Dermatology Practical & Conceptual

Lenalidomide Augments Differentiation of Cultured Hair Follicle Derived Melanocyte Stem Cells Into Functional Melanocytes

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Key words: lenalidomide, melanocyte stem cells, melanocyte, differentiation

Citation: Kumari U, Pervaiz N, Kaur H, Sharma H, Parsad D, Kumar R. Lenalidomide Augments Differentiation of Cultured Hair Follicle Derived Melanocyte Stem Cells Into Functional Melanocytes. *Dermatol Pract Concept*. 2023;13(2):e2023077.
DOI: https://doi.org/10.5826/dpc.1302a77

Accepted: September 3, 2022; **Published:** April 2023

Funding: None.

Competing interests: None.

Authorship: All authors have contributed significantly to this publication.

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ABSTRACT

Introduction: Melanocyte progenitors are embryonically derived from the neural crest and subsequently get localized in hair follicles and epidermis to provide hair and skin pigmentation. These progenitor cells in hair follicles repeatedly proliferate and differentiate to maintain pigmentation. Vitiligo, a pigmentary disorder, is associated with loss of melanocytes. Repigmentation of vitiligo lesions mainly depends upon the proliferation, migration and differentiation of melanocyte stem cells (MelSCs) into functional melanocytes. The current study is designed to check the efficacy of lenalidomide, an imide drug in the differentiation of MelSCs into functional melanocytes.

Objectives: The aim of the study is to check the effect of lenalidomide in the proliferation, migration of cultured hair follicle derived melanocyte stem cells and their differentiation into functional melanocytes.

Methods: Primary culture of MelSCs was established from whisker hair of C57BL/6 mice. Proliferation and migration of cultured cells were done by MTT assay and Boyden's chamber migration assay, respectively. Effect of lenalidomide on the MelSCs differentiation was checked at gene level by qPCR and protein expression was checked by immunocytochemistry.

Results: A significant increase in the migration of MelSCs in comparison to control was also observed. Lenalidomide treatment significantly increased the expression of melanocyte specific genes in cultured MelSCs as compared to control.

Conclusions: From the results we concluded that lenalidomide induce the proliferation and migration of MelSCs and accelerate the differentiation of MelSCs into functional melanocytes.

Introduction

Hair follicle melanocyte stem cells (MelSCs) are present in bulge or sub-bulge regions of hair and are important reservoirs of melanocytes. They persist as self-renewing cells and play a very significant role in the pigmentation of hair and skin [1]. They regenerate mature melanocytes for hair and skin in response to some kind of melanocyte loss under normal conditions and ultimately maintain the pigmentation [2]. The regeneration of functional melanocytes is influenced by activation, proliferation and differentiation of these MelSCs [3]. Loss of functional melanocytes causes skin depigmentation and thus leads to depigmentory disorders like vitiligo [4,5]. Vitiligo is an autoimmune cutaneous disorder that has grave psychosocial impact on the patient's quality of life [6]. The mechanism of melanocyte disappearance in this disease still remains unclear [7]. Several treatment modalities are available like the conventional medical, surgical and physical means but none are completely satisfactory.

In the depigmented patches there is a loss of functional melanocytes but hair follicle MelSCs are preserved. Therefore this original source of regeneration in the hair follicle bulge is being targeted to repopulates the vitiligo-depigmented epidermis with melanocytes [8]. Various studies have shown that MelSCs in the outer root sheath played important part in the epidermal follicular repigmentation. However the activation, proliferation, migration and differentiation of MelSCs from the outer root sheath of the hair follicle require more novel and effective strategies.

Here we aimed at investigating the potential therapeutic efficacy of lenalidomide as the possible repigmentation strategy. Lenalidomide is an immunomodulatory drug that has shown a clinical effect in several autoimmune and inflammatory disorders. Recently, its effect was observed as the hyperpigmentation of skin in black/African-American patients suffering from multiple myeloma [9]. Interestingly, in a case study it also led to progressive hair repigmentation in an old multiple myeloma patient [10]. Pervaiz et al [11] also reported the inhibitory influence of this drug on the progression of depigmented lesions in a vitiligo mouse model. Therefore, the current study was designed to check the role of lenalidomide on the differentiation of murine hair follicle derived melanocyte stem cells into pigment producing melanocytes.

Objectives

The aim of the study is to check the effect of lenalidomide in the proliferation, migration of cultured hair follicle derived melanocyte stem cells and their differentiation into functional melanocytes.

