in the Transplantation group revealed a thinner epidermis at the lesion site, reduced inflammation infiltration, and an increased number of hair follicles.

in the cytoplasm, while SOX10 was expressed in both the cell nucleus and cytoplasm. In addition, it was found that HFNCSCs showed positive staining for nestin and SOX10 (Fig. 1d).

### 3.2. Characterization of HFNCSC-derived melanocytes

For subculturing of HFNCSCs, the majority of HFNCSCs exhibited a spindle-shaped morphology (Fig. 2a). Upon changing to a specialized melanocyte culture medium for three days, a significant portion of the cells exhibited a bipolar or multipolar dendritic-like morphology. (Fig. 2b). L-DOPA assay revealed a positive reaction, with the cytoplasm and dendrites appearing in dark brown or black (Fig. 2c). After 14 days of differentiation, the melanocytes were further characterized for specific melanocyte markers: C-KIT, MITF, S–100B, TYRP1, TYRP2, and tyrosinase. Most of the differentiated melanocytes expressed these markers, which are the minimal essential criteria for melanocyte identity. MITF expression was observed in the cell nucleus, tyrosinase was expressed in the cytoplasm, and both C-KIT and S-100 were expressed in both the cell nucleus and cytoplasm. TYRP1 and TYRP2 were expressed in the cell membrane (Fig. 2d). In comparison to the HFNCSC group, the fluorescence expression of C-KIT, TYRP2, and tyrosinase significantly

