RESEARCH ARTICLE

In vitro Differentiation of Melanocyte Stem Cells Derived from Vitiligo Patients into Functional Melanocytes

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Abstract

BACKGROUND: Melanocyte stem cells (MelSCs) residing in the hair follicle bulge act as melanocyte reservoir for skin and hair, and may serve as an autologous source for treating vitiligo. Therefore, the study aimed to evaluate the *in vitro* differentiation ability of MelSCs derived from vitiligo patients into melanin-producing melanocytes for potential cellular therapy.

METHODS: MelSCs from the vitiliginous (V-MelSCs), non-vitiliginous (NV-MelSCs) regions of vitiligo patients, as well as from control subjects (C-MelSCs) were established for evaluating their differentiation potential into melanin producing cells. The differentiation abilities were compared at the cellular and molecular levels. MelSCs were differentiated *in vitro* into induced-melanocytes (iMCs) by supplementing the culture medium with melanogenic factors. iMCs were analyzed by quantitative polymerase chain reaction (qPCR) for the expression of key melanogenic markers, including *tyrosinase* (*TYR*), *tyrosinase-related protein 1* (*TYRP1*), *dopachrome tautomerase* (*DCT*), *microphthalmia-associated transcription factor* (*MITF*), *c-KIT* and *S100*. iMCs were also stained with L-DOPA to assess TYR activity in cells. Intracellular melanin content in iMCs was evaluated and compared among the vitiligo and control groups.

RESULTS: MelSCs induced into iMCs displayed morphological changes with longer dendrites and prominent nuclei. iMCs stained positive for L-DOPA with an average intracellular melanin content of 30 pg/cell. iMCs expressed key melanogenic genes and the relative expression did not differ significantly among the groups.

CONCLUSION: NV-MelSCs were unaltered by disease pathogenesis and capable of differentiating into melanocytes compared to V-MelSCs. Hence, these cells might offer a reliable source of melanocytes for vitiligo repigmentation therapy by autologous cellular transplantation.

KEYWORDS: melanocyte stem cells, differentiation, melanocytes, vitiligo, *in vitro*

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Introduction

Vitiligo is the most common depigmentation disorder characterized by loss of function of melanocytes within the skin lesions.(1) Currently, non-invasive techniques including ultraviolet radiation and topical corticosteroids are often chosen as first-line treatment for localized vitiligo, although the success rate of which is just slightly greater than 50%. (2) Non-responsiveness to these treatments is commonly observed in a more widespread condition, hence, surgical techniques, such as tissue and cellular transplantation have

recently gained more interest.(3-6) Tissue grafts offered impressive results in most patients, but only limited area of affected skin could be treated. Cellular grafting is performed using non-cultured epidermal cellular suspensions or autologous cultured melanocytes suspension isolated from unaffected donor skin biopsy. The advantage of cellular graft over tissue graft is that it permits to treat skin lesions much larger than the donor site.(5,6) Nevertheless, culturing epidermal melanocytes *in vitro* is expensive and time-consuming due to their low proliferative property. Hence, there is still further scope for enhancing the efficiency of cellular grafting techniques for vitiligo treatment.

It is known that the outer layers of the skin epidermis are replaced every 30-40 days in adults. This is maintained by epidermal stem cells residing in the basal membrane as well as hair follicle stem cells (HFSCs) exist in the bulge area of the outer root sheath of hair follicles.(7,8) A distinct population of HFSCs termed melanocyte stem cells (MelSCs) serve as melanocyte reservoir for skin and hair. Hair follicles are believed to have an immune privilege and therefore these cells are spared from the autoimmune attack in vitiligo pathogenesis.(9) MelSCs remain in a state of quiescence until the beginning of the anagen phase of hair cycle or until there is an external stimulus, such as trauma. Consequently, MelSCs proliferate, migrate toward hair bulb and differentiate to replenish functional melanocytes. Repigmentation following therapy, especially involving ultraviolet radiation, is chiefly from the proliferation and migration of MelSCs to the epidermis, by the activation of Wnt signaling pathway.(10,11) Due to these distinctive properties, MelSCs pose as potential candidates for cellular therapies against pigmentary disorders such as vitiligo.

Recently, we established human hair follicle-derived MelSCs and compared their characteristics obtained from both vitiligo patients and normal subjects to understand the effect of the disease on MelSCs integrity.(12) In continuation, the present study attempted to unravel the ability of MelSCs to differentiate *in vitro* into melanin-producing cells for prospective vitiligo treatment. MelSCs derived from the skin of vitiligo patients as well as of unaffected individuals were induced into melanocytes (induced-melanocytes/ iMCs) and their differentiation efficiencies were compared.