Methods

Ethical Statement

The study design was based on the guidelines set by the Institutional Animal Ethics committee, Panjab University, Chandigarh, India (Approval No: PU/45/99/CPCSEA/IAEC/2017/31). C57BL/6 mice were maintained as per the guiding principles of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Reagents

The reagents used in the study include Dulbecco's Modified Eagle Media (Cat. No. BE12-604F), Fetal Bovine Serum (Cat. No. 10270106), and Recombinant Human FGF-Basic protein (Cat. No. PHG0264). The antibodies include Anti-mouse AF488-conjugated CD34 (Cat. No. sc-7324AF488), PE-conjugated TRP2/ DCT (Cat.No. sc-74439PE), primary anti-mouse tyrosinase (Cat. No. sc-20035) and nestin (Cat. N0 sc-23972) antibody and secondary antibody anti-mouse IgG-FITC (sc-2010), purchased from Santa Cruz Biotechnology.

Isolation and Culture of MelSCs

MelSCs culture was established from whisker hair cells of C57BL/6 mice at 4 weeks of age. Mice were euthanized and the upper lips containing vibrissae were dissected as described by Gilanchi [12].It was rinsed with betadine and 70% ethanol for 2 minutes and placed in phosphate buffer saline (PBS). Hair follicles were separated from lip pad under sterile conditions and were incubated overnight at room temperature in collagenase IV (1mg/ml). After incubation the digested hair follicles were pipetted thoroughly to obtain single cell suspension. Afterwards, the cell suspension was centrifuged at 1000 rpm for 5 minutes and the obtained pellet was resuspended in a stem cell specific culture medium which contains 80% KnockOut™ DMEM/F12 medium, 20% KnockOut™ serum replacement, 200 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids and 4 ng/ml basic fibroblast growth factor (bFGF) [13].

Characterization of Cultured Spheres

Cultured spheres were characterized for the presence of nestin which is a neural crest marker. Spheres were fixed in chilled paraformaldehyde for 10 minutes and then washed in PBS. Blocking was performed overnight at 4° C with 3% BSA in a humidified chamber. For nestin immunostaining, primary antibody (dilution 1:100) and secondary antibody (dilution 1:200) were used. After antibody incubation PBS washing was done and spheres were then counterstained with DAPI. Spheres were washed again with PBS and examined using confocal microscope.

Subculturing of Cells From Cultured Spheres

Subculturing was done for further propagation of cells. For that, spheres were easily detached by agitating the culture plate. Spheres were then transferred to new culture wells, where they were cultured in media having DMEM, FBS and recombinant human FGF-basic protein [14]. Next day cells started coming out of the sphere and when the cells reached to 80% confluency, they were further subcultured by trypsinization method using trypsin-EDTA.

Characterization of Cells Obtained From Spheres

Cultured cells obtained from the spheres were characterized by nestin, CD34, DCT and TYR immunostaining and DOPA staining. For immunostaining, cells were fixed in chilled paraformaldehyde for 10 minutes and then washed in PBS. Blocking was performed overnight at 4°C with 3% BSA in humidified chamber. Next day, the fixed cells were incubated with fluorochrome conjugated antibodies CD34 and DCT for 3 hours at room temperature (dilution 1:150). For nestin and tyrosinase immunostaining, primary antibody (dilution 1:100) and secondary antibody (dilution 1:200) were used for 3 hours and 2 hours respectively. After PBS washing, cells were then counterstained with DAPI. Cells were washed again with PBS and examined using confocal microscope. For DOPA staining, washing of cultured cells was done with 1X PBS and then fixed with chilled paraformaldehyde for 10 minutes. 10 mM L-DOPA was added to the cells and then incubated at 37° C. Cells were examined hourly under the microscope for 4 hours.

Cell Proliferation Assay

Cell proliferation was checked by MTT assay by following the modified procedure of Mosmann [15].Cultured MelSCs (5×10^3) were plated in the 96-well cell culture plate. Lenalidomide treatment was given to the cells at 2.5 µM and 5 µM concentrations, the untreated cells were considered as control. After 24 hours of lenalidomide treatment, culture media was removed from each well and 100µl of MTT (0.5 mg/ml) solution was added. The cells with MTT (0.5mg/ml) solution were incubated for 4 hours at 37°C till the formation of formazan crystals. After incubation 100 µl of DMSO was added to each well and proper dissolution of formazan crystals was done. Optical density was evaluated using microplate spectrophotometer (EPOCH) at 560 nm.

Boyden Chamber Migration Assay

Cultured MelSCs were starved for about six hours in serum free culture media. After the starvation period, 3×10^4 cells were plated in serum-free medium (no chemoattractant) in the upper chamber. To the lower chamber, medium with serum for control group and serum plus lenalidomide for treatment groups was added. Cells were kept at 37° C in CO_2 incubator for 18 hours. Afterwards, cells were fixed with 4% formaldehyde and stained with 0.5% crystal violet. The inside of each insert was swabbed using cotton swabs. Randomly selected fields were photographed, and the cells that had migrated were counted.