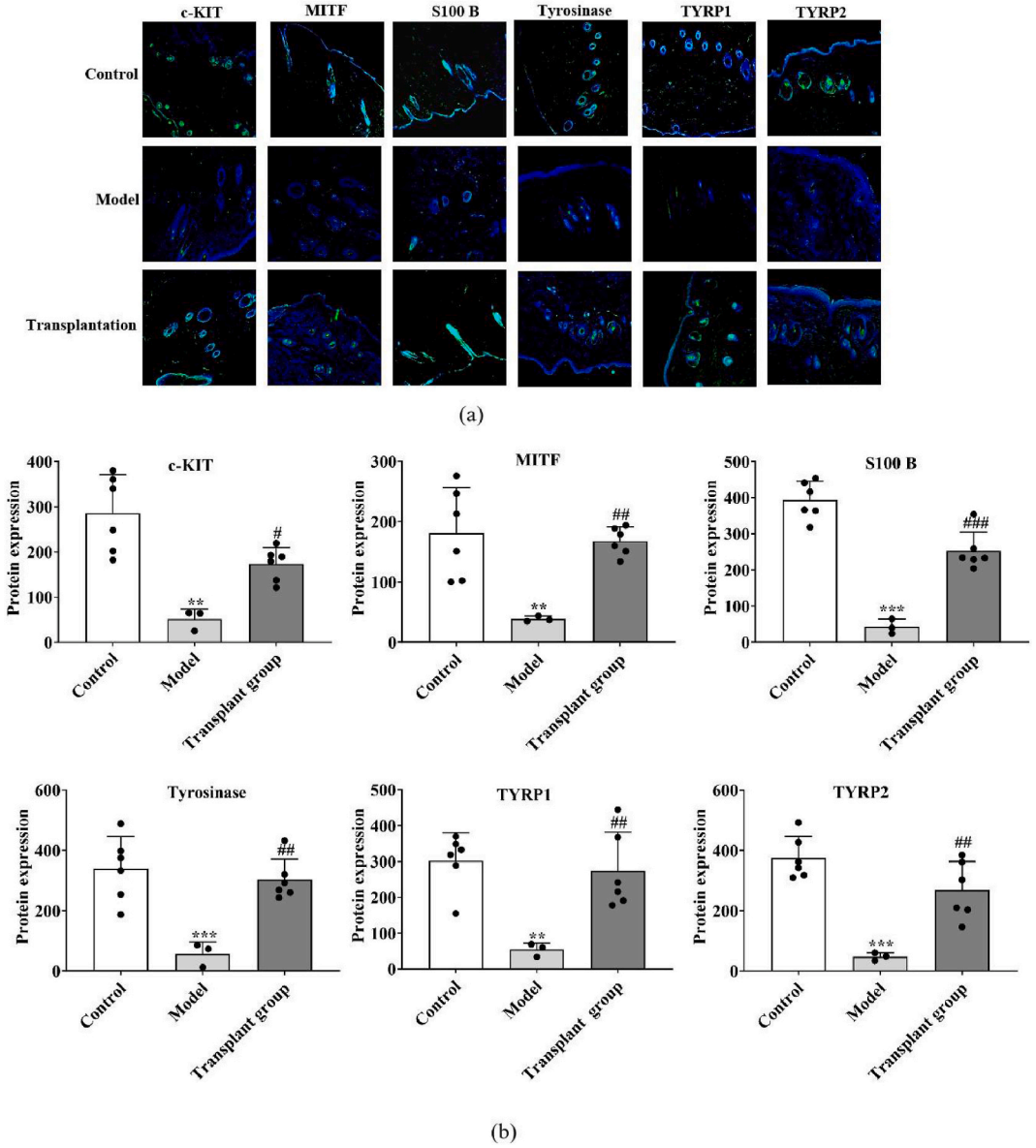


**Fig. 4.** Expression of antibodies in skin tissues. (a) Expression of c-KIT, MITF, S100 B, tyrosinase, TYRP1, and TYRP2 in skin tissues. (b) The fluorescence was quantified using ImageJ (n = 3). **: $P < 0.01$ vs Control, ***: $P < 0.001$ vs Control; ##: $P < 0.01$ vs Model, ###: $P < 0.001$ vs Model.

increased with HFNCSCs-derived melanocytes. The fluorescence expression of MITF, S-100, and TYRP1 in melanocytes also exhibited a notable increase (Fig. 2e).

### 3.3. The application of HFNCSCs-derived melanocytes to the skin of mice

A vitiligo mouse model was established using an $H_2O_2$ depigmentation method. On the 36th day after modeling, the modeling area on the skin of C57BL/6 mice in the Model group and the Transplantation group exhibited obvious depigmentation, with some fur turning white and skin thickening, indicating the successful construction of the vitiligo mouse model. In contrast, the skin and fur color in the Control group mice were normal (Fig. 3a). On the 37th day, melanocytes were injected subcutaneously into the Transplantation group. On the 52nd day, the number of melanocytes increased in the Transplantation group, and repigmentation was observed in skin lesions. (Fig. 3b). Microscopy image results showed that in the mice of the Control group, the epidermal margin was continuously intact, and the skin showed no thickening or infiltration of inflammatory cells. Compared to the Control group, the mice of the Model group exhibited a fractured epidermal margin, obvious epidermal hyperplasia at the lesion site, thickness higher than the Control group, thickening of the spinous layer, a reduced and dispersed number of hair follicles, and infiltration of inflammatory cells. Compared to the Model group, the Transplantation group exhibited thinning of the epidermis at the lesion site, reduced inflammation infiltration, and an increased number of hair follicles (Fig. 3c).

### 3.4. Expression of c-KIT, MITF, S100 B, tyrosinase, TYRP1, and TYRP2 in skin tissues

This study determined the expression of C-KIT, MITf, S–100B, TYRP1, TYRP2, and tyrosinase in HFNCSCs-derived melanocytes in vivo in the mouse skin and discovered that most differentiated melanocytes expressed C-KIT, MITF, S–100B, TYRP1, TYRP2, and tyrosinase at the protein level (Fig. 4a). The statistical analysis of immunofluorescence results revealed a significant decrease in the expression of c-KIT, MITF, S100 B, tyrosinase, TYRP1, and TYRP2 in the Model group compared to the Control group. Conversely, when compared to the Model group, the Transplantation group exhibited an increased expression of c-KIT, MITF, S100 B, tyrosinase, TYRP1, and TYRP2 (Fig. 4b).

## 4. Discussion

Melanocytes are responsible for pigment production in the epidermis and hair follicles and play a crucial role in providing melanin to surrounding keratinocytes. Research indicates that melanocytes in vertebrates originate from the neural crest of the ectoderm [18–20]. These neural crest stem cells exhibit a remarkable ability to differentiate into diverse cell lineages, including peripheral neurons, endocrine cells, bone, cartilage, connective tissue, and melanocytes [21,22]. Subsequently, differentiated melanocytes migrate to the skin [23,24]. Neural crest stem cells, located at the bulge of hair follicles, can be cultured in vitro and possess the potential for differentiation [25]. Importantly, neural crest stem cells derived from hair follicles can be induced to differentiate into melanocytes in vitro [26]. HFNCSCs offer advantages such as convenient sourcing, abundant yield, autologous nature, and absence of immunogenicity or ethical concerns, rendering them highly promising for clinical applications. This study focuses on inducing the in vitro differentiation of HFNCSCs into melanocytes, followed by transplantation for the treatment of a mouse model of vitiligo.

SOX10, a neural crest transcription factor, serves as a crucial molecular marker for neural crest stem cells, playing a vital role in regulating the differentiation and survival of these stem cells and maintaining their potential for pluripotent differentiation [27,28]. Immunohistochemical detection of SOX10 has become a significant diagnostic marker for determining cell lineages [29]. Nestin, a type of cytoskeletal protein classified within the intermediate filament family, was originally identified as a marker for neural stem cells [30,31]. In this study, the primary culture of HFNCSCs was performed, and immunofluorescence staining confirmed the positive expression of both SOX10 and Nestin in the cells. Furthermore, after induction of differentiation using a melanocyte-specific culture medium, positive reactions were observed in the cells through DOPA staining, indicating the directed differentiation of HFNCSCs into melanocytes in vitro.