Methods

Sample Collection and Culture of MelSCs

Skin biopsies from both impacted and unaffected regions of vitiligo patients, as well as, full thickness skin biopsy from control subjects (n=2 each) were collected as described previously.(12) Further, MelSCs from the vitiliginous (V-MelSCs), non-vitiliginous (NV-MelSCs) regions of vitiligo patients, as well as from control subjects (C-MelSCs) used in the present study were culture expanded *in vitro* following a previously explained procedure.(12) Briefly, the expansion of cells was carried out in minimum essential medium-α (MEM-α, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 0.5% human melanocyte growth supplement (HMGS, Gibco) and 2 ng/mL epidermal growth factor (EGF, Biolegend, San Diego, CA, USA). The culture medium was changed every 3 days.

Induction of Melanogenesis

For differentiation, MelSCs were cultured in M254 media supplemented with 1% HMGS (Gibco), 2 nM endothelin 1 (Sigma-Aldrich/Merck, St. Louis, MO, USA), 0.5 mM dibutyryl cyclic adenosine monophosphate (DBcAMP, Sigma-Aldrich) and 100 nM α-melanocyte-stimulating hormone (α-MSH, Sigma-Aldrich) for 12 days with fresh media replaced every 3 days.

L-DOPA Stain Assay

The iMCs differentiated from MelSCs were fixed in 3.7% paraformaldehyde for 15 minutes. Following a wash with DPBS, the cells were incubated in 5 mM L-DOPA (Sigma-Aldrich) at 37ºC for 3 hours. iMCs treated with L-DOPA produced brown precipitates owing to the activity of key melanogenic enzyme, TYR and its formation was observed under phase-contrast microscope (Olympus, Tokyo, Japan).

Melanin Content Assay

Intracellular melanin content was measured in uninduced MelSCs, iMCs as well as epidermal melanocytes (MCs) isolated from the same donor tissue samples. MCs were used in the present study to make a comparative evaluation of melanogenesis in induced cells. To measure intracellular melanin content, cell pellet containing 5×10^5 cells was heated in 100 µL of 1N NaOH with 10% dimethyl sulfoxide (DMSO, Himedia, Maharashtra, India) at 80ºC for 2 hours. Following centrifugation to pellet out the cell debris, the absorbance of the supernatant containing melanin was recorded at 450 nm using a Microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). To translate the absorbance value to absolute quantity of melanin, a standard curve was established from serial dilutions of synthetic melanin from 0-500 µg/mL dissolved in 1N NH_4OH.

Quantitative Real-Time PCR (qPCR)

iMCs functionality depends on the expression of key melanogenic markers, such as *TYR*, *TYRP1*, *DCT*, *c-KIT*, *MITF* and *S100*. To assess the marker expression, iMCs were harvested and total RNA was extracted from 5×10^5 cells using RNAiso Plus kit (Takara, Tokyo, Japan). RNA was quantified and 1 µg of RNA was used to synthesize 20 µL of complementary DNA (cDNA) by PrimeScript™ RT reagent kit (Takara). The qPCR was performed using SYBR™ Green master mix on StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) to evaluate and compare relative expression (RE) of selected markers. The primer sequences were presented in Table 1.

Immunofluorescence Assay

iMCs derived from C-MelSCs, NV-MelSCs and V-MelSCs were seeded on chamber slides at a density of 5000/well and allowed to attain 70% confluence. Cells were then fixed with 3.7% paraformaldehyde overnight at 4ºC. Cells were then treated with 0.2% Triton-X for 20 min followed by treatment with 1% bovine serum albumin for 45 min. Subsequently, 200 µL of primary antibodies against TYR, TYRP1 and MITF (Biolegend) were added to respective wells and incubated overnight at 4ºC. Following which, 100 µL of fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Biolegend) was added to each well and incubated for 1 hr at 37ºC. Cells were counterstained with 250 µL of propidium iodide (PI) for 10 min in the dark followed by a wash with DPBS and allowed to dry. Imaging was performed using a fluorescence microscope (Nikon, Tokyo, Japan).

Statistical Analysis

The data were presented as mean±SD and analyzed by using GraphPad Prism (GraphPad Software, San Diego, CA, USA). The data were subjected to one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test. A $p<0.05$ was considered as the significant value of difference.

Results

Melanogenic Induction of MelSCs Caused Distinct Morphological Changes

MelSCs possessed a spindle shape with single or double dendritic extensions (Figure 1, left picture). MelSCs from controls were designated as C-MelSCs and MelSCs from vitiligo patients were designated as NV-MelSCs and V-MelSCs for non-vitiliginous and vitiliginous skin, respectively. By day-9 in melanogenic induction media, the number and length of dendrites were visibly larger (Figure 1, rigth picture). Induced melanocytes possessed more noticeable nuclei. Cells were then designated as C-iMCs, NV-iMCs and V-iMCs.

