Differentiation of CD34+ Human Hair Follicle Stem Cells into Functional Melanocytes

Ihab Q.Ali 1,2, Shahlaa M.Salih 1 and Galawish A.Abdulla 3

1 Molecular and medical biotechnology department, Al-Nahrain University, Iraq-Baghdad.
2 Pathological analyzes department, Al-Esraa university college, Iraq-Baghdad.
3 Al-Kindy medical colleges, Baghdad University, Iraq-Baghdad.

Abstract. Skin degenerative diseases, which lower the quality of life has become a colossal challenge across the globe. Despite significant number of such diseases including vitiligo and psoriasis, there has been no great progress in finding a cure to these diseases. Mesenchymal Stem Cells (MSCs) seem promising in this front as MSCs possess unique immunomodulatory properties. There have been preclinical and clinical studies suggesting the use of MSCs for the treatment of atopic dermatitis (AD), a prominent inflammatory skin disease. MSCs can be derived from multiple sources including adipose tissue, dental pulp, umbilical cord, placenta, peripheral blood, endometrium, synovium, and skin. However, stem cells isolated from hair follicle seem to be the closest to the original melanin producing cells. The differentiation of these stromal cells into melanin producing cells holds a promising cellular therapy for skin degenerative diseases. This study aims to differentiate human CD34+ melanocyte stem cells into melanin producing cells. The differentiation has been characterized by probing for the expression of proliferation markers dopachrome tautomerase (DCT) and microphthalmia associated transcription factor (MITF) and differentiation markers melanocytic antigen (PNL2) and tyrosinase (TYR) through the differentiation process. The results confirm the differentiation of the enriched CD34+ melanocytes into melanin producing cells. Melanin production was confirmed by ELISA to estimate melanin content.

Keywords: Melanocyte stem cells, Melanin producing cells, melanocyte stem cell markers, hair follicles, vitligo.

1. Introduction
Melanocytes are melanin-producing neural crest-derived cells that migrate to hair follicles and basal layer of the epidermis [1]. These cells provide a physiological protection against harmful UV rays. A lack of melanocytes and melanocyte producing stem cells can result in various skin disorders including psoriasis. They are also implicated in the occurrence of melanoma, an aggressive type of cancer [2]. Mesenchymal stem cells isolated from different sources including adipose tissue, hair follicle, dental pulp etc. can be differentiated into various cells types. Of these, the stem cell population in the epidermis and dermis has melanocyte producing stem cell [3]. Understanding the biology of melanocytes is important in understanding and treating these disorders. It has been reported that melanoma stem cells are genetically and phenotypically similar to melanocyte stem cells and therefore understanding their mechanism is critical for therapeutic purpose [4]. Autologous cultured melanocyte cells may be used in the treatment of various disorders like vitiligo, graying and albinism [5]. Owing to the above mentioned, it is desirable to find a source of melanocytes and expand the cells in vitro for cell based therapies.
Hair follicles undergo a lifelong hair cycle that involves a process of stem cell proliferation and quiescence [6]. Nishimura et al. first reported the reservoir of melanocytes in the lower permanent portion of mouse hair follicles. The study demonstrated the capacity of hair follicles to serve as a melanocyte stem cell (MSC) niche [7]. MSCs are the progenitors of pigment-producing melanocytes during each hair cycle to maintain hair pigmentation[7]. Melanocyte differentiated from other sources of stem cells like embryonic stem cells[8][9], bone marrow stem cells[10], induced pluripotent stem cells [11][12] etc. have already been reported earlier. Hair follicle is an adult mesenchymal stem cell source and hence compared to other sources of stem cells, are ethically naïve and less invasive [13].

Poblet et al. first reported CD34 expression in human hair follicle [14]. Since markers that are exclusive for McSCs have not been specified the best identification method available is locating CD34+ of these cells in the bulge area of the hair follicle [15]. The aim of the study is to investigate the potential of hair follicle stem cells to differentiate into melanocytes induced by melanocyte differentiation medium. In this direction, we have established an in vitro experiment to enrich CD34+ stem cells from hair follicle and differentiate into melanocytes.

2. Materials and Methods

2.1. Isolation of CD34 positive cell from human hair follicle cells

Primary cells were isolated from hair follicle culture and were cultured in DMEM (Life Technologies, USA) with 10% FBS in a humidified incubator at 37°C with 5% CO2. The cell suspension was enriched for CD34+ cells using MagCellect technology. The enriched cells were cultured and expanded in DMEM with 10% FBS.

