

## REVIEW.

# THE STORY OF MELANOCYTE: LONG WAY FROM BENCH TO BEDSIDE

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**Keywords:** Cultured Melanocyte, Regenerative Therapy, Vitiligo, Cell Transplantation

**Abstract.** Skin is composed of major layers, a superficial epidermis and a deeper dermis. The color of skin is influenced by a number of pigments, including melanin. Melanin is produced by cells called melanocytes. Most skin disorders are relatively benign, but a few, including melanomas, can be fatal. A couple of the more noticeable disorders, namely albinism and vitiligo, affect the appearance of the skin and its accessory organs. Vitiligo is associated with significant psychosocial morbidity and profound effect on quality of life.

Topical steroids, calcineurin inhibitors, phototherapy and surgery are most common treatments. However, there are many patients who do not respond to any of these modalities. The transplantation of cultured or non-cultured melanocyte is the most important treatment for hypopigmentary disorders. In this study, we are going to assess the history of melanocyte cultivation and evaluate the effectiveness of transplantation cultured cells.

We examined the beginning process of isolation, characterization, and transplantation of epidermal cells. This review, summarize our current understanding of the cutaneous pigmentary

system from the start of synthesis in the pigment cells, along with the response of repigmentation. During the production of melanin, melanosomes are transferred to neighboring keratinocyte in order to form perinuclear melanin caps. The objective of this review is to analyze the melanocytes transplantation in the last century up to now, and how epidermal cells can increase pigmentation in hypo-pigmented areas in skin disorders. Also, we focus on the story of the melanocyte back to 1950.

In addition, prior systemic therapy was associated with a significant increase based on combined additional therapy, achieving desired results and improved outcomes. Despite the short study of a long way of melanocyte assessment and follow up patient's treatment, results of the all reports were confirmed the efficacy of the used method in the treatment of stable vitiligo who did not respond to the common algorithms of non-invasive treatments.

### **1- Introduction**

With a relatively low prevalence rate, vitiligo is an acquired pigmentary disorder of skin, clinically characterized by the presence of circumscribed depigmented macules resulted from impaired function of melanocytes (1). Amongst multiple theories proposed for unraveling the complex pathogenicity of vitiligo, theories based on the pivotal role of autoimmune and oxidative stress are the most accredited theories (2), as they are in agreement with different clinical and experimental findings. Although no specific therapeutic guideline has been established for vitiligo yet, certain approaches exist which can slow down the progress of the disease and promote pigmentation of depigmented areas (2, 3). These approaches are mainly based on three strategies, including modification of immune responses, suppressing melanocytes stress and promoting regeneration of melanocytes (4). Recently, cultured melanocyte transplantation has been evolved as a promising modality against extensive depigmentation in vitiligo, which is resistant to conventional treatments. Over the last three decades, melanocyte transplantation has been evolved as a promising modality against vitiligo patches which are resistant to conventional treatments. Firstly, Lerner et al. introduced the transplantation of autologous cultured melanocytes for vitiligo in 1987. Since then, several clinical trials have shown the safety and efficacy of transplantation of cultured autologous melanocyte in spite of marked safety concerns. We reviewed research studies reporting on different procedures of identification, determination, and isolation of melanocyte. Studies illustrated, the activity of cultured melanocytes in vitiligo patients' evolution of different isolation methods for depigmentation in Vitiligo lesions occurs mostly in a perifollicular pattern, which suggested that melanocyte repopulates from the depigmented epidermis (5). In this study, we are going to assess melanocyte preparation for transplantation. A multitude of plausible theories have been put forward to provide different kinds of combination treatments on vitiligo patients, lead to the increase in epidermis melanocytes activity (6). Here we explain First-line vitiligo treatment includes moderate-to-high response as well as cell transplantation several recent studies comparing the use of medical modalities, including steroid drugs, Lasers (Erbium, excimer),

growth factors and nanoparticles Apart from, skin biopsy procedures and injection cells playing an important role in betterment of Hypo pigmented macules.

