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Title: The regenerative potential of autologous stem and somatic cells in vitiligo

Short title: Cell-derived autologous melanocytes

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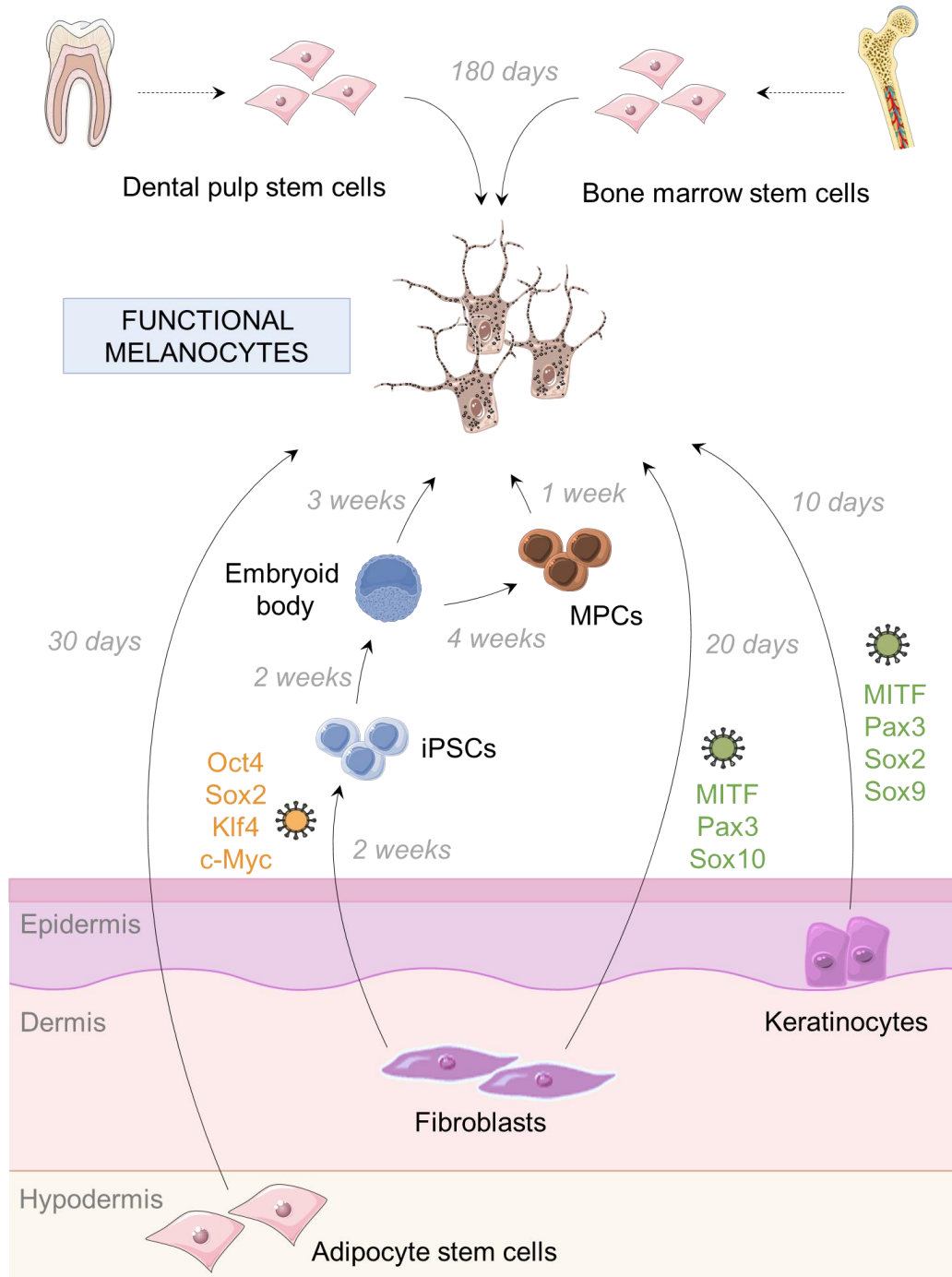
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Abstract