2.2. Induction of melanocyte differentiation

The enriched cells were cultured in 6 well plates coated with 10 ng/ml fibronectin (Sigma, USA) at an initial seeding density of 2.5 x 10^5 cells/ml. After 24 hours of culture, cells were treated with medium containing the required melanocyte differentiation factors in 2 ml of culture medium. The medium included 0.05 mM dexamethasone, 1X insulin-transferrin-selenium (ITS), 1 mg/ml linoleic acid-bovine serum albumin (LA-BSA), 30% low-glucose DMEM, 20% MCDB201, 100mM L-ascorbic acid, 50 ng/ml Wnt3a, 100 ng/ml, stem cell factor (SCF), 100 nM endothelin-3 (EDN3), 20 pM cholera toxin, 50 nM phorbol 12-Otetradecanoylphorbol-13-acetate and 4 ng/ml bFGF (All the chemicals were procured from Sigma, USA). The cells were maintained in this medium for 21 days. At the end of the treatment (for every 7 days), the cells were trypsinised and taken for cytoplasmic marker studies using Flow Cytometry and qPCR.

2.3. Flow Cytometry

The trypsinised cells were fixed and permeabilized by treating them with ice-cold 70% ethanol at -20°C for 60 min and the ethanol was removed by centrifugation at 1600 rpm for 20 min. The cells were then washed with 1X PBS and were incubated with primary antibody. For un-conjugated antibodies (DCT, MITF, PNL2), cells were treated with primary antibody for 30 min in the dark and then with the respective secondary antibodies for 30 min in the dark. Conjugated antibodies (Tyr - PE) were added directly to the cells after ethanol fixation and were incubated for 30 min in the dark. The cells were then washed with 1X PBS containing 0.1% sodium azide and were analyzed in BD FACS Calibur. The data was analysed using Cell quest pro software.

2.4. RNA extraction, cDNA synthesis and qPCR

The total RNA from differentiated and undifferentiated cells was extracted by Trizol (Takara, Japan) method and the pellet was finally re-suspended in RNase free water. RNA was treated with DNase and was reverse transcribed using oligo dT (Sigma Aldrich, USA) and Reverse Transcriptase (Biolabs, New England). The mixture was incubated at 45°C for 90 minutes and reaction was terminated by incubating at 70°C for 15 minutes. Gene-specific primers for quantification analysis were designed using Primer3 software. The primers designed were as follows:
Table 1. The primers designed

| Primer | Sequences (3’-5’)       |
|--------|-------------------------|
| CD34 FP | AAAACGTGTGGCCTTGAAACC   |
| CD34 RP | TGGCCCCAGAGAGACTAGAA   |
| DCT FP  | GGTTCTTTTCTTCCCTCCAG   |
| DCT RP  | AACCAAAGCCACCAGTGTTC   |
| MITF FP | CTCGAGCTCATGGACTTTCC   |
| MITF RP | CCAGTTCCGAGGTGTTGTTT   |
| TYR FP  | TACGGCGTAATCCTGGAAC    |
| TYR RP  | ATTGTCATGCTGCTTGGAG    |

The relative quantification of gene expression was done using Applied Biosystems Step One Real Time PCR. The gene expression of target gene relative to the control was determined by the Ct values using Step one Software v2.2.2.

2.5. Estimation of melanin content

The melanin content was estimated by Melanin ELISA Kit (Life Span Biosciences, Inc.). All the reagents, standards, and samples were prepared as per manufacturer’s instructions. One hundred µl of sample, standard, or blank was added to each well and incubated for 1 h at 37°C. This was aspirated and 100 µl of Detection Reagent A was added and incubated for 1 h at 37°C. This was then aspirated and the wells were washed 3 times. Then, 100 µl of Detection Reagent B was added and incubated for 30 min at 37°C. After aspiration and 5 times washing, 90 µl of TMB Substrate solution was added and incubated for 10-20 minutes at 37°C. 50 µl of Stop Solution was added and read at 450 nm. The data was linearized by plotting the log of the target antigen concentration versus the log of the OD and the best fit line was determined by regression analysis.

