2.1 INTRODUCTION

Vitiligo is a hypopigmentary skin disorder caused due to selective destruction of melanocytes, and its etiology is poorly understood. Melanocytes are neural crest derived cells residing at the basal layer of the epidermis where they are surrounded by keratinocytes along with dendritic cells (DC), fibroblasts, antigen presenting cells (APC) etc. (Plonka *et al*., 2009). To develop a treatment modality for melanocyte related disorders such as vitiligo, melanoma etc., it is essential to understand the melanocyte biology, its various biochemical pathways, and factors affecting its different functions. Melanocytes are cells which synthesize melanin pigments by the activity of different melanogenic enzymes including tyrosinase (Hirobe, 1995).

Epidermal melanin unit is a structural and functional unit of epidermis where single melanocyte is surrounded by ~36 keratinocytes. Melanosomes, synthesized within melanocytes are transferred to surrounding keratinocytes *via* dendritic projections to impart skin coloration (Cichorek *et al*., 2013; Nordlund, 2011). The proliferation, differentiation, melanogenesis, and dendritogenesis of melanocytes in the epidermis and hair follicles of mammalian skin are primarily regulated by paracrine factors derived from keratinocytes. The paracrine regulation of melanocyte function by keratinocytes plays a key role in regulating the homeostasis of the epidermis and hair follicles (Hirobe, 2014). As keratinocytes secrete melanotropic molecules for regulating the growth and functioning of cells, melanocytes exhibit variations among different individuals as well as in different body regions of the same individual. The variation is attributed due to the size, number, composition and distribution of melanosomes as well as melanin synthesis capacity. Melanocytes are typically large, slow growing cells of ~19µm and their growth depend on the anatomical position of the source of cells as compared to other epidermal cells. The percentage of melanocytes in the epidermal layer is around 8- 10 % (Ivanova *et al*., 2006). Culture techniques for normal human melanocytes (NHM) have been developed over the last three decades. *In vitro* model for studying pigment-producing cells is expected to provide the knowledge of cell-to-cell and cell-to-matrix interactions, melanocyte and melanin biology, pathophysiology of pigment disorders, and malignant melanomas (Lacour *et al.*, 1992). However, melanocyte culturing and *in vitro* studies have become a challenge due to following reasons:

- Melanocytes undergo very little replication as compared to keratinocytes because they are rarely shed from the epidermis.
- Limitation for a good source of tissue, which contains melanocytes as the major component
- Slow growth and proliferation of melanocytes
- The presence of faster-growing cells in the skin such as keratinocytes and fibroblasts as compared to melanocytes.

In vitro studies of human melanocytes are complicated due to the presence of contaminating populations of rapidly multiplying cells other than melanocytes. This prevents sub culturing and long-term study of melanocytes in culture.

The imbalance between pro and anti-inflammatory cytokines play an important role in the pathogenesis of various autoimmune and inflammatory disorders. A complex melanogenic cytokine network between skins cells, which regulate melanocyte activity was demonstrated (Imokawa, 2004). Keratinocytes present in the vicinity of melanocytes secrete additional cytokines, such as IL-6 and TNF-α, which function as paracrine inhibitors of growth and proliferation of melanocytes (Swope *et al.,* 1991). Altered levels of keratinocyte derived mediators have been described in vitiligo epidermis, suggesting an important role of epidermal cytokines in vitiligo pathogenesis (Moretti *et al.,* 2002; Lee *et al.,* 2012). Though cytokine imbalance has been well documented in vitiligo patients, the exact mode of its action on melanocyte biology is still not well explored.

The proposed study aims to explore the role of cytokines in vitiligo pathogenesis and to address the response and downstream signaling in *in vitro* cultured melanocytes (established from human skin biopsies) exposed to various cytokines. This study will pave the way to identify the role of cytokines in the pathogenesis and progression of vitiligo. There is an imperative need for a proper *in vitro* model system to establish the role of different cytokines in melanocyte biology.

2.2 MATERIALS AND METHODS

2.2.1 Ethics statement

The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all subjects before performing the studies.