In Vitro Treatment of Melscs With Lenalidomide and Its Effect on Differentiation Into Functional Melanocytes

Cultured MelSCs were treated with lenalidomide at concentrations of 2.5 µM and 5 µM for 2 weeks. The media was changed in plate after every 48 hours. After 2 weeks of lenalidomide treatment, its effect on the differentiation of MelSCs into melanocytes was checked at both gene and protein level.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR was performed to analyze the transcriptional gene expression. RNA was isolated using Trizol reagent (Sigma) by following manufacturer's guidelines. Gel electrophoresis was performed after RNA isolation to assess its quality. Absorbance at 260 and 280 nm measured on Nano-drop was used to determine the concentration and purity of RNA. cDNA was synthesized from the isolated RNA using iScript™ c-DNA synthesis kit (BIO-RAD). qPCR was performed using SYBR(R) Green JumpStart (TM) TaqReadyMix (TM) master mix on a real time PCR system (Roche LC480) according to manufacturer's guidelines. The forward and reverse sequences of primers are given in Table 1. The relative levels of mRNA expression were calculated by following the method of Livak and Schmittgen [16], normalized by β-*actin*.

Immunostaining of Differentiated Melanocytes

After 14 days of differentiation assay MelSCs cells were checked for melanocyte marker TYR and DCT by immunostaining (protocol discussed in characterization of cells obtained from spheres section). The fluorescence was quantified using image-J software.

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism 6 for windows version 6.01 (GraphPad Software, Inc.). Final results were shown as mean with standard deviation (mean ± SD). The data was examined for normality by the Shapiro–Wilk test. Two-tailed Student t test was done to calculate the statistical significance between two groups, whereas One-way analysis of variance (one-way ANOVA) followed by post hoc Tukey test was used to calculate statistical significance for more than two groups. P-values less than 0.05

Table 1. Primers used for qPCR, their Amplicon Length and Annealing Temperature.

Gene	Forward primer	Reverse primer	Amplicon length	Annealing temperature
β-Actin	5' GAATTGCTATGTGTCTGGGT 3'	5' CATCTTCAAACCTCCATGATG 3'	257bp	58°C
TYR	5' GGGCCCAAATTGTACAGAGA 3'	5' ATGGGTGTTGACCCATTGTT 3'	174bp	58°C
TYRP1	5' AAGTTCAATGGCCAGGTCAG 3'	5' TCAGTGAGGAGAGGCTGGTT 3'	157bp	58°C
DCT	5' AGCAGACGGAACACTGGACT 3'	5' GCATCTGTGGAAGGGTTGTT 3'	180bp	58°C
MITF	5' GGAACAGCAACGAGCTAAGG 3'	5' TGATGATCCGATTCACCAGA 3'	170bp	58°C
SOX9	5' CGACTACGCTGACCATCAGA 3'	5' AGACTGGTTGTTCCCAGTGC 3'	188bp	58°C
SOX10	5' AGCCCAGGTGAAGACAGAGA 3'	5' ATAGGGTCCTGAGGGCTGAT 3'	175bp	58°C

(*P <0.05, **P <0.01, ***P <0.001 and ****P <0.0001) were considered as significant.

Results

Primary Culture and Characterization of MelSCs

MelSCs were cultured from whisker hair cells of C57BL/6mice. On the 5th day of cell plating, some of the cells started aggregating and forming spheres. A well-marked sphere took around 10 to 15 days in formation (Figure 1A).

Spheres were characterized for neural crest stem cells marker nestin, and it was found that the cells of the spheres showed positive staining for nestin (Figure 1D). For subculturing of MelSCs, spheres were taken out and plated in a new culture plate. The next day, the cells began to migrate out of the spheres gradually (Figure 1B). These cells were than further characterized for specific stem cell marker nestin, CD34, and melanocyte stem cell marker, DCT and melanocyte specific marker TYR. We observed that these sphere-derived cultured cells were nestin (Figure 1E) and DCT (Figure 1G) positive and were negative for CD34 (Figure 1F) and TYR (Figure 1H). Further, DOPA staining was performed to check the presence of melanocyte contamination in MelSCs culture. The results indicated that cells were negative for DOPA, compared to melanocytes taken as positive control (Figure 1C).