3. Results

3.1 Isolation and enrichment of CD34 positive cells

The harvested cells were cultured and maintained in DMEM with 10% FBS. The non-adherent cells were eventually removed. The remaining cells have spindle shaped, fibroblast-like appearance.

3.2. Relative quantification of gene expression

The gene expression profile of cells grown in differentiation medium was quantified relative to the control cells grown in growth medium (DMEM with 10% FBS). The decreasing mRNA levels of CD34, with increasing incubation time, in the melanocyte differentiation medium confirmed cell differentiation (Figure 1A). The real time quantification of the proliferation markers DCT and MITF also showed a gradual decrease as seen in Figures 1B and 1C. Further, the differentiation was confirmed by the increased expression of TYR, a well-known melanocyte differentiation marker (Figure 1D). The overall pattern therefore confirms the differentiation of CD34+ hair follicle stem cells into differentiated melanocytes.
A. CD34 expression

Relative mRNA expression (fold change, normalized)

Day 0  Day 7  Day 14  Day 21
Melanocyte differentiation - Incubation time

Growth Medium (Control)  Melanocyte Diff Medium

B. DCT expression

Relative mRNA expression (fold change, normalized)

Day 0  Day 7  Day 14  Day 21
Melanocyte differentiation - Incubation time

Growth Medium (Control)  Melanocyte Diff Medium

C. MITF expression

Relative mRNA expression (fold change, normalized)

Day 0  Day 7  Day 14  Day 21
Melanocyte differentiation - Incubation time

Growth Medium (Control)  Melanocyte Diff Medium
Figure 1. Quantitative real-time Polymerase Chain Reaction of CD34+ hair follicle cells grown in growth and melanocyte differentiation media. The charts show expression profile of markers (A) CD34 (B) DCT (C) MITF (D) TYR in both the type of cells through their 3-week incubation time.

3.3. Flow Cytometry

During the course of differentiation, the cells were assessed for proliferative and differentiation markers using flow Cytometry. The mean intensity values of proliferation markers like CD34, DCT and MITF saw a gradual decrease on treatment with differentiation medium with increasing time in culture. On the other hand, the mean intensity values of differentiation markers like PNL2 and TYR saw a gradual increase as indicated in Figure 2, suggesting that the cells were being differentiated into melanocytes with time when cultured in differentiation medium.
Figure 2. Flow Cytometry of cells cultured in normal growth medium and in differentiation medium. The graph shows the mean intensity values of proliferation markers like CD34, DCT and MITF and differentiation marker PNL2. The mean intensity values of CD34, DCT and MITF saw a gradual decrease with time when cultured in differentiation medium, but the mean intensity of PNL2 showed a gradual increase thus indicating that melanocyte differentiation was successful.
3.4. Estimation of melanin content

The melanin content estimated by Human Melanin ELISA kit showed an increase in melanin concentration with differentiation. As cells differentiated into melanocytes when cultured in
differentiation medium, melanin concentration was found to increase indicating that melanin production increased with differentiation of the CD34+ cells into melanocytes as shown in Figure 5.

![Melanin Concentrations](image)

**Figure 4.** A graph of melanin concentration plotted against culture conditions. With increasing exposure to differentiation medium, melanin concentration was found to increase indicating that melanin production increased with differentiation of the CD34+ cells into melanocytes.

4. **Discussion**

The major reasons that back the development of an *in vitro* melanocyte differentiation system are to understand the melanocyte biology, to learn about the various underlying mechanisms of pigment production and disorders and to help understand their therapeutic implications. There are various sources of stem cells that may be able to differentiate into melanocytes. A few sources like bone marrow and embryonic stem cells and even autologous iPSCs have been already reported. Tsutomu Motohashi *et al.* studied the induction of pigment cell differentiation from embryonic stem cells [16]. It facilitated our knowledge of the precise underlying molecular mechanism.

Melanocyte stem cells and progenitor stem cells in the hair follicle bulge express DCT and TRP1, but lack TYR and hence do not produce melanin. The mature melanocytes differentiated from these progenitors however express TYR at the anagen phase of the hair cycle [17]. Our results also in line increasingly express TYR in the differentiation media.