2.2.2 Culture establishment of primary normal human melanocytes (NHM)

Melanocytes were isolated from normal human skin biopsy samples and cultured successfully using the standard protocol with a few modifications (Hsu *et al*., 2005; Sungbin *et al*., 1992; Czajkowski *et al*., 2007). Briefly, the epidermis was separated from the dermis after an overnight incubation of skin biopsies in 0.25% Dispase II protease (Sigma Aldrich, USA) prepared in phosphate buffer saline (PBS at 4˚C. In order to separate epidermal cells, the epidermis was incubated at 37˚C for 10 min, in a solution of 1X trypsin phosphate versene glucose solution. Cellular suspension was centrifuged at 1,300 rpm for 5 min at room temperature to harvest cells. Melanocytes were selectively cultured in M254 medium with human melanocyte growth supplements (Gibco® life Technologies™, Cascade Biologics™, Portland, Oregon) and 1X antibiotic-antimycotic solution (Himedia, India). The cells were maintained in a humidified incubator with 5% CO_2 at 37˚C and media replenishment was given after every 2-3 days. Melanocyte-keratinocyte mixed population starts appearing around days 4-9. Melanocytes were purified from keratinocytes by differential trypsinisation, which is based on the more sturdy and adherent property of keratinocytes. In addition, melanocytes were given 100µg/ml G418 (Geneticin® Disulfate Salt, MP Biomedicals, France) treatment for gradual removal of fibroblasts to obtain primary human melanocytes and further were split at 1:3 ratio, when cell confluency reached 80%. Media was replenished after every 48-72 hrs; melanocytes cultured up to the fifth passage were used for the experiments.

2.2.3 Protocol for Cultivation of Normal Human Epidermal Melanocytes (Hsu *et al*., 2005)

- The skin samples were soaked in 70% ethanol for 1 min and transferred to the Petri dish containing HBSS to rinse off ethanol.

- For foreskin samples the skin-ring was cut opened and fat was trimmed off along with other tissue, with scissors. Skin samples were cut into pieces (approx 5 × 5 mm^2) using the surgical scalpel blade.
- The skin pieces were transferred into the tube containing epidermal isolation solution (M254 basal media and HMGS). Further, the tube was capped, inverted, and incubated in the refrigerator at 4°C for 18–24 hrs.
- The tube containing the sample was removed from the refrigerator and incubated at 37°C for 5 min.
- The tissue was poured into a Petri dish containing epidermal isolation solution. The epidermis (thin, brownish, translucent layer) was separated from the dermis (thick, white, opaque layer) with the help of the forceps. The dermal part of the skin piece was held with one pair of forceps, and the epidermal side with another forceps.
- The epidermis was gently teased apart from dermis. Immediately the dermis was discarded.
- The harvested epidermal sheets were transferred to an empty Petri dish, drops of Ca^{2+} and Mg^{2+}free HBSS was added to prevent the tissue sample from drying. The above described steps were repeated for each piece of tissue and further minced them into smaller pieces (approx. 2 × 2 mm^2) with the help of a surgical scalpel blade.
- The collected epidermal sheets were transferred from the Petri dish to the centrifuge tube containing 5 mL of cell-dispersal solution. The tube was incubated at 37°C for 5 min.
- The tube was shaken vigorously. The resulting single-cell suspension was washed once with 10 mL of Ca^{2+} and Mg^{2+} free HBSS.
- The suspension in was centrifuged for 5 min at 800g at room temperature.
- The supernatant was carefully aspirated, which may contain remaining stratum corneum. The pellet was re-suspended in 5 mL culture media. The resulting epidermal cell suspension was plated in a T25 cell-culture vessel and further incubated at 37°C in 5% CO_2 for 48–72 hrs without disturbance.

Antibiotic cocktail 1X (Penicillin, Gentamycin, Tetracycline, Streptomycin, Geneticin)

Human Melanocyte Growth Supplement (HMGS) is an ionically balanced supplement containing bovine pituitary extract (BPE), fetal bovine serum, bovine insulin, bovine transferrin, basic fibroblast growth factor, hydrocortisone, heparin, and endothelin-1.

2.2.4 Confirmation of melanocytes by DOPA staining

The pure culture of melanocytes was confirmed by L-DOPA (L-3,4-dihydroxy phenylalanine, Hi-Media, India) staining (Iijima and Watanabe, 1957). NHM were trypsinized, plated on coverslips and cultured for ~3 days before detection of tyrosinase activity. For DOPA reaction, culture media was removed and cells were rinsed twice with PBS, fixed for 20 min in 4% formaldehyde solution in PBS, washed three times with PBS and then incubated at 37°C for 18 hrs in the dark with 10 mM L-DOPA. After incubation, the cells were rinsed with distilled water, dehydrated, mounted and the number of cells positive for tyrosinase activity was observed using light microscopy.