Increased TYR Enzyme Activity in iMCs

L-DOPA stains functionally active melanocytes into brown colour due to the action of tyrosinase enzyme. iMCs from all MelSCs stained L-DOPA visibly with brown pigmentation compared to MelSCs (Figure 2). The percentage of cells that were found functionally active was 65±4%, 67±2% and 60±7% for C-iMCs, NV-iMCs and V-iMCs, respectively.

Melanogenic Induction Resulted in Higher Levels of Intracellular Melanin Content

Intracellular melanin content was evaluated in MelSCs before and after induction as an additional representation of the functionality of iMCs. Significantly higher levels of melanin were observed in all iMCs post induction with a mean value of 30±1 pg/cell (Figure 3).

Upregulation of Melanogenic Markers in iMCs

Expression levels of key melanocytes markers of MelSCs before and after induction were compared by qPCR analysis (Figure 4). Relative expression of melanocyte markers, such as *TYR*, *TYRP1* and *S100* was significantly higher ($p<0.01$)

Table 1. Primer sequences for marker genes used in the study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
TYR	CTTGTGAGCTTGCTGTGTCG	GTGAGGTCAGGCTTTTTGGC
TRP1	GTGCCACTGTTGAGGCTTTG	ATGGGGATACTGAGGGCTGT
DCT	TCTTTTGGGCACTCAGCATGTA	CTTCACCTGAGGAAAGGCTGG
MITF	GCCTGTCTCGGGAAACTTGA	GCCAGTGCTCTTGCTTCAGA
c-KIT	GCTCTGCTTCTGTACTGCCA	GGTGTGGGGATGGATTTGCT
S100	TGTAGACCCTAACCCGGAGG	TGCATGGATGAGGAACGCAT
β-ACTIN	TCCTTCCTGGGCATGGAG	AGGAGGAGCAATGATCTTGATCTT

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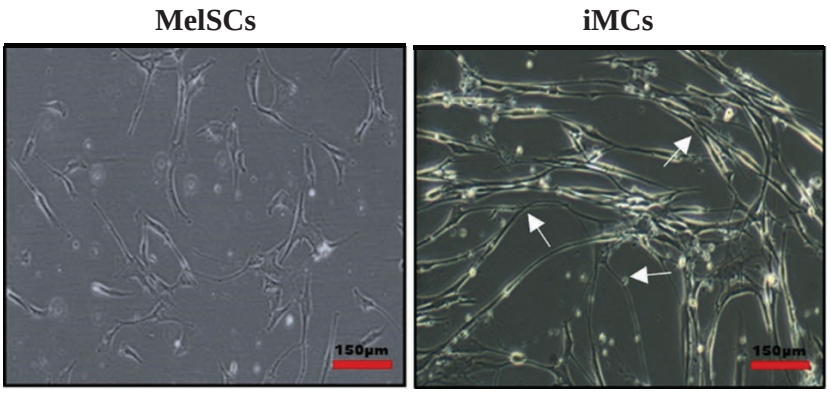

Figure 1. Morphological features of MelSCs and iMCs. Left pic: MelSCs derived from hair follicles displayed spindle shape with single or double dendritic extensions. Right pic: MelSCs were differentiated into melanin-producing cells (iMCs) exhibiting increased number as well as longer dendrites (white arrows).

in all iMCs compared to their MelSC counterparts. However, the relative expression of *c-KIT* and *DCT* did not increase significantly (p>0.01) in induced cells. Relative expression of *MITF* was observed to be much higher in non-induced MelSCs derived from control subjects and non-vitiliginous region of vitiligo subjects. Further, the expression of key melanocyte markers at protein level in iMCs was evaluated by immunofluorescence assay. The results showed that all iMCs positively expressed *TYR* (Figure 5), *TYRP1* (Figure 6) and *MITF* (Figure 7) markers.

