Chapter 6
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Isolation and Cultivation of Hair Melanocytes

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ABSTRACT

Hair melanocytes have gained significant attention for their utility in research particularly in the cosmetic industry. Studies have highlighted their applications in pigmentation research and disease modeling, making them an indispensable tool for understanding melanin biosynthesis and disorders associated with pigmentation. A reliable protocol for isolating and culturing hair melanocytes ensures consistency in cell yield, viability, and functionality. Coating of plates with foetal bovine serum (FBS), the inclusion of selective trypsinization and the use of G418 further ensure the removal of contaminating cell types, enhancing culture purity. This methodology ensures a high yield and >90% pure population of melanocytes.

Keywords: Hair melanocytes; biosynthesis; dysfunctions; pigmentary disorders.

1. INTRODUCTION

Hair melanocytes are specialized pigment-producing cells located in the hair follicle, primarily in the bulb region. These cells have been widely studied for their role in pigmentation and as a model system for cellular and molecular studies (Yamaguchi et al., 2007; Tobin et al., 1995). Recent advances have further emphasized their relevance in understanding genetic regulation and environmental interactions influencing pigmentation. These cells are responsible for the synthesis and distribution of melanin, the pigment that determines hair color. Melanin production in hair melanocytes is tightly regulated by genetic, environmental, and hormonal factors (Tobin et al., 1998). The functional integrity of melanocytes is critical not only for pigmentation but also for their roles in

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oxidative stress regulation and cellular homeostasis. Dysfunctions in melanocyte biology have been linked to conditions such as vitiligo, melanoma, and hair graying (Tobin et al., 1995; Abdel-Malek, 2001). Additionally, emerging studies point to their role in systemic metabolic disorders and oxidative stress-related diseases. Dysfunctions in melanocyte biology are associated with various conditions, such as premature hair graying, alopecia, and pigmentary disorders (Botchkareva et al., 2003).

Hair melanocytes have garnered significant attention for their utility in research and therapeutic applications. Studies have highlighted their applications in pigmentation research and disease modeling, making them an indispensable tool for understanding melanin biosynthesis and disorders associated with pigmentation (Yamaguchi et al., 2007; Abdel-Malek, 2001). Their use extends to elucidating mechanisms underlying hair follicle biology and developing interventions for pigmentary conditions (Slominski & Paus, 1993). They serve as a model system for studying pigmentation pathways, drug screening for pigmentary diseases, and regenerative medicine applications (Ghasemi et al., 2020). A robust and reproducible protocol for isolating and culturing hair melanocytes is essential to ensure their utility across these domains.

1.1 Applications of Hair Melanocytes

1. Hair melanocytes provide a primary cell system to explore melanin biosynthesis and its regulation under physiological and pathological conditions.
2. These cells are instrumental in developing *in vitro* models for pigmentary disorders like vitiligo and albinism.
3. Hair melanocytes are used to evaluate the efficacy and safety of compounds aimed at modulating pigmentation.
4. The potential of melanocytes in tissue engineering and regenerative therapies is being explored, particularly for skin grafts in patients with depigmentation disorders.

1.2 Importance of a Robust Protocol

A reliable protocol for isolating and culturing hair melanocytes ensures consistency in cell yield, viability, purity and functionality. Challenges such as contamination, low cell yield, and loss of phenotypic characteristics necessitate meticulous optimization of each step—from sample collection to cell expansion.

2. MATERIALS

- **Carrier media:** DMEM (Dulbecco's Modified Eagle Medium) with high glucose (Gibco - 11965092), 10% fetal bovine serum (FBS), and 10X antibiotic-antimycotic (Ab-Am).
- **Washing buffer:** 1X Dulbecco’s phosphate-buffered saline (DPBS) with 1X Ab-Am.

- **Enzymes:** Collagenase Type V (Stemcell Technology - 07431) (working stock:0.5%) and Trypsin (0.05%).
- **Culture media:** MGM Gold media (Lonza - CC-3249)
- **Consumables:** Petri dishes, 6-well plates, cell strainers (40–70 µm), and T75 flasks.
- **Selective agent:** G418 (Sigma - 4727878001) (working stock: 100 µg/mL).
- **Selective marker:** S100β (ThermoFisher - 6285-MSM2-P1ABX)
- **Instruments:** Centrifuge, CO_2 incubator, hemocytometer, brightfield microscope, flow cytometer, and confocal microscope.

2.1 Protocol

2.1.1 Sample preparation

1. Store hair follicles in carrier media under chilled (4°C) conditions.
2. Wash follicles with a washing buffer (5 mL) in a 15 mL Falcon tube. Shake for 1 minute and allow the follicles to settle. Discard the supernatant.
3. Repeat the washing process 7–8 times to ensure thorough cleaning.