### **1-1 Skin Biology and Melanin Biosynthesis:**

Skin is the largest organ in our body, the largest complex organ that accounts for about 15% of the total body weight. Loss of skin integrity may cause a substantial physiological imbalance, significant disability or even death. An in vitro study has shown, during development, neural crest cell migration occurs from the neural tube to the skin tissue then passes into the melanocyte Lineage thereby giving rise to the pigment system. Melanin, as the major pigment in the skin, provides protection against radiation-induced photo-carcinogenesis. Melanosome produces melanin, which polymerizes and settles on the basal layer of the epidermis and hair follicle, as well as mucous membrane (6, 7) (8). Two major forms of melanin exist. Eu melanin and pheomelanin, which are responsible for different skin colours (e.g., black, brown, yellow). Pigmentation is regulated by genetic and environmental factors. Pigment cells, with a density of 500-2,000 cells per mm<sup>2</sup>, are localized in the basal layer of the epidermis. Each melanocyte is surrounded by approximately 36 keratinocytes(9, 10) (11) melanocyte dendritic make contact with the neighboring cells for transferring melanin pigmentation, Melanin is produced in lysosomes-like organelles called melanosomes that store the pigments and transfer to keratinocyte to move further towards the surface of the skin (12) (13). Abnormalities in the division of melanocytes, with potentially oncogenic growth, are usually followed by cell senescence producing benign naevi (moles) or occasionally melanoma (14). In melanogenesis process, Tyrosinase-Related Protein I (TRP1) and Tyrosinase-Related Protein II (TRP II) play crucial roles. Tyrosinase is an oxidase enzyme leading to melanin production from tyrosine (15). Pigmentation in skin depends on melanin production of the cutaneous melanocytes. Darker Skin has larger melanocytes which contain more melanosomes, than those in white skin. Pigmentation disorders include numerous heterogeneous conditions characterized by melanocyte density and melanin concentration. Melasma (Hyperpigmentation disorders) or vitiligo (Hypopigmentation disorders), are extremely common skin disorders (16) (17). Hypopigmentary disorders can be result of mutation in many of the respective encoding genes can disrupt the process of melanogenesis and apoptosis leading to increasing melanocytes in number and size and a reduction of melanin synthesis. A mutation in many of the respective encoding genes can disrupt this process of melanogenesis and can result in hypopigmentary disorders (18, 19) (13) Different classes of topical Corticosteroids, and surgery are most common treatments. no responding was provided to patients to use the platform. Today's modern and noninvasive techniques like cell and tissue grafting help to achieve repigmentation in hypopigmentation disorders. In these techniques, autologous melanocytes obtained from a small donor biopsy, cells can be diluted or expanded, are transplanted to the depigmented area. Moreover, there are several drawbacks in currently established techniques for autologous transplantation of melanocytes in vitiligo including

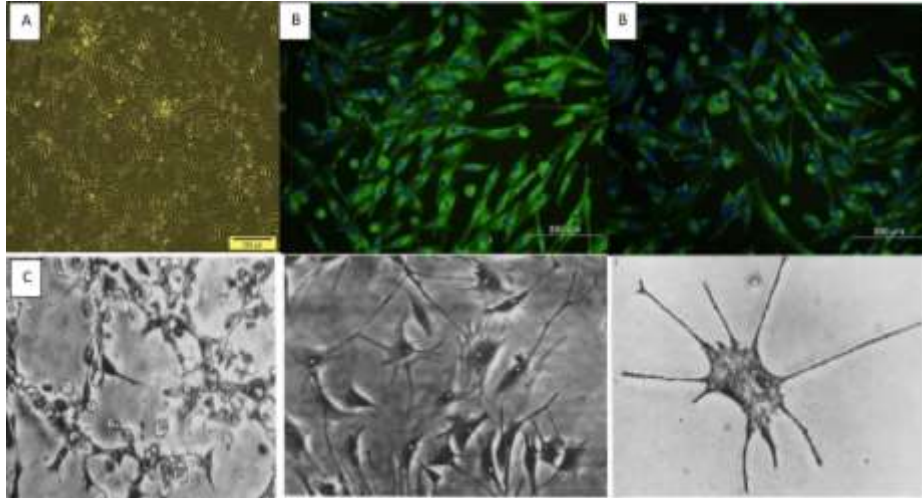
prolonged treatment, high cost and inevitable adverse effects (e.g., Koebnerization) (20). These types of interventions are especially helpful in subjects with stable vitiligo with undesirable effectiveness of existing medications (7, 18). In the cases of stable vitiligo with no response to usual treatments, whole tissue grafting methods including split thickness grafting, punch grafting, suction blister and cellular grafting techniques are the main surgical approaches. Results of the study performed by Bao H et.al in the treatment of stable vitiligo in a mutual self-control study confirmed previously stated (18) superiority of blister roof grafting (BG) with 91% improvement in repigmentation as compared to cultured melanocyte transplantation (CMT) and non-cultured epidermal cell suspension transplantation (NCES) with 82% and 81% repigmentation respectively. But, the need for 1:1 donor site to recipient skin size ratio (DR ratio) undermines its superiority against CMT and NCES with 1:20 and 1:5 DR ratio, respectively (18). Cellular transplantation employs cultured melanocytes and non-cultured cellular suspensions (melanocytes and keratinocytes). Previous studies have reported that transplantation of autologous cultured melanocytes can successfully repigment vitiliginous skin. ...In our research study, the maximum response has been reported. in subjects with focal (segmental) vitiligo which is consistent with the results reported by Olsson and Juhlin who achieved complete repigmentation in all patients with segmental vitiligo enrolled in their study (18). In addition, Mulekar et al. also reported that the maximum response (95% repigmentation) to the autologous melanocyte keratinocytes transplantation were observed in subjects with segmental vitiligo (18). The moderate to fair response of generalized vitiligo patients reported in our study was also consistent with the finding of study performed by Huggins et al. (21). Despite the consistency of the results of abovementioned studies with the present review, it's noteworthy to mention that no need for dermabrasion in the all procedures make it more preferable. During this report, cells derived from dermo-epidermal junctions of an autologous thin skin graft sample taken from subject are transferred into albumin containing vehicle and the obtained final then transplanted into the recipient's lesion site (22). As non-cultured techniques are more practical, cultured procedures require more complex laboratory equipment, (23, 24) and are more preferable since significant therapeutic outcomes and high satisfaction rates reported with cultured methods in the treatment of small depigmented areas (25). Furthermore, application of these approaches in treatment of acral vitiligo have been reported to result in poor response and relatively reduced success rate (26, 27). The current study, explain the efficacy and complications of injection autologous cultured melanocytes and effect on repigmentation of patches in subjects with stable vitiligo have been reported since half a century follow up (13, 28).