2.2.5 Human Melanocyte Cell lines

Immortalized human melanocyte cell lines PIG1 (derived from a healthy individual) and PIG3V (derived from vitiligo patient) (a gift from Dr. I.C. Le Poole, Loyola University, Chicago, Illinois) were cultured as described by Le Poole (Le Poole *et al*., 1997; 2000).

2.2.6 Melanoma derived cell lines

G361 and SK Mel28 melanoma derived cell lines were also included in our studies. The G361 is pigment producing malignant melanoma cell line which is derived from 31 years old Caucasian male and was cultured in McCoy's 5A Medium supplemented with 10% Fetal Bovine Serum (FBS) and 1X Antibiotic supplementation (Himedia, A002-20 ML, Antibiotic Antimycotic Solution 100X). Moreover, SK Mel 28 is also a malignant melanoma cell line derived from 51 years old male. However, it exhibits poor pigmentation phenotype and is cultured in Eagle's Minimum Essential Medium supplemented with 10% FBS and 1X Antibiotic supplementation.

2.3 RESULTS

2.3.1 Isolation and culturing of melanocytes from human skin biopsies: Melanocytes were successfully isolated and cultured from human skin biopsies (Figure 1). During initial three days, we observed ~ 5-10% of epidermal cell suspension got adhered to the culture flask and most of them were possessing dendrites. After media change around day 4-9, numerous polygonal and cobble shaped cells (keratinocytes) were observed surrounding dendritic melanocytes. In this mixed culture of the melanocytes and keratinocytes, the keratinocytes are more adherent as compared to melanocytes. With the help of two rounds of differential trypsinisation, we obtained keratinocyte free melanocyte culture. During the culturing of the melanocytes, Gentamicin was used to eradicate fast growing fibroblasts (Figure 1). The melanocytes were confirmed by DOPA staining which is based on Tyrosinase, a melanocyte specific enzyme which catalyzes the conversion of tyrosine to DOPA and DOPA to DOPAchrome (Figure 2).