2.1.2 Enzymatic digestion

1. Prepare a 0.5% Collagenase Type 5 solution.
2. Incubate hair follicles in 10 mL of collagenase solution in a petri dish for 1 hour at 37°C in a CO_2 incubator. (NOTE - Working stock in 10mL DMEM high glucose)
3. Wash the digested tissue 4 times with 1X DPBS, allowing a 1-minute incubation during each wash.

2.1.3 Trypsinization

1. Add 3 mL of 0.05% trypsin to hair follicles and incubate at 37°C for 5 minutes.
2. Collect the spent trypsin, neutralize with MGM Gold media supplemented with 10% FBS, and pool in a single tube.
3. Repeat the trypsinization process two additional times.
4. Pass the pooled suspension through a 40–70 µm cell strainer.
5. Centrifuge at 300 × g for 5 minutes at 22°C. Resuspend the pellet in 1 mL of MGM Gold media. Seed at a density of 10,000–20,000 cells/cm² in MGM Gold media.
6. To enhance melanocyte attachment pre-coating flasks overnight with 100% FBS yielded better results.

2.1.4 Selective trypsinization and G418 treatment

1. Keratinocyte cells can remove with selective trypsinization 0.05% for 2-3 min incubation at 37°C.
2. Once primary cultures are established, treat cells with G418 at a working concentration of 100 µg/mL for 48 hours to selectively eliminate fibroblast cells.

3. Monitor the culture daily under a brightfield microscope to ensure selective survival of melanocytes.
4. Wash cells thoroughly after G418 treatment to remove residual antibiotic.

2.1.5 Cell culture

1. Count the cells using Trypan Blue and a hemocytometer. Seed at a density of 10,000–20,000 cells/cm² in MGM Gold media.
2. Maintain the culture at 37°C in a CO_2 incubator. Do not change the media until Day 7.
3. Post Day 7, change the media every 3 days.
4. Subculture cells when they reach 80–90% confluence using the trypsinization 0.05% protocol (37°C/3-5 min).
5. Freeze cells in cryopreservation media (90% FBS + 10% DMSO) at a density of 1.5 × 10⁶ cells/vial.

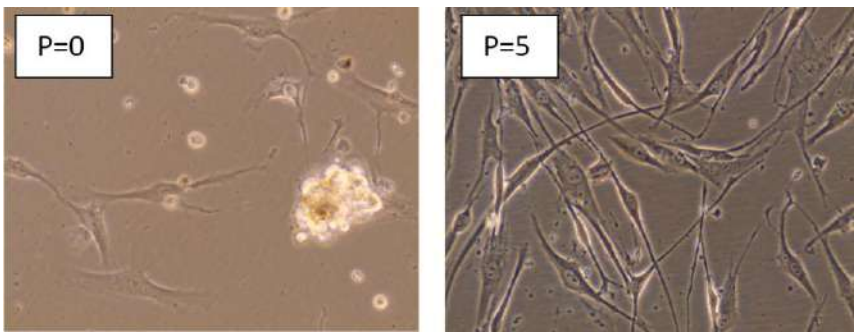
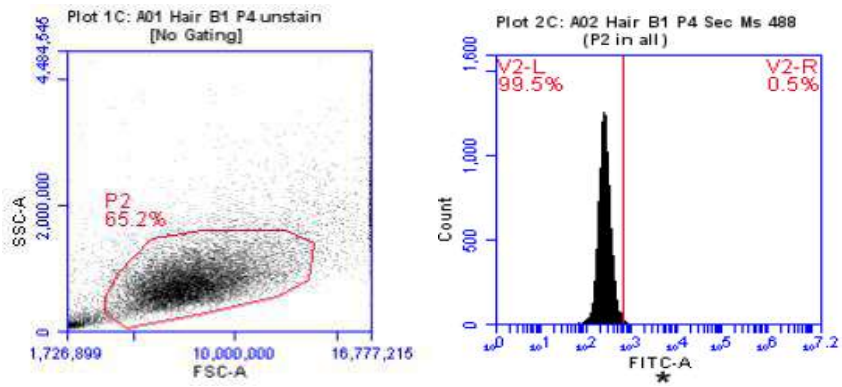
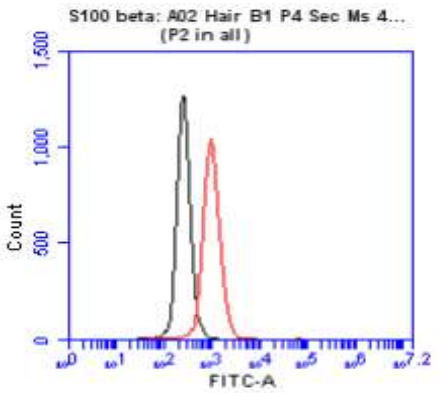