## **2- Technique evolution**

### **2-1 Finding a Safe Way for Melanocyte Proliferation**

Successful surgical approaches as treatment choices for vitiligo have been established for more than half of a century. Culturing melanocytes is a potential way of treating hypopigmentation

diseases. So, contrary to the whole tissue grafting methods, cellular grafting technique can easily treat large depigmented patches presented on almost any cutaneous surfaces only by applying a small donor sample skin. As a cellular grafting technique, the history of autologous cultured epidermal cell injection used during these decades were introduced as a modified technique firstly by Lerner et al. (29) which possess multiple advantages over previously developed techniques. First of all, splitting along the basal membrane through application of different enzyme, reduces the possibility of epidermal stem cells dissipation during isolation process. Second, intraepidermal administration of cells mostly restricts possibility of scar formation and induction of Kobner phenomenon and finally, use UV-phototherapy along with other technologies were applied during procedure, effect multi techniques on cell therapy can be observed and easily monitored. Here We report procedures developed for melanocytes culture. (18). Regenerative medicine is considered one of the viable, safe, and effective treatments of vitiligo (18). For the first time, in 1956, human melanocyte was studied; in this experiment, Funan Hu(30) and his partners worked on human melanocytes from benign pigmented nevi and utilized foreskin of white and black infants. They indicated the presence of two distinct different cells in normal human epidermis (i.e., epithelial cells and melanocytes), which differ morphologically, functionally, and biochemically. Also, they observed two types of melanocytes: the small type that was ordinarily seen in normal epidermal outgrowth and a large variety that was at least 2 to 4 times larger than the small type. The large type reacted strongly with Dopa and became filled with black granules. Interestingly, no apparent difference was reported in the number of melanocytes between cultures of white skin and those of colored skins, but the amount of melanin granules, and sizes and shapes of melanocytes differ between cultures of white and pigmented foreskins; also, a direct relationship between pigment-producing capacity and cellular size and complexity, was reported (18). Until 1960, no appropriate separation between adult epidermis and dermis was done. Then another research committee in the field of dermatology for the first time described a method for culturing adult epidermal cells based on preparing a cell suspension by using trypsinization: epidermal part consisted of two different types of cells; subsequently, cultivation in plasma clot gave rise to overgrowth of epithelial cells and fibroblast, demonstrating the possibility of cultivation of epidermal cell without dermal element ( **Figure 1** ) (18). During these years, considerable efforts were made to separate epidermal cells enzymatically (31) Wilkins and Szabo, by increasing  $Ca^{2+}$  levels in media, inhibited keratinocyte culture and achieved pure melanocytes (with 95% purity) scientists utilized high levels of trypsin to detach cells In 1978 add in 1981 (31, 32). In an iconic study conducted by Eisinger, for the first time a combination of factors, including cholera toxin (which inhibits the growth of fibroblasts) and phorbol 12-myristate 13-acetate (which is toxic for human keratinocytes



**Figure 1:**

Melanocytes are defined as dendritic cells located in the basal layer of the epidermis (Figure 1). The microscopic picture shows the appearance of melanocyte in different duration, Melanocytes, derived from neural crest cells, are involved in pigmentation with elongated shapes) or neuron-like cells (c, with long thin axon). Melanocyte were identified as a dendritic cell with different shapes large and huge cells like star cells, small cells and as it can be seen, melanocytes are skin attachment cells.