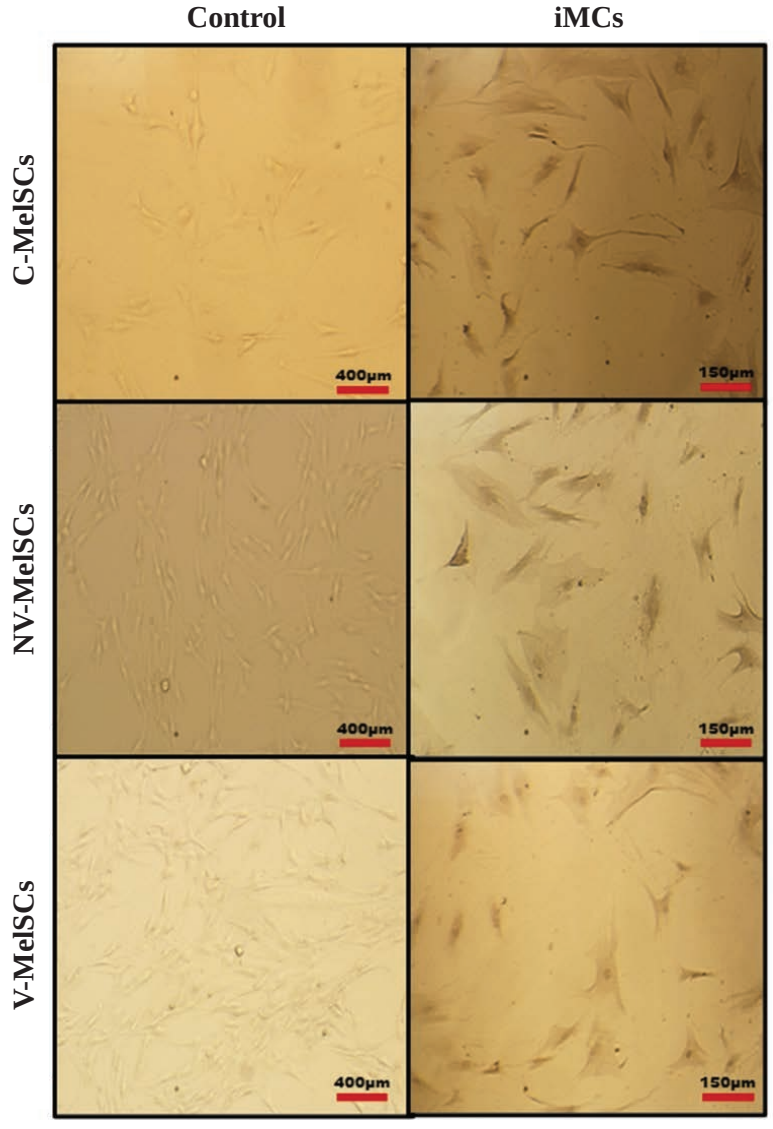

Figure 2. L-DOPA assay to assess TYR activity in cells. Brown precipitate indicates tyrosinase (TYR) activity.

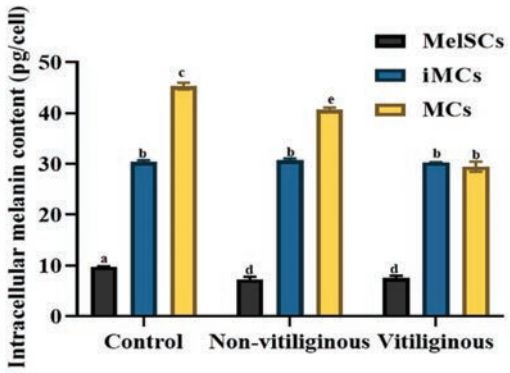

Figure 3. Assessment of intracellular melanin content. The melanin was measured in uninduced MelSCs, iMCs and melanocytes MCs isolated from the same donor tissue samples. All the values were represented as mean±SD. [a,b,c,d,e] indicate statistically significant differences in melanin levels between MelSCs, iMCs and MCs.

Discussion

The earlier studies on pigment cells suggested that the MelSC reservoir in the hair follicles are involved in repigmentation of epidermis and hair shafts whenever necessary.(7,13) MelSCs have been identified and characterized in several mammalian species, but the isolation of MelSCs from human hair follicles is lagging due to challenges regarding their marker expression and detection.(5) Previously, we reported the establishment of MelSCs *in vitro* using optimized media components and specific growth factors.(12) MelSCs were recognized by the expression of stem cell markers, such as *CD34*, *Pax3* and *Sox10* as well as melanocyte progenitor markers, including *MITF*, *TYRP1* and *DCT*. Although MelSCs from vitiligo patients were comparable with normal MelSCs in terms of morphology and colony forming ability, vitiligo-derived MelSCs displayed diminished proliferative capacity and downregulation of certain key genes.(14) In this study, we induced MelSCs to differentiate into melanin-producing cells with the expression of key markers of melanogenesis.