Vitiligo is a human pigmentary disorder characterized by autoimmune destruction of mature melanocytes in the skin. In addition to researches on the inflammatory component of the disease, current treatments tend to stimulate local melanocyte stem cells or transplant functional melanocytes from uninjured areas. However, in some cases of extensive depigmentation, only few healthy cells can be obtained. This review discusses examples in the literature of using different sources of autologous stem and somatic cells to obtain melanocyte progenitors or mature melanocytes, and compares the strategy of stem cell differentiation with that of somatic cell reprogramming. More specifically, this review illustrates the differentiation capabilities of stem cells from dental pulp, bone marrow, and adipose tissue; the reprogramming of pluripotent cells, and the transdifferentiation of fibroblasts and keratinocytes. Each of these approaches is capable of producing fully functional melanocytes, but all have advantages and disadvantages. Finally, the relevance for potential clinical application is discussed, along with the risks associated with each strategy and the major current barriers to their use.

Keywords: differentiation, melanocytes, reprogramming, stem cells, transdifferentiation, vitiligo

Graphical abstract



Summary outline of the different somatic cells (purple) or stem cells (pink) origins; and their differentiation mechanism to obtain functional melanocytes: differentiation medium (black arrows), cell reprogramming (yellow) or cell transdifferentiation (green). Comparison of the induction times (in italics). iPSCs: induced pluripotent stem cells; MPCs: melanocyte precursor cells; MITF: melanocyte inducing transcription factor; Pax3: paired box 3; Sox2,9,10: SRY-box transcription factor 2,9,10; Oct4: octamer-binding transcription factor 4; Klf4: Kruppel-like factor 4; c-Myc: avian myelocytomatosis virus oncogene cellular homolog.

The illustration was drawn using pictures from Servier Medical Art website available at <https://smart.servier.com/> (accessed 03 Aug 2023).

Introduction

Vitiligo is a chronic skin depigmentation resulting from massive destruction of melanocytes by specific cytotoxic CD8+ T lymphocytes and is classified as an autoimmune disease. Vitiligo is mainly divided into two types, segmental vitiligo (SV), and non-segmental vitiligo (NSV), the latter being the more frequent and thus encompassing various subtypes, among which generalized and acrofacial vitiligos are the most common. The difference between SV and NSV relies mainly in the clinical manifestation of the depigmented vitiliginous lesions, which is symmetrical in the case of NSV, and the prognosis for disease progression that remains more unfavorable in SV. Therapeutic management will therefore depend on the subtype of vitiligo, as well as the extent of the lesions, their distribution and the activity of the disease (active or stable). First-line treatments include phototherapy (ultraviolet A and psoralen (PUVA), or narrow-band ultraviolet B (NB-UVB)), and topical or systemic administration of immunosuppressants or immunomodulators [1]. Surgery is proposed only in cases of stable vitiligo, when conventional treatments have failed to induce a satisfactory response. Recognized surgical techniques include tissue grafting, transplantation of autologous epidermal cell (cultured or not), and hair follicle transplantation [2].