But not for melanocytes at pH 7.2 in IBMX (3-isobutyl-1-methylxanthine) and fetal calf serum was used. Then melanocytes were proliferated extensively and passaged serially (18). These methods were modified continuously, to achieve higher purities. Nielsen and Eisinger reported separation/purification of melanocytes in culture, and promotion of sustained growth of melanocytes in special media. They demonstrated that trypsin has more advantages over collagenase treatment because by using trypsin, melanocytes of higher purity and viability can be isolated. Nielsen and his colleagues at the end of 1980s proved that trypsin for separation of epidermis from dermis was better than collagenase, and fibroblast contamination was at minimum levels. Finally, they found that enriched melanocyte cultures could be obtained by seeding cells without or, and then transferring them to a fibroblast-conditioned medium containing horse serum and polyamines. Eventually, melanocytes were identified by their dendritic morphology, ultrastructure, and reaction to cholera toxin and pigment production after treatment with melanocyte stimulating hormone. By this method, pure melanocytes were cultured for more than 43 weeks (i.e., ten passages) (33, 34)

### **Tuning Melanocyte Transplantation in Clinical Trials**

Transplantation of healthy melanocytes in depigmented or hypopigmented areas, as a recent surgical intervention, has been considered as a promising approach in the treatment of stable vitiligo. Cellular grafting techniques are also another promising surgical intervention showing improvements causing re-pigmentations in vitiligo lesions (35). Most recently,

melanocyte transplantation has been introduced as a procedure which can be performed either in cultured or non-cultured forms. Cells derived from dermo-epidermal junctions of an autologous thin skin graft and the obtained final solution will be transplanted into the recipient's lesion site. During 1980 to 1990 replenishing melanocytes by autologous cultured melanocytes selectively in vitiliginous patients was a novel and promising treatment. The results explained MCDB 153 as a melanocytes culture medium can be adapted without serum to produce suitable epidermal sheets for skin grafting (**figure 2**),(36) (37) Advances in tissue culture, tissue engineering, and scaffold models have made the examination and precise measurements of ECM components in repigmentation healing possible. Likewise, the development of specific procedure models has created the opportunity to characterize the role of various ECM molecules in healing white patches (17-20, 28-33). In addition, the recent characterization of new ECM molecules, including multicellular proteins, and FACIT collagens (Fibril-Associated Collagens with Interrupted Triple helices) demonstrated our cursory knowledge of the ECM in coordinated with cultured epidermal cells(38) (39, 40). Also, upon progresses made in skin stem cells, Rafael Flabella conducted a new approach in the absence of feeding layers and growth factors by baring the recipient site before grafting with liquid nitrogen; in hence, the pigmentary graft was transferred to the intended areas of three stable vitiligo patients. Six months after transplantation, permanent repigmentation in the grafted areas, was obtained. As mentioned previously, there are several challenges in translating stem cells from laboratory to clinics. The combination of stem cell therapy and repigmentation must be further examined with development of transplant procedures, will accomplish optimal clinical benefits for vitiligo patients (35) Following resolution after transplantation and ten days after injection cultured melanocyte to nine vitiligo Patients with stable disease without added supplements such as antimicrobials and growth factors to the suspension medium excepting FBS (fetal bovine serum) results showed 80% repigmentation in three patients(41) On reviewing the literature reported Olsson, used trypsin inhibitor to stop tissue digestion, centrifuged the cells to obtain a cell pellet and added supplements such as antimicrobials and growth factors to the suspension medium. Mulekar made the procedure easier and more economical by using an ordinary incubator and DMEM/F12 without any additives for suspending the cell pellet He later replaced trypsin inhibitor by washing the epidermis several times PBS before separating it from the dermis, Later, PBS was used during suspension preparation to further cut Kumar et all introduced a four-compartment technique in 2014 in which pipettes, no dangerous growth factors were used and repigmentation was induced by grafting a small specimen of buttock skin to a large area (60 cm<sup>2</sup>) four segmental vitiligo patients. Cultured melanocytes were cry stored for 6-12 months, and cells with > 90 % viability were injected intra-epidermal into the vitiliginous areas. (19, 28-31) (3, 11-16, 42, 43) without applying any growth enhancers or growth factors and FBS; Results showed 80% repigmentation in three patients (9, 17-20, 28-33, 35, 44, 45). In another revolution reported by Olsson, no dangerous growth factors were used and repigmentation was induced by grafting a small specimen of buttock skin to a large area (60 cm<sup>2</sup>) four segmental vitiligo patients. Cultured melanocytes were cry stored for 6-12 months, and cells with > 90 % viability were injected intra-

epidermal into the vitiliginous areas (17-20, 28-33, 35, 45, 46). Actually, in another clinical trial 100 patients with vitiligo diseases were selected, about  $30 \times 10^6$  cultured melanocytes (from P2-P6) were transplanted to the depigmented areas. Based on the results, vitiligo was stabilized for at least 6 months. Moreover, after several years, these scientific group conducted a comparative study on different transplantation methods in 132 patients who underwent transplantation. It was found that transplantation is a suitable choice for treatment of stable forms of vitiligo and the best response was seen in patients with shorter duration of the disease while patients with hypothyroidism responded less well. All these findings can help in selection of an appropriate treatment for patients (17-20, 28-35, 38-40). Moreover, another research group in Korea conducted a clinical trial on 120 vitiligo patients and showed that geneticin eliminated keratinocytes and fibroblasts, and bFGF (Basic fibroblast growth factors) is a tumor promoter, as like as tetradecanoyl phorbol acetate (TPA). Importantly, they also found that active disease has a poor response (25). (35, 45) Besides, other scientific group proved that application of growth factors such as EGF, BPE, FBS, bFGF, ET-1 and  $\alpha$ -MSH in culturing media, increased melanocyte growth. Research collaboration in groups and networks studied repigmentation after transplantation of cultured melanocyte in 25 vulgaris and 2 segmental vitiligo cases. (41, 47)

Repigmentation was initiated after 2-3 weeks and completed in 6 months. Researchers pointed out that the best outcome was observed in 52.17% of the patients (**figure 3**).