TYR is considered the most significant enzyme involved in melanogenesis, and MelSCs lack the expression of TYR and hence are incapable of synthesizing the pigment. In an *in vivo* setting, MelSCs differentiate in response to a stimulus and gain the expression of TYR to become functional melanin-producing melanocytes.(15) Thus, the chief method of assessing the differentiation potential of MelSCs is by the acquisition of TYR enzyme activity and formation of subsequent metabolites in the cells post-induction. Thus, MelSCs were induced to differentiate into terminal melanocytes *in vitro* using induction media containing growth supplements, such as α-MSH, endothelin-1 and DBcAMP that promote melanogenesis. The α-MSH is a neuropeptide that is naturally synthesized by keratinocytes as well as melanocytes in response to sun UV light exposure. It activates the cAMP signalling pathway that induces the target genes encoding *TYRP1* and *DCT*.(16) Endothelins are keratinocyte-derived factors that display a mitogenic effect on melanocytes.(17,18) Hence, endothelin-1 along with DBcAMP were supplemented in the induction medium.

The results of this study showed that MelSCs cultured in melanogenic induction media acquired more dendritic features, and these iMCs attained TYR activity as demonstrated by the development of brown stain upon treatment with L-DOPA.(19) Intracellular melanin content significantly increased in iMCs compared to their MelSC counterparts, which sheds light on their acquired functionality post induction. Melanogenic yield was similar in all iMCs which ensures no loss of functionality in vitiligo derived cells due to disease pathogenesis. This agrees with the observation that hair follicle-derived MelSCs are not affected by the disease.(9) The expression of *TYR* in induced cells was also confirmed at the mRNA and protein levels.

TYRP1 and DCT (TYRP2) are tyrosinase-related proteins that share genetic sequence and structural resemblance with TYR, all being type 1 membrane proteins in the melanosomes. It has been hypothesized that TYRP1 was evolved from the duplication of TYR and later bringing about TYRP2/DCT.(20) DCT is involved in the tautomerization of dopachrome into DHICA while TYRP1 helps in the oxidization and subsequent polymerization of DHICA units into eumelanin.(21) Both of these tyrosinase related proteins assist in stabilizing TYR. In the present study, a significant upregulation of *TYRP1* in iMCs was observed. However, the *DCT* expression was not statistically significant, and there is evidence to suggest that *DCT* expression is observed markedly ahead of *TYR* and *TYRP1* during the development, making it one of the earliest melanoblast markers.(22,23) Both *TYRP1* and *DCT* expression did not significantly differ amongst the iMCs.

Stem cell factor (SCF) is a growth factor involved in regulating proliferation, differentiation and survival of various cells including melanocytes.(24) SCF binds to its receptor KIT or CD117 (encoded by gene *c-KIT*), which is a tyrosinase kinase receptor located on the cell surface. SCF/c-KIT signalling activates MAPK pathway that leads to the phosphorylation of *MITF* and hence stabilizing it.(24) *MITF* exhibits a dual function: activation of transcription of