Effect of Lenalidomide on the Proliferation and Migration of MelSCs

Effect of lenalidomide was checked on the proliferation of MelSCs and we found that after 24 hours of treatment, it increased the proliferation of MelSCs at both 2.5 µM and 5µM concentrations in comparison to untreated control, although the increase was insignificant (Figure 2,A- and B). Further it significantly enhanced the migration of MelSCs at both concentrations in comparison to control. The percentage of migrated cells increased by 88% and 95% in 2.5 µM and 5 µM treated wells respectively in comparison to control (Figure 2, C and D).

Effect of Lenalidomide on the Differentiation of MelSCs Into Functional Melanocytes

The cultured cells derived from spheres were treated with lenalidomide for 14 days to check its effect on the differentiation of MelSCs into functional melanocytes. After 14 days of treatment cell were checked for expression of melanocyte specific genes. qPCR results showed that lenalidomide significantly enhanced the expression of melanocyte specific genes (*DCT, TYRP1, TYR, MITF, SOX10, SOX9*) as compared to control. After lenalidomide treatment, the expression of *TYR* was found higher at 2.5 µM (1.902 ± 0.023 fold) and 5 µM concentrations (3.516 ± 0.269 fold) as compared to control. Similarly, we observed a significant elevation in the expression of *TYRP1* (2.543 ± 0.08 fold) and (1.715 ± 0.104 fold), *DCT* (1.786 ± 0.335 fold) and (3.516 ± 1.353 fold), *MITF* (1.640 ± 0.080 fold) and (1.498 ± 0.188 fold), *SOX9* (2.738 ± 0.129 fold) and (1.166 ± 0.470 fold), and *SOX10* (1.077 ± 0.408 fold) and (5.508 ± 2.203 fold) in 2.5 µM and 5 µM concentrations, respectively (Figure 3). Differentiation of MelSCs into functional melanocyte was further confirmed by protein expression of melanocyte specific genes TYR and DCT. It was found that lenalidomide treatment significantly increased the protein expression of tyrosinase in treated MelSCs. After 14 days of lenalidomide treatment, number of TYR positive cells were found significantly higher at both 2.5 µM (14.33 times) and 5 µM concentration of lenalidomide (7.33 times) as compared to control. Similarly, we observed a significant elevation in the expression of DCT (1.56 ± 0.1081 fold) and (1.33 ± 0.12 fold) in 2.5 µM and 5 µM concentrations, respectively (Figure 4). Hence, the results from immunocytochemistry also showed that the protein expression of TYR and DCT was significantly higher in lenalidomide treated cells in comparison to control (Figure 4).

Conclusions

Vitiligo is an autoimmune disease characterized by cutaneous depigmentation caused by autoimmune-mediated damage of melanocytes. Despite continuous research is being