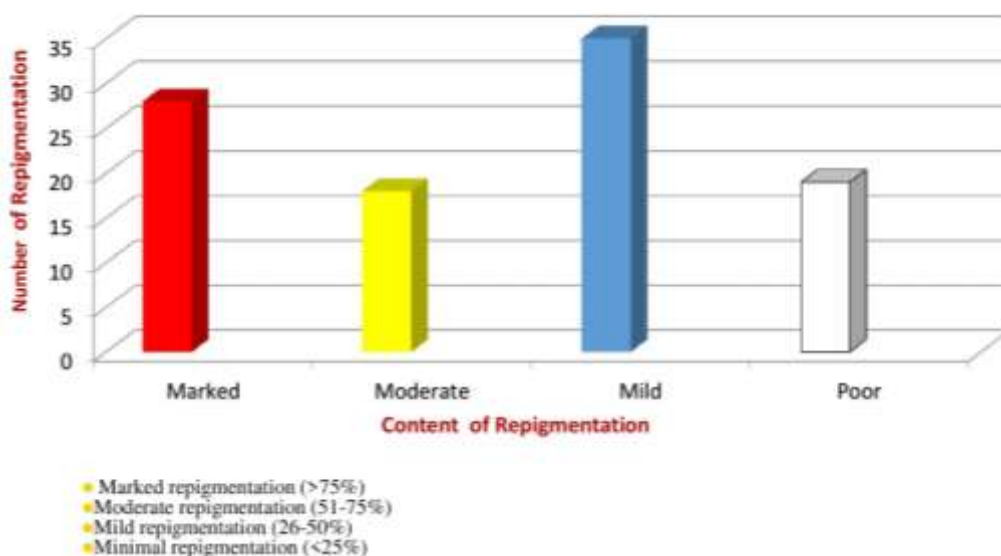
**Figure 2:**

Melanocyte Growth Medium component. Picture shows Melanocyte Growth Medium is designed to promote melanocyte proliferation in vitro. This medium consists of Fetal Bovine Serum, Melanocyte Growth Supplement, and Melanocyte Basal Medium, Fetal Bovine Serum with Melanocyte Growth Supplement and Antibiotic-Antimitotic



**Figure 3:**

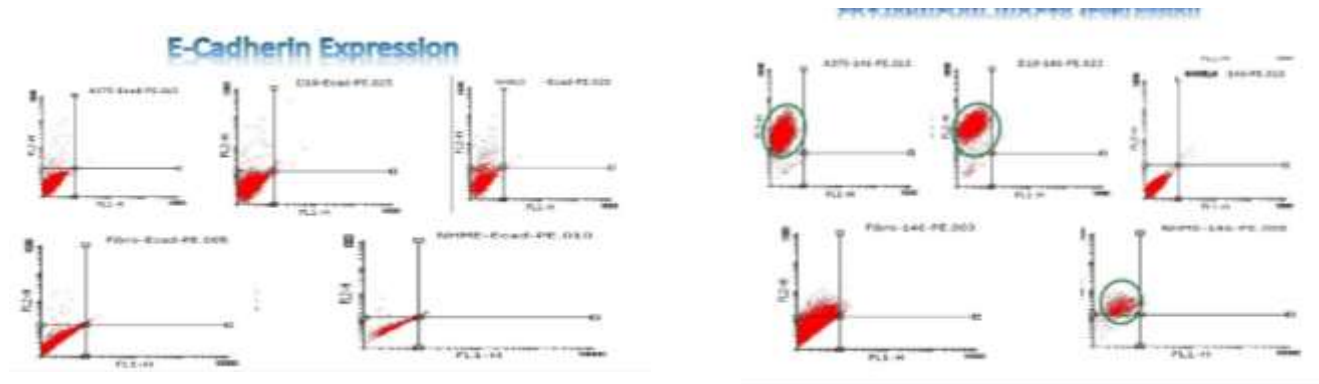
Autologous Cultured Melanocyte Transplantation. In this method, in vitro cultured melanocytes are used in the surgical treatment of vitiligo. The graph explained the repigmentation of transplantation melanocyte to vitiligo patients, as the picture show medical treatment of vitiligo includes the use of melanocyte transplant, all estimated graph including the average Response to treatment as: Excellent, Moderate, Mild and Poor.