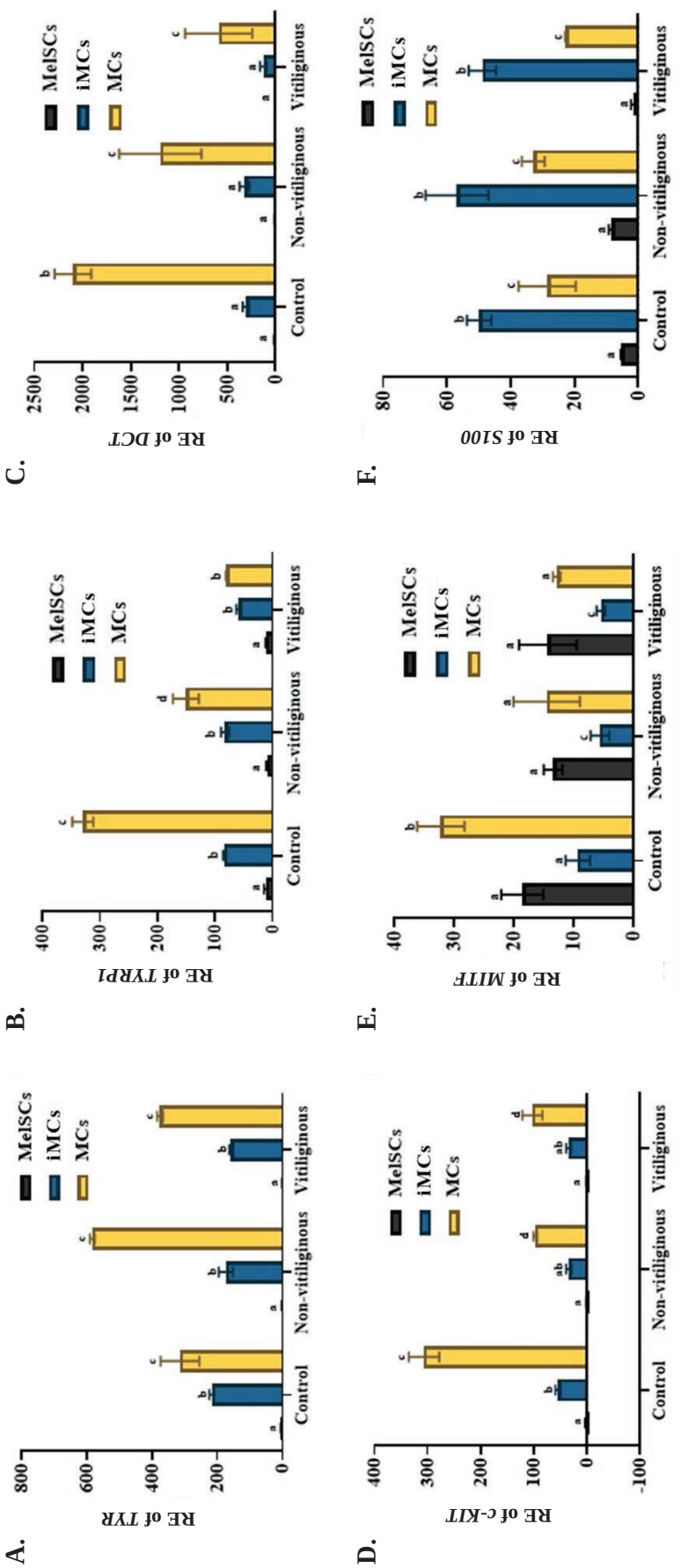

Figure 4. qPCR analysis of melanocyte marker genes. Relative expression of: *TYR* (A), *TYRP1* (B), *DCT* (C), *c-KIT* (D), *MITF* (E) and *S100* (F) in uninduced MelSCs, iMCs, and MCs isolated from the same donor tissue samples. ^{a,b,c} represent significant differences at $p<0.05$. Data were expressed as mean±standard deviation (SD).

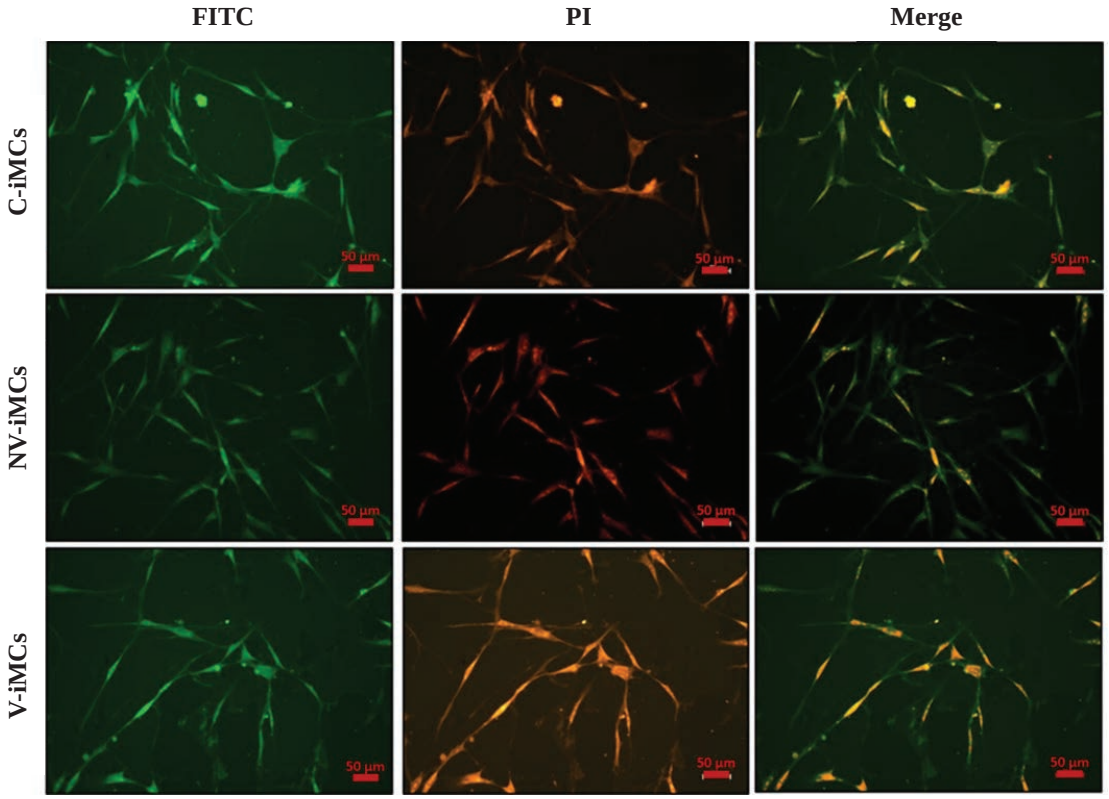

Figure 5. Immunofluorescence analysis of tyrosinase enzyme (TYR). Induced melanocytes from control (C-iMCs), non-vitiliginous (NV-iMCs) and vitiliginous (V-iMCs) MelSCs. FITC-conjugated (green) secondary antibody was used. Nuclei were counterstained with PI (red).