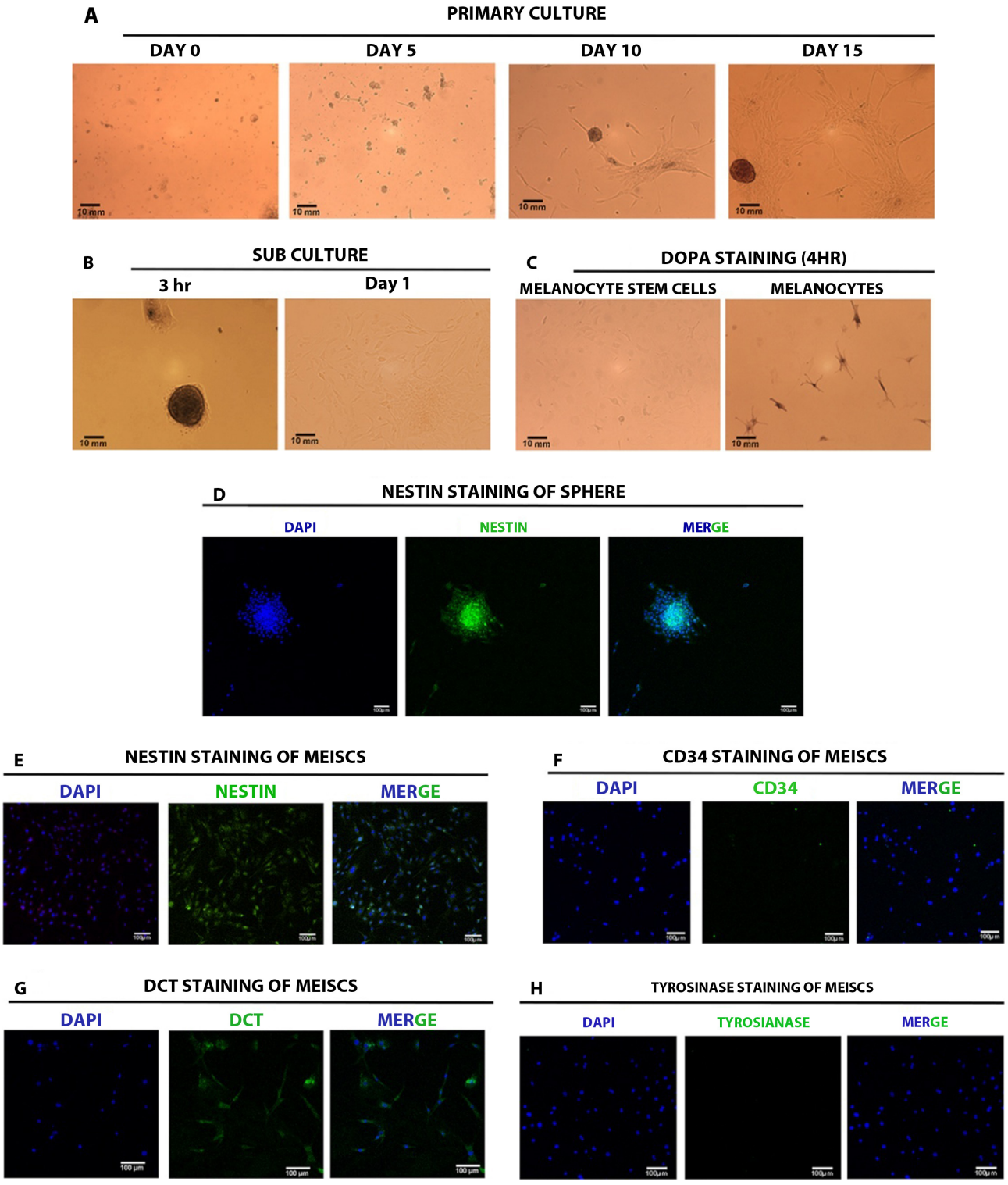

Figure 1. Representative images showing (A) primary culture of melanocyte stem cells at Day 0, Day 5, Day 10 and Day 15, (B) Subculture of MelSCs at 3 hours and Day 1, (C) Dopa staining of melanocyte stem cells and melanocytes (taken as positive control) after 4 hours, (D) Confocal imaging indicating nestin immunostaining for the characterization of MelSC spheres. Representative images from confocal microscopy showing the characterization of MelSCs by immunostaining with (E) nestin, (F) CD34, (G) DCT, and (H) tyrosinase.

elucidated, a definitive cure still remains elusive. Therefore, many newer therapeutic options are being explored to improve the outcome; immunomodulators being the most recent and exciting additions.

Lenalidomide is an FDA approved immunomodulatory imide drug which is currently being investigated in various disorders and has shown therapeutic effects on various autoimmune or inflammatory diseases. Dasanu et al [10] reported the first case of progressive hair repigmentation with the use of this drug in an elderly patient of multiple myeloma. Further they hypothesized that lenalidomide may be capable of stimulating migration and differentiation of melanocytes