While all melanocytes in the lesion area are destroyed, some hairs sometimes retain their pigmentation, demonstrating the existence of a melanocyte reservoir protected from T lymphocytes by the immune privilege of the hair follicle. In mammal development, melanocytes derive from the neural cell crest and acquire the Sox10 (SRY-related HMG-box 10) transcription factor to become a bi-potent progenitor common to melanocyte lineage and glial cells. In a next stage, some specific markers are expressed to give rise of melanoblasts, the melanocyte precursors, such as the MITF (microphthalmia-associated transcription factor), DCT (DOPAchrome tautomerase) and KIT (proto-oncogene receptor tyrosine kinase). Melanoblasts undergo terminal differentiation into functional melanocyte as they gain the machinery of melanogenesis through the TYR (tyrosinase) enzyme, and acquire a dendritic morphology. Besides, melanoblast are also able to dedifferentiate into melanocyte stem cells in the hair follicle by the loss of KIT expression (KIT- cells). Under specific stimuli such as the release of SCF (stem cell factor) from keratinocytes in response to UV radiations, these quiescent stem cells (located in the bulge), differentiate in transit amplifying cells (KIT+ cells), and then in mature melanocytes (TYR+ cells) [3]. Many treatments have therefore focused on phototherapy to stimulate the KIT- stem cells to obtain functional melanocytes *in situ* [4]. In order to improve the pool of melanocytes, others are experimenting with transplanting melanocytes from skin and hair follicles from uninjured areas [5, 6]. However, depending on the size of the patient's lesions, only few melanocyte stem cells can be harvested from healthy areas, in addition to the risks of donor site infection, scarring, and sometimes irregular pigmentation. In culture, if mature melanocytes can be preserved, they do not proliferate spontaneously, as observed with basal keratinocytes for instance, and they gradually lose their dendritic morphology. This could be imputed to the signaling protein Wnt3a which induce melanogenesis while it inhibits cell proliferation [7]. Indeed, functional pigmentation is achieved at the expense of melanocyte proliferative capacity. Therefore, it is necessary to find other sources of cells capable of providing melanocyte progenitors and a reliable source of autologous melanocytes.

This review summarizes the different sources of autologous cells capable of melanocyte differentiation. Two main strategies are compared: stem cell differentiation and somatic cell reprogramming.

The first use of mesenchymal stem cells to restore melanocytes

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of cell types under certain conditions. They are found in dental pulp (DPSCs), bone marrow (BMSCs), and adipose tissue (ADSCs).

While DPSCs and melanocytes share a common embryonic origin derived from neural crests, BMSCs are of endodermal origin but retain the ability to differentiate into various mesodermal and ectodermal lineages.

In 2010, Paino and coworkers achieved spontaneous differentiation of human DPSCs into functional, mature melanocytes *in vitro* without any stimulation. Expression of MART-1 (Melanoma Antigen Recognized by T Lymphocytes), which is required for melanosome structure and maturation, was observed after 150 days, and melanosomes were detected after 180 days [8]. Shortly thereafter, Weimin Shi's group succeeded in differentiating BMSCs into melanocytes *in vitro* in 120 to 180 days using a medium containing hydrocortisone, FGF (fibroblast growth factor), and transferrin. Morphological changes and melanin production were observed, as well as the expression of TYR, TRP-1/2 (tyrosinase-related protein) and MITF [9].

Therefore, the use of these two cell sources is particularly interesting, as their collection is minimally invasive and a limited number of *in vitro* manipulations are required to induce their differentiation [8, 9]. However, because there are few *in vivo* manipulations, amplification is mandatory for clinical application. There remains a risk of loss of mitotic capacity or, conversely, induction of malignancy after very long induction times.

Faster differentiation with MSCs from adipose tissue

ADSCs were first tested in vitiligo for their anti-inflammatory properties when autologous melanocytes were transplanted into lesioned areas [10]. To this end, ADSCs are co-cultured with melanocytes prior to transplantation to prevent inflammatory cell infiltration six weeks after transplantation. This co-transplantation was recently used in conjunction with narrow-band UVB phototherapy to revitalize post-transplant pigmentation in a murine vitiligo model [11]. In particular, this study highlighted the importance of Nr2 (nuclear factor erythroid 2 like 2) in the protective effect of ADSCs against intracellular calcium release and further oxidative stress.

In addition to the protective effect of ADSCs, other authors have shown that these cells can be directly differentiated into melanocytes in only 30 days [12]. The *in vitro* induction medium promotes the differentiation of these stem cells into melanocytic precursors that no longer express ADSC-specific markers (CD105 and CD73), but then display melanocyte-specific features, including morphology, the molecular markers Pax3 (paired box gene 3), MITF, TYR and PMEL (premelanosome protein), melanin synthesis and the presence of melanosomes. Complementary *in vivo* tests also showed restoration of pigmentation in the skin of albino mice, with melanin synthesis and transfer to keratinocytes within 14 days [12]. This technique would therefore represent a major advance in medicine, combining high quality and quantity of melanocytes obtained with low risk in their use.