The optimal outcome in their study was observed when the number of cells were more than 1000 cells/mm<sup>2</sup>, the site of grafting in the recipient was also an important factor, and generally, transplantation of cultured melanocytes in the thickness part of skin was more A comparative study was conducted among children, adolescents, and adults; better transplantation outcome was achieved in children and adults, but no statistical difference was observed Therefore, they concluded that the technique was suitable and similarly effective for children and adolescents. In different studies, cultured melanocytes were combined and co-cultured with other issues, like scaffolds, ECM, feeder layers, hyaluronic acid and amniotic membrane (AM) (1, 2, 4-10, 41, 48) In particular, several studies explained during 2011 and 2020 using AM (Amniotic Membrane) as a scaffold for melanocyte transplantation were replaced melanocytes into the basement membrane side of AM and cultured on amniotic membrane as a protective basement membrane for 3-4 days, Moreover, cell combination with membrane transplanted onto the white patch skin.(9, 41) This review highlights recent work and current knowledge on the application of tissue-engineered products to enhance the efficacy of cell-behavior. taken together, there is an

urgent scaffold for more attention on native human AM and tissue-engineered products are necessary to maintain biological functions such as angiogenesis and anti-inflammatory, anti-fibrotic and antibacterial activity. It appears that employment of AM as a scaffold offers a unique, simple and successful treatment (9, 41, 45). Based on the literature, the most important factor for a successful treatment, is to select patients with stable vitiligo an immunological study on melanocyte Transplantation was undertaken by immunohematologists. In this study, researchers showed that to obtain the best outcome in melanocyte transplantation, the percentage of CD8 cells should be considered in 2012, a study was conducted using Adipose-derived Stem Cells (ADSCs) as a substitute and keratinocytes as a cell combination in co-culturing. Cell-to-cell interactions between melanocytes and stem cells, contribute to induction of melanocytes proliferation and migration because of the growth factors, cytokines and condition media derived from cells. Thus, combination of cultured melanocyte with other stem cells like MSC turned out to be effective in treating vitiligo. Findings from this study establish combination stem cells as a novel cell therapy approach in stable vitiligo with superior repigmentation outcome over epidermal cell suspension, even in inherently resistant lesions Our review showed that melanocytes could be selectively proliferated in the medium. Subsequently, pure melanocytes (i.e., without contamination of fibroblast and keratinocyte) are harvested and finally by intraepidermal transplantation, 70% of white patches can be depigmented. (35, 44, 45) (9, 44, 49). In 2014, a comparative study between autologous cultured melanocytes and autologous melanocyte-keratinocyte cell suspension (non-cultured) showed marked differences between the two groups. Although both therapeutic modalities were beneficial in more than 50% of the cases, the (Cultured Melanocyte Transplantation) CMT method had better results as 69% patches were improved compared to the (Non-Cultured Melanocyte Transplantation) NCMT method By improving laboratory settings, scientists also cultured a suspension of epidermal cells in serum-free M2 medium using bFGF (TPA), CT (Cholera Toxin), IMBX and bovine pituitary extract; after transplantation, repigmentation was observed in 36 patients (20 cases in the developmental stage and 16 cases in the stable stage). Furthermore, using immunofluorescence to detect antibodies located in the cytoplasm of the melanocytes, in all patients (stable and progressive), the melanocyte-specified marker was checked (8). Excimer laser is one of the best inducers of repigmentation with melanocytes. Electroconvulsive therapy (ECT) appears to be a more feasible, less time-consuming, and more comfortable treatment for both physicians and patients. Further randomized controlled clinical trials in larger sample sizes and with longer follow-up durations showed higher repigmentation and patient satisfaction. Nevertheless, the current evidence does not indicate a significant difference between the combination of cultured melanocyte and Excimer laser, and transplantation of cultured melanocyte alone. The regulation of melanogenesis is highly complex and a UV radiation-induced in number of cellular factors are involved in this process. Different studies have examined gene expression in cultured melanocytes. Important studies during 2011 and in 2015, showed mutations of genes in RAS/RAF/MEK/ERK signaling pathway and CDKN2A genes. The expression of the above-mentioned genes was confirmed in the early stages of human melanoma cells (SK-Mel-2, SK-

Mel-5), but not in cultured melanocytes. More recently, in 2019, gene therapy scientists checked the mutation using whole genome sequencing in cultured melanocytes, and found no mutation in gene sequencing. (Figure 4A, B).



**Figure 4:**

In these pictures we can see the expression of genes, E- Cadherin and CD-146 in cultured melanocytes. Important studies showed mutations of genes in RAS/RAF/MEK/ERK signaling pathway and CDKN2A genes. The expression of the above-mentioned genes was confirmed in the early stages of human melanoma cells (SK-Mel-2, SK-Mel-5), but not in cultured melanocytes.