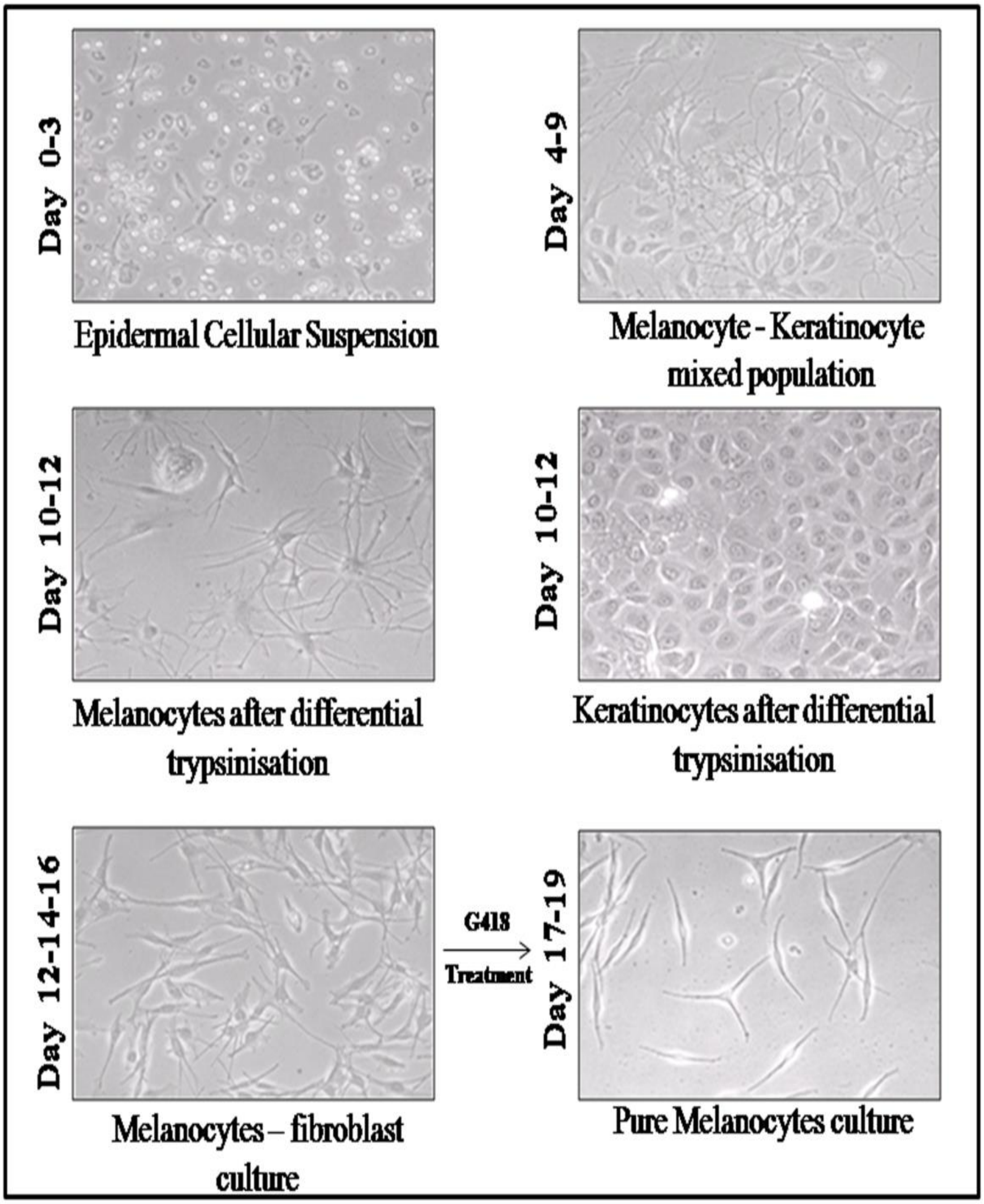

Figure 1: Establishment of primary cultured human skin melanocytes: Melanocytes were isolated and cultured successfully. Images were taken at different stages of differentiation under an inverted microscope (Magnification 10 X; scale 100μm).

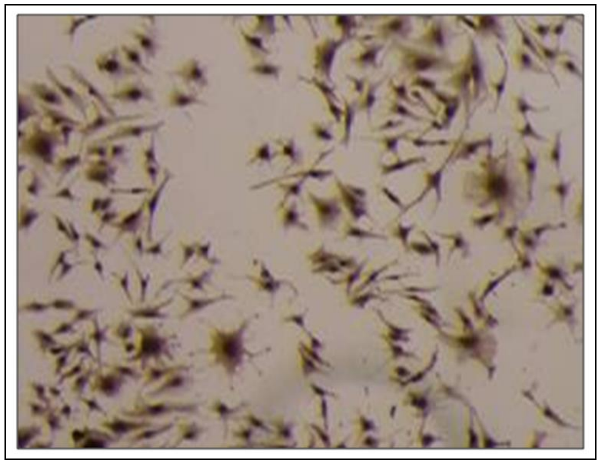

Figure 2: Confirmation of cultured melanocytes by DOPA staining as observed under an inverted microscope (Magnification 10 X; scale 100μm).

2.3.2 Melanocytes derived from different sources:

In addition to primary melanocytes, melanocyte culturing was also done from different sources. Viral transformed melanocytes derived from healthy persons PIG1 as well from vitiligo patients PIGV, (a gift from Dr. I.C. Le Poole, Loyola University, Chicago, Illinois) were also used for our preliminary experiments. PIG1 and PIGV are used as a substitute for melanocytes derived from healthy control and vitiligo patients due to the limitation of human samples. PIG3V vitiligo melanocytes showed a tendency to be more sensitive to 4-TBP as compared to control PIG1 melanocytes reflecting the restoration of intrinsic sensitivity of vitiligo subjects even after several times of subculturing (Kroll *et al.*, 2005). Moreover, melanin producing and non-producing cell lines derived from malignant melanoma patients *viz* G361 and SKMel 28 cell lines respectively were also cultured (Figure 3). NHM showed higher melanin production as compared to PIG1 and G361 melanin positive cell line. Also, slender morphology and multiple dendrites were observed in NHM as compared to PIG1, PIGV, G361 and SK Mel 28 cell lines. On the other hand, the rest of the other cells exhibited bipolar dendrites while NHM exhibited multiple dendrites.