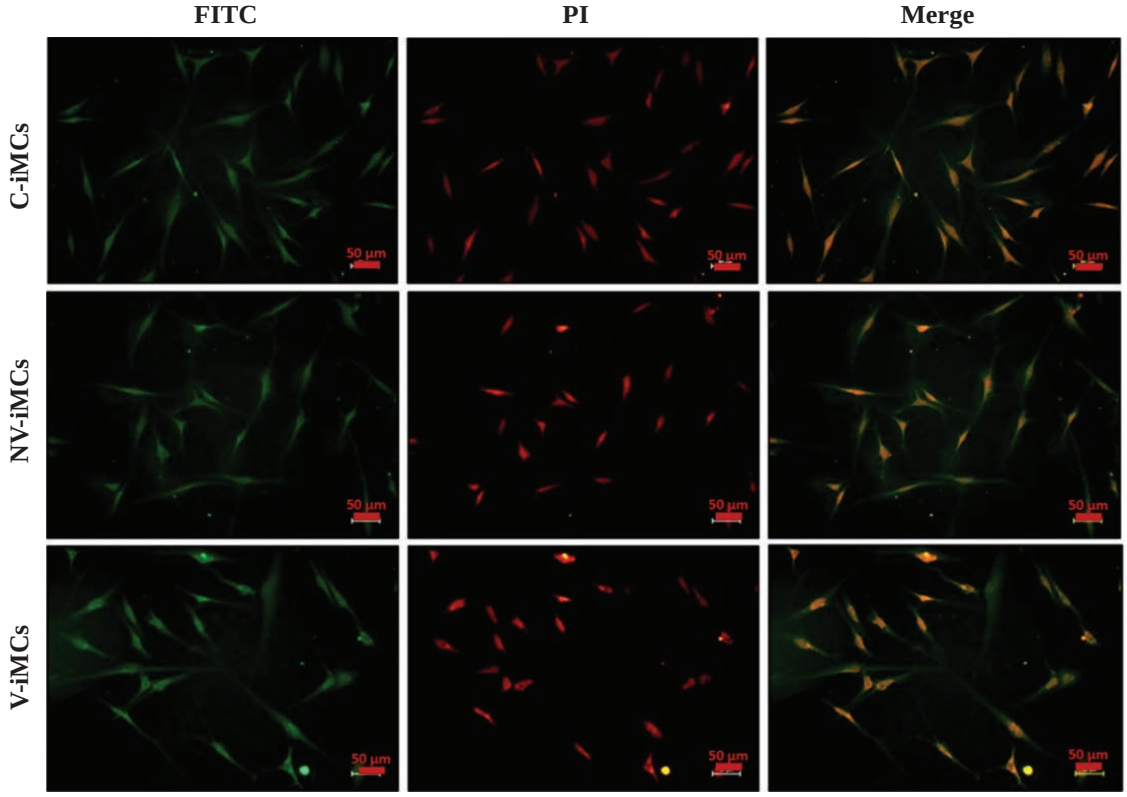

Figure 6. Immunofluorescence analysis of tyrosinase related protein 1 (TYRP1). Induced melanocytes from control (C-iMCs), non-vitiliginous (NV-iMCs) and vitiliginous (V-iMCs) MelSCs. FITC-conjugated (green) secondary antibody was used. Nuclei were counterstained with PI (red).