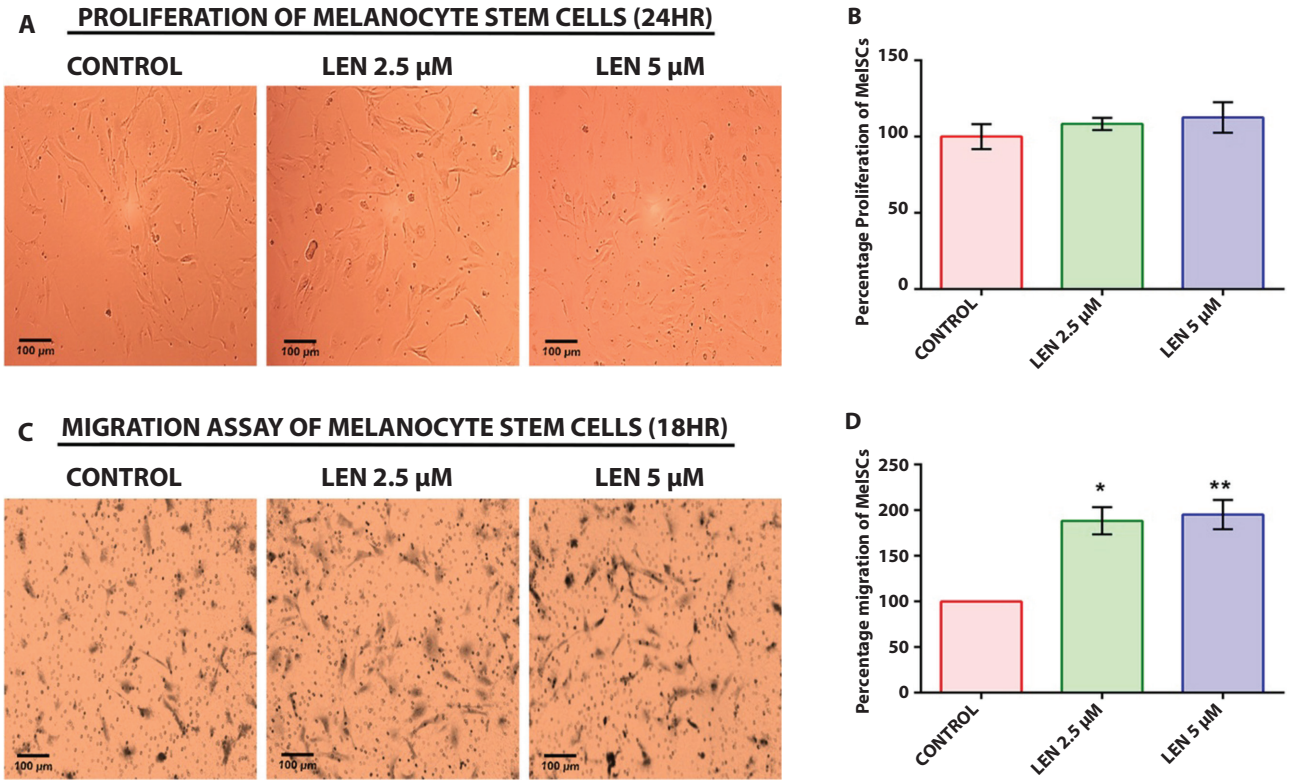

Figure 2. (A) Representative pictures of cultured MelSCs. (B) Bar diagram showing the effect of lenalidomide (2.5 µM and 5 µM) on the proliferation of MelSCs as compared to untreated control cells after 24 hours of treatment. (C) Light microscopic images depicting the migration of MelSCs in control, 2.5 µM and 5 µM lenalidomide treated cells at 18 hours. (D) Bar diagram displaying the effect of lenalidomide (2.5 µM and 5 µM) on migration (at 18 hours) of MelSCs as compared to control. Data are presented as mean ± standard deviation; statistical significance is shown by *P < 0.05, **P < 0.01.