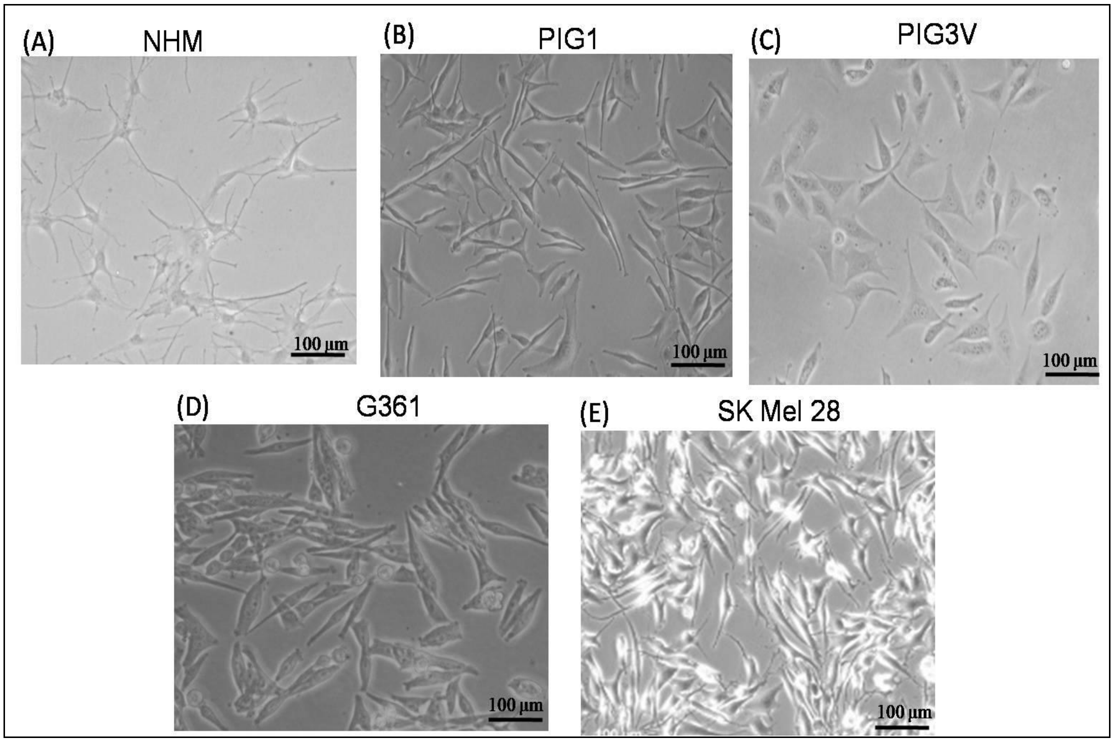

Figure 3: Differences in cell morphology were observed in the melanocyte culture obtained from different sources (A) NHM (B) PIG1 (C) PIGV (D) G361 (E) SK Mel 28 at 10 X magnification.

2.4 DISCUSSION

An important approach to study the molecular mechanisms of normal, diseased, and malignant cells is their growth profile under *in vitro* conditions. Due to ethical issues, and the scientific concern as well as limitation of human samples, it becomes difficult to investigate the molecular mechanisms of diseases on human subjects directly. Therefore, mice/animal models, primary cultures and cell lines (mostly cancerous, viral immortalized etc.) have been developed to overcome these constraints.

Cytokine imbalance is well reported in vitiligo patients, but their exact mode of action on melanocytes is still not well explored. Therefore, to study the effect of candidate cytokines on melanocyte biology, it is very essential to establish the *in vitro* system, as the *in vivo* or animal model system is not apt to study the role of cytokines *per se*.

Moreover, cytokines affect melanocyte biology by the activation of their respective receptors. To address melanocyte biology under cytokine imbalance it is essential to design of an ideal *in vitro* system.

Vitiligo is a multifactorial, polygenic and hypomelanotic skin disorder caused due to selective destruction of melanocytes of unknown etiology (Laddha *et al.*, 2013). As it involves melanocyte destruction, melanoma derived cell lines or viral transformed primary cell lines would not be the appropriate model system to investigate/validate hypothesis of our study. Hence, developing a proper *in vitro* model system is a prerequisite to study and understand the complex interplay of cytokines and oxidative stress in melanocyte biology.