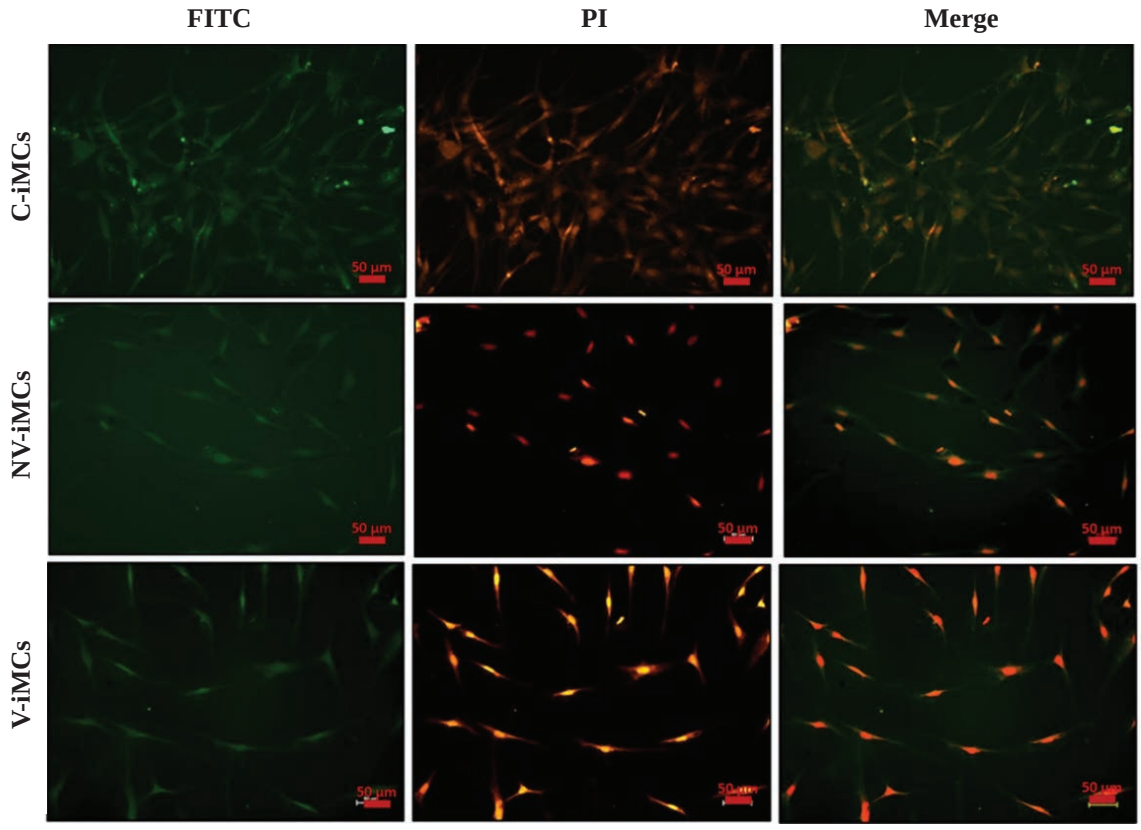

Figure 7. Immunofluorescence analysis of melanocyte inducing transcription factor (MITF). Induced melanocytes from control (C-iMCs), non-vitiliginous (NV-iMCs) and vitiliginous (V-iMCs) MelSCs. FITC-conjugated (green) secondary antibody was used. Nuclei were counterstained with PI (red).

melanogenesis genes *TYR*, *TYRP1* and *DCT* in melanocytes; and survival of melanocyte precursors in the bulge region (25), facilitated by targeting anti-apoptotic gene *BCL-2* (26,27). In this study, *c-KIT* expression was significantly increased post induction, but *MITF* expression was downregulated. A similar decrease in the expression levels of *MITF* in induced cells was also reported previously.(28) *MITF b*eing known as the master regulator of melanogenesis in melanocytes, this downregulation may imply its altered primary role in hair follicle-derived MelSCs aiming at their survival and maintenance of the undifferentiated state.(29)

In this study, MCs were simultaneously isolated and characterized along with iMCs to compare their efficiencies as melanin-producing cells. Intracellular melanin content in C-iMCs and NV-iMCs was lower to their MCs counterparts. However, V-iMCs and V-MCs had comparable levels of intracellular melanin, owing to the disease pathogenesis affecting epidermal melanocytes ability to synthesize melanin. Similarly, iMCs failed to display equivalent expression of key genes of melanogenesis compared to MCs. Further studies on MelSCs could involve the evaluation of immunoregulatory properties which enables in predicting their long-term therapeutic effects on vitiligo remission.

Conclusion

Hair follicle-derived MelSCs isolated and established *in vitro* were successfully differentiated into melanin-producing cells, and the iMCs were able to express key enzymes and proteins involved in melanin biosynthesis. Further, there were no noticeable variations in the differentiation ability of NV-MelSCs compared with MelSCs from the normal subjects. However, melanin-producing propensity of iMCs was slightly lower in comparison with MCs derived from epidermis of the same skin and thus, warrants further studies for enhancing the differentiation efficiency of MelSCs.

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Authors Contribution

MV, SS, and MKB were involved in the design of the research concept, data acquisition and results interpretation. MV, VS and MKB analyzed the data and drafted the manuscript. NS and JP were involved in the sample collection and data analysis. SKY and PP were involved in data and images composition and providing critical revisions.

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