More recently, in 2019, gene therapy scientists checked the mutation using whole genome sequencing in cultured melanocytes, and found no mutation in gene sequencing. In the present work, we studied the functionality of melanocytes as we focused on keratinocytes, endothelial cells and fibroblasts, our method established melanocyte cultures from small amount of tissue. Melanocyte-keratinocyte co-culture, as expected, the majority of melanocytes remained bipolar and increased proportion of dendritic, oval and clustered cells was recorded. Boyce with his collaboration showed,  $10^7$  cells/ml cultured melanocyte transplanted into nude mice but no tumor, nodule or macules was observed (Table 1).

**Table I:**

Summary of the history of cultured melanocyte, reported during early days until now. This table explaining finding a safe way for melanocyte proliferation in a short but complete package during in vitro culture.

<b>Sample</b>	<b>Method</b>	<b>Result</b>	<b>Reference/Year</b>	<b>Final Report</b>
Human Skin	Flotation Skin Layers In Surface Of A Special Fluid Medium.	Epithelial Cells and Fibroblasts Like Cells Growth	1950/Pomerat	The Successful Pure Cultivation of Epidermis
Foreskin Black/White Infants	The Tissue Culture	Two Types of Melanocytes; Large/Small Growth	1956 / Funan Hu	No Apparent Difference in The Number Of Melanocytes Found In The Cultures Of White Or Colored Skins
Human skin of Kidney	Trypsinization Methods For the Cultivation	Two Different Types of Cells were seen Both Epithelial and Dendritic Cells like	1960/Bellingham	Cultivation Of Epidermal Cells Free From Dermal Elements, Might Be Possible
Guinea Pig Ear Skin	Epidermal Cell Trypsinization	Both Epithelial and Dendritic Cells, Were Two Different Types of Cells,	1960/Cruickshank	Cultivation Of Epidermal Cell Without Dermal Element Might Be Possible
Skin Of Adult Guinea Pig Ear	Separate Epidermal Cells Enzymatically	Utilized High Levels of Trypsin to Detached Cells	1981/Wilkins and Szabo	By Increasing In Ca <sup>2+</sup> In Media, Cultured Keratinocyte Were Inhibited and Pure Melanocyte (With 95% Purity) Were Achieved
Human Foreskin	Single-Cell Suspensions By Trypsinization, Cells	Used Combined Different Factors: Cholera Toxin, FBS, and ...	1982/Eisinger	Eisinger used a pH of 7.2 and fetal calf serum, by adding these materials melanocytes proliferated extensively and had been passages serially in vitro
Human Foreskin	Separation of epidermis from dermis with trypsin10X	By this method Pure melanocytes have been cultivated more than 43 weeks (Ten passages)	1984/Nielsen and Eisinger	Finally, they achieved enriched melanocyte cultures could be obtained by seeding cells without Mg <sup>2+</sup> and Ca <sup>2+</sup> and then transferring them to fibroblast-conditioned medium containing horse serum and polyamines.

The biopsy of mice not shown any tumorigenicity markers, Immunohistology Chemistry (IHC) did not lead to any carcinoma. (9, 17-20, 28-33, 44, 49, 50) All to gather a complete study has been done on 2283 vitiligo patients as a retrospective study of long-term follow-up of treated by autologous, non-cultured melanocyte-keratinocyte transplantation. The analysis has been comprised after 12 months and the result explained 2171 patients (94.4%) were followed up for at least 2 years. In this study the maximum area in one individual patient was 200cm<sup>2</sup> and the minimum was 2 cm<sup>2</sup>. In general, the modified melanocyte cultured method performed during these decades this report has been introduced as an effective and well tolerated method in the treatment of stable vitiligo. As the process of repigmentation were ongoing at the end of the 50 years follow up, better outcomes may be predictable with further follow up as seen in similar studies. Subjects with all types of focal, general and universal stable vitiligo may benefit from this approach especially in cases whom other medicinal interventions were shown to be ineffective (51, 52).

Initiation of repigmentation were evident in transplantation sites at the end of the second month. At this point 60-80 % of subjects demonstrated a mild and a moderate response. At the end of the fourth month, skin surface repigmentation were demonstrated in almost 90% of subjects ranging from a mild to excellent improvement. This trend of skin repigmentation were more tangible at the end of the 6-month follow up period since repigmentation in all depigmented patches of all subjects were evident. The process of repigmentation were also ongoing in cases with moderate or mild repigmentation. the site of tissue grafting or Epidermal grafts on full thickness skin, which contain epidermis and isolated cells, can be achieved with by two methods cultured and non-cultured cell. surely the best way to utilize method is physically and enzymatically to achieve cells with more than 90 % viability (checked by trypan blue). Clinical applications of all important expansion cell isolation techniques have demonstrated promising results. The benefits are manifold in treating white patches. Transplanted sites re-pigmented with Low thickness is beneficial outcome (53, 54). With particular attention on cultured melanocyte to repair the lesion of vitiligo. In this review, we explained the state of the art of treatments based on regenerative medicine and its current limitations and challenges, we focused on emerging beneficial roles of different combination therapy, like: Laser excimers, Scaffolds and Stem Cells to accelerate treatment and improvement of this combination strategy. Gathering all, understanding how careful integration of different approaches presented in this review could help to potentiate therapeutic results in preclinical models and their good manufacturing practice (GMP) implementation for future clinical trials. Our literature review indicated that, in many studies, over 50% success has been observed and cultured melanocyte transplantation is still regarded as the most viable method for the treatment of vitiligo.