Human epidermal melanocyte isolation and culturing has been attempted since 1957 (Hu *et al.,* 1957***;*** Yukio, 1976; Mayer, 1982; Wllkms and Szabo, 1981), but since 1982 only pure NHM cultures have been established to yield cells in adequate quantity for biochemical and molecular studies (Eisinger and Marko, 1982). Selective growth of the melanocytes, which comprises around 10% of epidermal cells, was initially attained by suppressing the growth of major populating cells (keratinocytes) and fast growing cells (fibroblasts) in epidermal cell suspensions. Previously, to suppress the growth of keratinocytes and fibroblasts tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) and the intracellular cyclic adenosine 3′, 5′ monophosphate (cAMP) enhancer cholera toxin were used respectively, both of which also act as melanocyte growth promoters. However, phorbol ester is metabolically stable and has prolonged effects on multiple cellular responses **(**Eisinger and Marko, 1982**)**. Therefore, in the present study, we established TPA, phorbol 12-myristate 13-acetate (PMA)-free culture medium for selective growth and long-term maintenance of human melanocytes. Based on the difference in the adherence properties of melanocytes and keratinocytes, and with the help of two rounds of differential trypsinization, we were able to get rid of keratinocytes from our melanocyte-keratinocyte co-culture. Also culture medium supplementation with gentamicin helped us to obtain a fibroblast free growth in our melanocyte culture. Melanocytes in the culture were easily distinguished from keratinocytes by their morphological characteristics. Keratinocytes had a round or polygonal shape while melanocytes appeared bipolar or polydendritic. The detailed description of the present method will be useful in basic and applied biological research.

In our preliminary standardization experiments, we used melanoma derived cell lines: SKMel28 (low melanin producing Caucasian origin cell line) and G361 (melanin producing cell line). Also, viral transformed primary melanocytes PIG1, PIG3V derived from healthy individuals and vitiligo patients respectively (Le Poole *et al*., 1997). Our results showed that primary cultured normal human melanocytes exhibited increased sensitivity to exogenous stimulation of cytokines and oxidative stress as compared to melanoma and viral transformed cell lines. NHM exhibit close resemblance in terms of number of dendrites and melanin synthesis capacity to melanocytes residing in the skin as compared to melanoma and viral transformed cell lines. The difference could be attributed to the incorporation of mutations during the procedure of immortalization. Therefore, NHM appears to be a better model for exploring the effect of various immnoregulatory molecules on melanocyte biology. The NHM culture system used for our studies is free of PMA, TPA and serum which minimizes the artifact of cytokine mediated sensitive studies. Nevertheless, the NHM observed were multi dendritic during initial plating, however after subsequent sub-culturing most of the cells became bipolar and 10-15% of cells were tripolar. This is suggestive of the importance of keratinocyte & its derived factors for growth and functioning of melanocytes and this could also explain the decrease in melanin synthesis and growth properties of NHM with further passaging of cells.

In conclusion, isolation and culturing of primary melanocytes has been established in our lab which will facilitate research in melanocyte biology to understand the etiology of pigmentary disorders (vitiligo) and melanoma. The culture model developed is apt to study the effect of cytokines or immune modulators on melanocyte biology as the culture system is devoid of TPA and serum.

2.5 REFERENCES

- Cichorek, M., Wachulska, M., Stasiewicz, A. and Tymińska, A., 2013. Skin melanocytes: biology and development. *Advances in Dermatology and Allergology/PostępyDermatologii I Alergologii*, *30*(1), p.30.