Vitiligo is associated with high morbidity and psychiatric complications. The infiltration of CD4+ and CD8 T lymphocytes into the skin causes this autoimmune disease by the loss of melanocytes [18]. A number of studies using stem cells in various cell-based therapies show that regenerative medicine is revolutionizing [19]. Although stem cells have high therapeutic applications, complex procedures and ethical issues still concerns the medical fraternity. Recent reports suggest the use of iPSCs to generate melanocytes for the treatment of skin degenerative disorders. However, melanocytes derived from simpler sources of stem cells would clearly address this issue of accessibility and help extend to a larger crowd.

Hair follicle and melanocyte biology, because of its highly complex structure, has always remained a topic of new problems. Biological models of melanocyte stem cells and melanocytes help in developing effective applications. Information about their structure, differentiation, self-renewal, their role in disorders, melanoma development and all its accompanying mechanisms are important for this.

5. **Conclusions**

We conclude that human Hair follicle stem cells positive CD34 are successfully differentiated into functional melanocyte and the Induction approach used in this study is fast, efficient and it is able to build basis for future clinical application.
6. References

[1] Ohta S et al. 2011 Generation of Human Melanocytes from Induced Pluripotent Stem Cells. *PLoS One* 6, e16182

[2] Grichnik, J M Melanoma and Nevogenesis 2008 Stem Cell Biology. *J. Invest. Dermatol.* **128**, 2365–2380

[3] Macrin D, Joseph J P, Pillai A A and Devi A 2017 Eminent Sources of Adult Mesenchymal Stem Cells and Their Therapeutic Imminence. *Stem Cell Rev. Reports* **13**, 741–756

[4] Sabatino M, Stroncek D F, Klein H, Marincola F M and Wang E 2009 Stem cells in melanoma development. *Cancer Lett.* **279**, 119–25

[5] Czaklowski R et al. 2007 Autologous Cultured Melanocytes in Vitiligo Treatment. *Dermatologic Surg.* **33**, 1027–1036

[6] Yang R and Xu X 2013 Isolation and culture of neural crest stem cells from human hair follicles. *J. Vis. Exp.* doi:10.3791/3194

[7] Nishimura E K et al. 2002 Dominant role of the niche in melanocyte stem-cell fate determination. *Nature* **416**, 854–860

[8] Yamane T, Hayashi S I, Mizoguchi M, Yamazaki H and Kunisada T 1999 Derivation of melanocytes from embryonic stem cells in culture. *Dev. Dyn.* **216**, 450–458

[9] Fang D et al. 2006 Defining the Conditions for the Generation of Melanocytes from Human Embryonic Stem Cells. *Stem Cells* **24**, 1668–1677

[10] Mei X et al. 2015 In vitro-induced differentiation of bone marrow mesenchymal stem cells into melanocytes. *Cell Biol. Int.* **39**, 824–833

[11] Callahan S J, Mica Y and Studer L 2016 Feeder-free Derivation of Melanocytes from Human Pluripotent Stem Cells. *J. Vis. Exp.* e53806. doi:10.3791/53806

[12] Nissan X et al. 2011 Functional melanocytes derived from human pluripotent stem cells engraft into pluristratified epidermis. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 14861–6

[13] Macrin D, Joseph J P, Pillai A A and Devi A 2017 Eminent Sources of Adult Mesenchymal Stem Cells and Their Therapeutic Imminence. *Stem Cell Rev. Reports* 1–16 (2017). doi:10.1007/s12015-017-9759-8

[14] Poblet E, Jimenez-Acosta F, and Rocamora A 1994 QBEND/10 (anti-CD34 antibody) in external root sheath cells and follicular tumors. *J. Cutan. Pathol.* **21**, 224–228

[15] Nishimura E K 2011 Melanocyte stem cells: A melanocyte reservoir in hair follicles for hair and skin pigmentation. *Pigment Cell Melanoma Res.* **24**, 401–410

[16] Motohashi T, Aoki H, Yoshimura N and Kunisada T Induction of melanocytes from embryonic stem cells and their therapeutic potential. doi:10.1111/j.1600-0749.2006.00317.x

[17] Sarin K Y and Artandi S E 2007 Aging, graying and loss of melanocyte stem cells. *Stem Cell Rev.* **3**, 212–7

[18] Badri A M T Al et al. 1993 An immunohistological study of cutaneous lymphocytes in vitiligo. *J. Pathol.* **170**, 149–155

[19] Hoffman R M 2000 The hair follicle as a gene therapy target. *Nat. Biotechnol.* **18**, 20–21