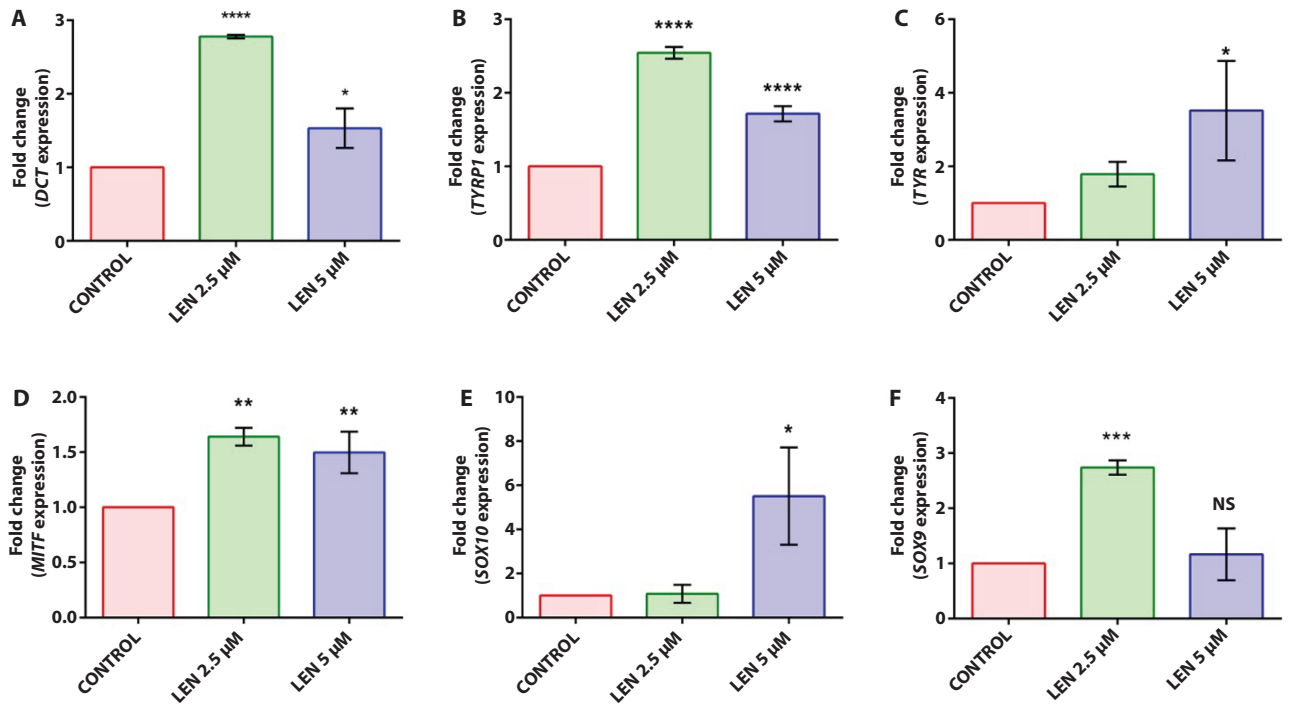

Figure 3. Bar diagram showing relative mRNA expression of (A) DCT (B) TYRP1 (C) TYR (D) MITF (E) SOX10 and (F) SOX9 in control, 2.5 µM lenalidomide and 5 µM lenalidomide treated MelSCs. The relative level of target gene expression was calculated by Livak and Schmittgen method and normalized with β-actin. Data are presented as mean ± standard deviation; statistical significance is shown by (*P <0.05, **P <0.01, ***P <0.001 and ****P <0.0001).