- Czajkowski, R., Placek, W., Drewa, T., Kowaliszyn, B., Sir, J. and Weiss, W., 2007. Autologous cultured melanocytes in vitiligo treatment.*Dermatologic Surgery*, *33*(9), pp.1027-1036.
- Eisinger, M. and Marko, O., 1982. Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. *Proceedings of the National Academy of Sciences*, *79*(6), pp.2018-2022.
- Hirobe, T., 1995. Structure and function of melanocytes: microscopic morphology and cell biology of mouse melanocytes in the epidermis and hair follicle. *Histology and histopathology*, *10*(1), pp.223-237.
- Hirobe, T., 2014. Keratinocytes regulate the function of melanocytes. *DermatologicaSinica*, *32*(4), pp.200-204.
- Hsu, M.Y. and Herlyn, M., 1996. Cultivation of normal human epidermal melanocytes. *Human Cell Culture Protocols*, pp.9-20.
- Hsu, M.Y., Li, L. and Herlyn, M., 2005. Cultivation of normal human epidermal melanocytes in the absence of phorbol esters. *Human Cell Culture Protocols*, pp.13-28.
- Hu, F., Staricco, R.J., Pinkus, H. and Fosnaugh, R.P., 1957. Human Melanocytes in Tissue Culture1. *Journal of Investigative Dermatology*, *28*(1), pp.15-32.
- Iijima, S. and Watanabe, K., 1957. Studies on DOPA reaction. II. Effect of chemicals on the reaction. *The Journal of investigative dermatology*,*28*(1), p.1.
- Imokawa, G., 2004. Autocrine and paracrine regulation of melanocytes in human skin and in pigmentary disorders. *Pigment Cell & Melanoma Research*, *17*(2), pp.96-110.
- Ivanova, K., Block, I., Das, P.K. and Gerzer, R., 2006. Role of cyclic GMP signaling in the melanocyte response to hypergravity. *Signal Transduction*, *6*(6), pp.406-413.
- Kroll, T.M., Bommiasamy, H., Boissy, R.E., Hernandez, C., Nickoloff, B.J., Mestril, R. and Le Poole, I.C., 2005. 4-Tertiary butyl phenol exposure sensitizes human melanocytes to dendritic cell-mediated killing: relevance to vitiligo. *Journal of investigative dermatology*, *124*(4), pp.798-806.

- Lacour, J.P., 1992. Culture of human melanocytes. Its contribution to the knowledge of melanocyte physiology. *Pathologie-biologie*, *40*(2), pp.114-120.
- Laddha, N.C., Dwivedi, M., Mansuri, M.S., Gani, A.R., Ansarullah, M., Ramachandran, A.V., Dalai, S. and Begum, R., 2013. Vitiligo: interplay between oxidative stress and immune system. *Experimental dermatology*, *22*(4), pp.245-250.
- Le Poole, I.C., Boissy, R.E., 1997. Vitiligo. *Semin Cutan Med Surg*. 16:pp3–14.
- Le Poole, I.C., Boissy, R.E., Sarangarajan, R., Chen, J., Forristal, J.J., Sheth, P., Westerhof, W., Babcock, G., Das, P.K. and Saelinger, C.B., 2000. PIG3V, an immortalized human vitiligo melanocyte cell line, expresses dilated endoplasmic reticulum. *In Vitro Cellular & Developmental Biology-Animal*, *36*(5), pp.309-319.
- Lee, A.Y., 2012. Role of keratinocytes in the development of vitiligo.*Annals of dermatology*, *24*(2), pp.115-125.
- Mayer, T.C., 1982. The control of embryonic pigment cell proliferation in culture by cyclic AMP. *Developmental biology*, *94*(2), pp.509-514.
- Moretti, S., Spallanzani, A., Amato, L., Hautmann, G., Gallerani, I., Fabiani, M. and Fabbri, P., 2002. New insights into the pathogenesis of vitiligo: imbalance of epidermal cytokines at sites of lesions. *Pigment Cell & Melanoma Research*, *15*(2), pp.87-92.
- Nordlund, J.J., 2011. Vitiligo: A review of some facts lesser known about depigmentation. *Indian journal of dermatology*, *56*(2), p.180.
- Plonka, P.M., Passeron, T., Brenner, M., Tobin, D.J., Shibahara, S., Thomas, A., Slominski, A., Kadekaro, A.L., Hershkovitz, D., Peters, E. and Nordlund, J.J., 2009. What are melanocytes really doing all day long…?.*Experimental dermatology*, *18*(9), pp.799-819.
- Sungbin Im, S.K.H., Park, Y.K. and Kim, H.I., 1992. Culture of melanocytes obtained from normal and vitiligo subjects. *Yonsei Medical Journal*, *33*(4).
- Swope, V.B., Abdel-Malek, Z., Kassem, L.M. and Nordlund, J.J., 1991. Interleukins 1α and 6 and tumor necrosis factor-α are paracrine inhibitors of

human melanocyte proliferation and melanogenesis. *Journal of investigative dermatology*, *96*(2), pp.180-185.

- Wllkms, L M., Szabo, G. C., 1981. Use of mycostatin-supplemented media to establish pure epidermal melanocyte culture (abstract) *J invest Dermatol* **76**, 332.
- Yukio, K., 1976. Stimulation by melanocyte stimulating hormone and dibutyryl adenosine 3′, 5′-cyclic monophosphate of DNA synthesis in human melanocytes in vitro. *Archives of Dermatological Research*, *257*(1), pp.47-52.