**(Table 2) Table II:**

During 21 century, different studies were conducted on replenishing melanocytes by autologous cultured epidermal cells. With expertise in transplantation, it has now become possible to treat larger recipient areas with smaller skin samples. In vitiliginous patients is a novel and promising treatment here we presented the story of replenishing melanocytes by autologous cultured melanocytes cells

<b>Study Design</b>	<b>Type Of conducted replenishing melanocytes</b>	<b>determine the relative efficacy</b>
A. Taieb 1992-2000	Grew cultured melanocytes in MCDB 153 without serum supplementation/ grew them on collagen-coated substrate as a good extracellular matrix (ECM)	To optimize conditions in serum-free MCDB 153 for safe transplantation. To assess the effect of sheets to proliferation and of cultured melanocyte.
Rafael Flabella 1989-2000	Cultured melanocytes without feeding layers, growth enhancers, or hormones but by denuding the recipient site with liquid nitrogen freezing 48 hours before grafting, and then transferring the pigmentary graft immediately to the intended areas, handling with similar manner as with epidermal grafts.	After one to six months of transplantation, Permanent repigmentation of refractory areas under medical therapies was obtained.
Rafael Flabella 2008-2010	cultured melanocyte without applying any growth enhancers or hormones but utilized FBS 10%	A high percentage of success was observed in outcomes, which showed a 80% repigmentation in three patients
Olsson MJ 1993-2011	Used a new method without any dangerous growth factors, and showed a new repigmentation by a small specimen of buttock skin, to the large area (60 cm <sup>2</sup> ) four segmental vitiligo patients were utilized this method.	Cultured melanocytes were cryostorage for 6-12 months, cells with more than 90 % viability were injected intra-epidermal into the vitiliginous areas
Yu-Fu Chen et al 2004	Yu-Fu Chen et al with his team discovered Geneticin used to eliminate keratinocytes and fibroblasts, but not affected on melanocyte.	Finally, he found patients with active disease take a poor response. And adding Geneticin used to proliferating melanocyte better
Pedro Redondo Sara Leal-Marin 2011-2020	was used as a scaffold for melanocyte transplantation, melanocytes were replaced into the basement membrane side of AM and cultured for 3-4 days, then transplanted onto skin	Native hAM and tissue-engineered products are necessary to maintain biological functions such as angiogenesis, anti-inflammation, anti-fibrotic and antibacterial activity. It appears AM as a scaffold is a unique, simple and successful treatment
Gunjan Verma 2015	A comparative study between autologous cultured melanocyte and autologous melanocyte-keratinocyte cell suspension Was done	The CMT (cultured melanocyte) method showed better result 70% patches were better in, compared with NCMT (Non- cultured melanocyte) method

Results of the all reports were confirmed the efficacy of the used method in the treatment of all subjects with stable vitiligo who did not respond to the common algorithms of non-invasive treatments (**Figure 5**) (55).



### 3- Conclusion

The rapid progress in the field of stem cell research has laid strong foundations for their use in regenerative medicine applications of skin disorders, like hypopigmentation diseases. Growing evidences indicate that some observed therapeutic outcomes of stem cell-based therapy are due to survival of transplanted cells. In the decade of 1950s, the methods for isolation of epidermal skin cells like melanocyte introduced and transplanted successfully for vitiligo patients (47). Finally, as transplantations were applied in diverse regions, extent of repigmentation were also varied depending on the site of transplantation. This is mainly due to the fact that based on the location of vitiligo, the response to different medications is different. For instance, based on literature, lesions presented on head and neck are usually the most responsive ones to all kind of vitiligo treatment modalities. Vitiligo lesions on the lips or the tips of the fingers or toes (also mentioned

as lip-tip vitiligo) usually fairly respond to the treatments and finally, vitiligo lesions of hands and feet (also referred as acral vitiligo) are the most resistant ones to the medications.

### Conflict of interest

The authors declare no conflict of interest.

### Contributors

AS, contributed to the study design, data collection, cell preparation, and writing of the manuscript. SJZ contributed to data collection and analysis. SJZ contributed to data analysis and writing the paper. SJZ contributed with patient evaluation and clinical data collection. AS contributed to study performance, and writing the manuscript. AS contributed data analysis and writing manuscript AS contributed in study design and performance and writing the paper. SJZ contributed to the study design, data collection, data analysis, and writing of the manuscript and financing the project. All the authors contributed to interpretation of the results and to the final preparation of the article.

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