Fig. 1. 40X Phase Contrast microscopy images of hair melanocytes at passage 0 and passage 5

2.1.6 Flow cytometry



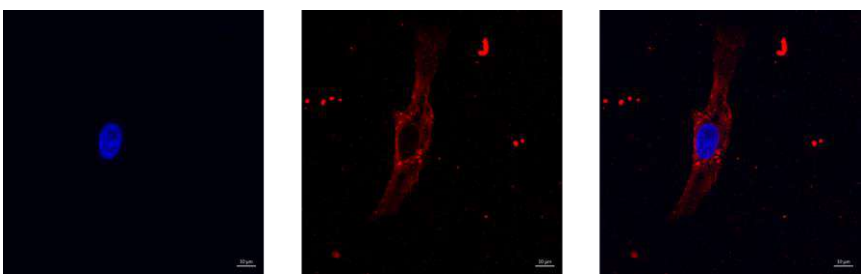
Unstained Secondary Alexa 488



S100β (82.5%)

Fig. 2. Flow cytometric analysis using the BD Accuri C6 Plus shows 82.5% expression of the hair melanocyte marker S100β

2.1.7 Confocal microscopy



DAPI | Alexa Fluor 647 -S100β | Overlay

Fig. 3. Confocal microscopy images (63X) of passage 5 hair melanocytes show S100β expression labelled with Alexa Fluor 647 and Nucleus with DAPI

3. RESULTS AND DISCUSSION

The isolation of functional hair follicle melanocytes is pivotal for advancing research in pigment biology, regenerative medicine, and dermatological therapeutics. Our protocol builds upon foundational methodologies established by Tobin et al. (1995), who first demonstrated the feasibility of isolating human hair follicle melanocytes and maintaining their viability in long-term culture. Like Dieckmann et al. (2010), we utilized plucked anagen hair follicles as a minimally invasive source, which preserves melanocyte stem cell populations critical for regeneration ((Rachmin et al., 2021; Nishimura, 2011). However, our approach introduces key modifications to enhance purity and functionality. For instance,

selective trypsinization and G418 treatment effectively eliminated keratinocyte and fibroblast contamination, a persistent challenge noted in earlier studies (MA et al., 2006; Baker & Thornton, 2020).

The high post-thaw viability (89.4%) and sustained morphological integrity up to passage 6–7 observed in our protocol align with findings by Yu, H et al. (2006), who highlighted the resilience of hair follicle-derived melanocytes. Flow cytometry confirmed S100β expression, a hallmark of melanocyte identity (Tobin, 2011), while confocal imaging validated S100β expression. Pre-coating culture surfaces with FBS significantly improved cell attachment, addressing adhesion challenges reported by Magerl et al. (2001) during hair follicle cell culture.

The clinical relevance of our protocol is underscored by its potential applications in vitiligo treatment. Therapeutic efficacy of transplanted hair follicle melanocytes have been demonstrated by earlier studies (Donaparthi & Chopra, 2016; Mohanty et al., 2011), which exhibit superior proliferative capacity compared to epidermal counterparts (Tobin & Bystryn, 1996). Furthermore, advances in iPSC-derived melanocytes (Ohta et al., 2011) and disease modeling (Coutant et al., 2024) highlight the need for standardized isolation protocols to generate reproducible *in vitro* systems.

Limitations persist, including donor variability in melanocyte yield and the finite proliferative capacity of primary cells. Future studies could integrate 3D culture systems or CRISPR-edited iPSC-derived melanocytes (Rachmin et al., 2021) to overcome these barriers. Nevertheless, our protocol provides a robust framework for isolating functional melanocytes, bridging gaps between basic research and clinical translation.

4. CONCLUSION

This chapter provides a comprehensive guide to the isolation and culture of hair melanocytes, emphasizing their utility in research and clinical applications. The described protocol offers a robust framework for obtaining high-quality melanocytes with consistent yield and viability. Future studies can leverage these protocols to explore advanced applications in pigment biology, drug discovery, and regenerative medicine. These protocols provide a foundation for advanced investigations into melanocyte biology, supporting the development of therapeutic interventions and novel drug targets (Yamaguchi et al., 2007; Abdel-Malek, 2001). This methodology can further support advancements in stem cell-based regenerative therapies and the development of precision medicine approaches.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

ETHICAL APPROVAL

Ethical approval for this study was obtained from the Institutional Ethics Committee (IEC) of Yashraj Biotechnology, Maharashtra, India.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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