Melanin production is a complex physiological process involving various paracrine factors. Skin cells such as keratinocytes, fibroblasts, and melanocytes communicate through secreted regulatory factors, thereby regulating the biological functions of melanocytes. Stem Cell Factor (SCF) is a paracrine factor produced by fibroblasts, and its receptor, c-kit, is expressed in melanocytes. The binding of SCF to c-kit activates its own catalytic activity of phosphorylation and tyrosine kinase, completing its signal transduction [32–34]. Most paracrine factors regulate melanin production by upregulating or downregulating the expression of MITF. MITF is a transcription factor for tyrosinase, TRYP-1, and TRYP-2 [35]. S100B is a member of the S100 protein family, expressed in various tissues, including melanocytes, astrocytes, oligodendrocytes, and Schwann cells [36]. In this study, immunofluorescence staining was used to detect the expression of MITF protein in the cell nucleus, tyrosinase in the cytoplasm, and both C-KIT and S100B in the cell nucleus and cytoplasm in induced differentiated melanocytes. TYRP1 and TYRP2 were expressed on the cell membrane. Compared to the HFNCSC group, the fluorescence expression of c-KIT, MITF, S100B, tyrosinase, TYRP1, and TYRP2 in induced differentiated melanocytes increased, indicating that the differentiated melanocytes possess the functions of normal melanocytes.

HfNCSCs are found in the bulge of hair follicles and possess the ability to expand in vitro and display effective differentiation potential. Exosomes produced by stem cells play a crucial role in supporting axonal regeneration following peripheral nerve injuries. Exosomes derived from HfNCSCs contribute to decellularized nerve allografts for repairing facial nerve defects, potentially serving as a clinical alternative to autologous transplantation [37]. Treatment involving hair follicle stem cells, and their conditioned medium has demonstrated improvements in nerve damage and a reduction in infarct size in mice with ischemic stroke [38]. While studies have

shown that HfNCSCs can differentiate into melanocytes in vitro [39], research on the function of differentiated melanocytes remains limited. In this study, melanocytes induced from HfNCSCs were transplanted into depigmented areas of vitiligo mice. HE staining results indicated that, compared to the Control group, the Model group exhibited epidermal discontinuity, significant epidermal hyperplasia, increased thickness compared to the Control group, thickening of the spinous layer, reduced and dispersed follicle counts, and inflammatory cell infiltration. In contrast, the Transplantation group showed a thinner epidermis, reduced inflammatory infiltration, and an increase in the count of hair follicles. Immunofluorescence results indicated an upregulation in the expression of c-KIT, MITF, S100B, tyrosinase, TYRP1, and TYRP2 in the Transplantation group compared to the Model group. This suggests that transplanting melanocytes from induced differentiation of HfNCSC into the depigmented areas of vitiligo mice can promote repigmentation.

### *4.1. Limitations of the study*

This study provided an initial exploration into the potential of transplanting melanocytes from induced differentiation of HFNCSCs for treating vitiligo, establishing a theoretical foundation for further investigations into the repigmentation mechanisms of hair follicles and stem cell therapy for vitiligo. However, this study did not verify the toxicity of induced melanocytes. In future studies, additional research can be conducted on cell toxicity and functionality to ascertain whether the differentiated melanocytes exhibit the functions of normal melanocytes.

## 5. Star methods

Detailed methods are provided in the online version of this paper and include the following:

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- RESOURCE AVAILABILITY

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Materials availability.
Data and code availability.

- EXPERIMENTAL MODEL AND SUBJECT DETAILS

Isolation and cultivation of HFNCSCs.
The differentiation of HFNCSCs into melanocytes.
Establishing a mouse model of vitiligo-like skin lesions.

- METHODS DETAILS

Fluorescence microscopy imaging.
DOPA staining.
Immunofluorescence staining.
Immunohistochemistry.
Hematoxylin-eosin staining.

## Ethics approval

This study was reviewed and approved by Ethics Committee of Xinjiang Uygur Autonomous Region People's Hospital Ethical with the approval number: KY2021052645, Date: May 26th, 2021.

## Data availability

Raw data supporting the conclusions of this paper will be provided by the authors on request, without undue reservations.

## CRediT authorship contribution statement

**Hongjuan Wang:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Wen Hu:** Methodology, Data curation, Conceptualization. **Fang Xiang:** Resources, Formal analysis. **Zixian Lei:** Methodology. **Xiangyue Zhang:** Resources. **Jingzhan Zhang:** Validation. **Yuan Ding:** Validation, Supervision. **Xiaojing Kang:** Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare no competing interests.

## Acknowledgments

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