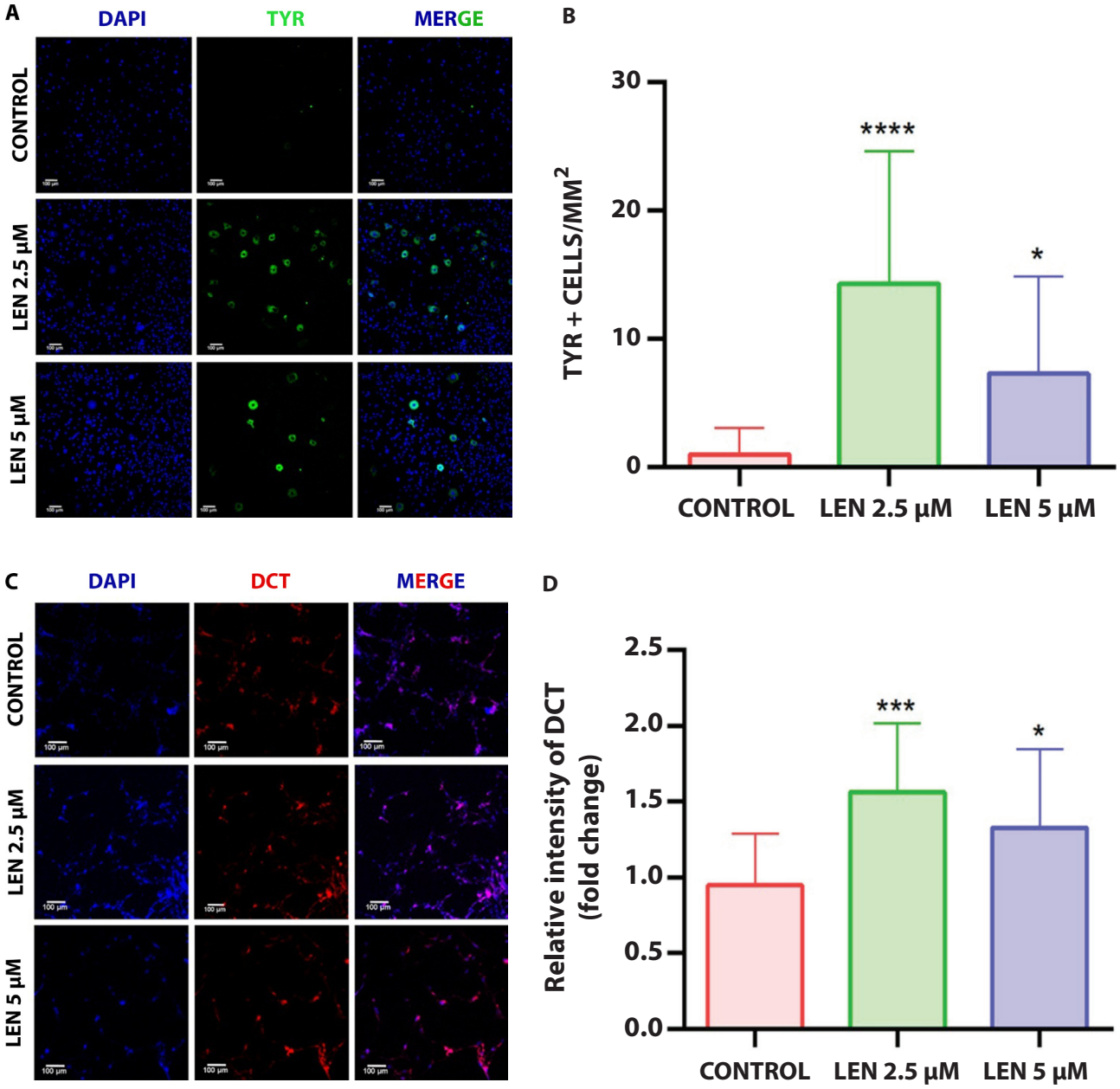

Figure 4. Representative images from confocal microscopy showing the effect of lenalidomide at 2.5µM and 5µM concentrations on melanocyte differentiation markers (A) TYR and (C) DCT in MelSCs after 14 days of treatment. (B) Bar diagrams depicting the TYR positive cells after lenalidomide (2.5 µM and 5 µM) treatment in MelSCs. (D) Bar diagrams display the relative fold change of DCT in control and lenalidomide (2.5 µM and 5 µM) treated MelSCs after 14 days of their differentiation. Representative images of both proteins are from 3 independent experiments that were quantified by using ImageJ software. Data are presented as mean ± standard deviation; statistical significance is shown by *P < 0.05, ***P < 0.0001, ****P < 0.0001.

to promote repigmentation. Also, in a previous study from our laboratory we have shown that lenalidomide inhibited the vitiligo progression in a vitiligo mouse model [11]. For the repigmentation of vitiliginous skin, melanocyte reservoir in the hair follicles must be activated, differentiated and migrated toward depigmented epidermis of vitiliginous skin [17,18].

In this study effect of lenalidomide was directly checked on the cultured hair follicle derived melanocyte stem cells. Cells from the murine whisker hair were isolated and cultured. These cells formed spheres and were found positive for neural crest cell marker, nestin. Nestin-positive neural crest-derived stem cells have the ability to form neurospheres [19,20]. The cultured spheres were later transferred to new culture and cells started migrating out of these spheres and were characterized for the various markers like nestin, CD34, tyrosinase, DCT and DOPA. The results showed that these cells expressed nestin, a marker for neural progenitors that is also expressed by stem cells of the hair follicle bulge [21]. Cultured cells were also positive for DCT, which is a marker of MelSCs in hair matrix [22] but were negative for CD34. CD34 negative neural crest derived melanocyte stem cells

from hair follicle are primed for melanocyte differentiation [23]. As these sphere derived cells were negative for functional melanocyte marker tyrosinase and DOPA, it confirmed that these cells were not functional melanocytes. Melanocytes are also derived from the neural tube and neural-crest, and then play an important role in pigmentation [24].

For repigmentation process, the most important step is the proliferation and migration of stem cells from hair dermis to the lesional areas [25]. Recovery from vitiligo is initiated by the subsequent migration of the melanocyte stem cells from the hair matrices to produce melanocytes [26]. Therefore, we checked the effect of lenalidomide in proliferation and migration of MelSCs. The results revealed that there was a non-significant rise in the proliferation of lenalidomide treated MelSCs in comparison to control. On the other hand, very importantly a significant increase in migration of lenalidomide treated MelSCs in comparison to control was observed. The proliferative role of lenalidomide has been earlier reported in the hematopoietic progenitor cells by Verhelle et al [27].

Role of lenalidomide in the differentiation of MelSCs into functional melanocytes was further analyzed. Differentiation of MelSCs into functional melanocytes was investigated through gene expression and immunocytochemical studies, which indicated that lenalidomide treatment enhanced the differentiation of MelSCs into melanocytes confirmed by their increased expression of *TYR, DCT, MITF, SOX10, SOX9* genes. *MITF* is the master gene that regulates the pigmentation through activation of downstream genes like *TYR* which ultimately determines pigmentation of skin as well as hair [28,29]. *SOX10* is one of the key genes which play its role in coordinating melanocyte differentiation by upregulating expression of genes required for melanogenesis [30]. TYR initiates the melanogenesis by oxidizing tyrosine to L-DOPA while DCT is required down the pathway in synthesis of melanin [31,32]. The increased expression of DCT and TYR by lenalidomide treatment was also observed at protein level, which further verified the differentiating effect of lenalidomide in the MelSCs into pigment producing melanocytes.

In conclusion, our data indicated that lenalidomide had very encouraging effects on the *in vitro* differentiation of MelSCs. It enhanced the proliferation and migration of cultured MelSCs. Further, it augmented the differentiation of hair follicle derived MelSCs into functional melanocytes that endorses its therapeutic use in the repigmentation process of vitiligo.

Acknowledgements

We acknowledge the Science and Engineering Research Board, Department of Science and Technology, New Delhi, India for financial support (ECR/2016/000671) to Ravinder Kumar and Council of Scientific and Industrial Research, New Delhi, India (09/135(0735)/2016-EMR-I) to grant fellowship to Uma Kumari.

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