In general, the use of MSCs is sometimes limited by the invasiveness of the harvest or the limited number of cells obtained. In such cases, other methods propose the use of more accessible cells through a reprogramming step.

Cell reprogramming: an effective but risky alternative

Inducible pluripotent stem cells (iPSCs) can be generated from adult fibroblasts (or other) by introducing viral vectors that express a combination of transcription factors that allow reprogramming to the stem cell state: Oct3/4 (octamer-binding transcription factor), Sox2, Klf4 (Kruppel-like factor 4), and c-Myc (avian myelocytomatosis virus oncogene cellular homolog). iPSCs are then typically cultured for two weeks to obtain embryoid bodies (EBs), and these structures are then placed in the presence of cocktails of soluble factors to commit the cells to a specific differentiation pathway for approximately three weeks. While differentiation of EBs into melanocytes is possible, it generally results in an inhomogeneous population of pigment cells. In 2019, Hosaka and colleagues developed melanocyte progenitors with low expression of melanocyte markers and no melanin production, but with a stable and homogeneous phenotype [13]. These precursors could then be differentiated into mature melanocytes on weekdays in the presence of a GSK3 β (glycogen synthase kinase 3 beta) inhibitor.

Other protocols for optimizing iPSCs for pigment cell production have subsequently emerged, highlighting in particular the importance of FGF8 (fibroblast growth factor 8) as a replacement for the BMP-4 (bone morphogenetic protein 4), endothelin 3, Wnt3a and SCF cocktail in the culture medium to allow specific maturation of the melanogenesis machinery [14].

However, the genetic stability of iPS-derived cells remains an area for improvement. Recently, a new differentiation pipeline has been proposed by Saidani and coworkers that leads to a population of mature, functional and proliferative melanocytes by specifically passing through a neural crest cell lineage step from EBs via inhibition of the SMAD pathway and activation of the Wnt pathway [15]. The other advantage of this protocol is that it can also be directly applied to reprogramming human embryonic stem cells (hESCs), another source of pluripotent cells, into pigment cells.

To date, the derivation of pigment cells from iPSCs represents a major advance in the development of experimental models that allow us to reproduce and better understand certain pathologies associated with pigmentation disorders, including vitiligo. However, the production of pigment cells by this process for eventual transplantation into human tissues remains highly uncertain. The main shortcoming is related to the genetic instability caused by the introduction of one or more viral vectors required for the initial reprogramming. On the one hand, these vectors integrate relatively randomly into the genome, and on the other hand, the integrated transgenes are themselves potential inducers of deleterious pathologies. For example, a significant number of genes expressed in iPSCs are also found in tumor cells [16]. While hESCs have a much lower oncogenic risk, the ethical issues of using human material created specifically for the repair of an individual remain unresolved. Moreover, hESCs derived from *in vitro* fertilization remain heterologous cells and, unlike iPSCs derived by reprogramming the somatic cells of the individual to be treated, hES-derived cells do not overcome the immune problems of rejection in the event of transplantation.

Transdifferentiation: another way to reprogram somatic cells

Transdifferentiation is the transformation of one somatic cell type into another. Unlike iPSCs, this technique does not require a pluripotent cell.

In 2014, human and mouse fetal dermal fibroblasts were transdifferentiated into melanocytes via a retrovirus expressing MITF, Sox10 and Pax3 [17]. After 20 days, the resulting cells showed adequate morphology and expressed melanocytic markers as well as genes specific for melanin production. In addition, functional assays in skin organoids demonstrated their integration into the basal layer of the epidermis, as well as the production and transfer of melanin to neighboring keratinocytes. However, transdifferentiation of adult fibroblasts remained a failure with an efficiency of only 6%. A similar strategy was used with keratinocytes transduced with vectors encoding MITF, Pax3, Sox2 and Sox9 [18]. Under these conditions, a functional and stable melanocyte population was established in only 10 days.

Thus, different combinations of transcription factors are capable of producing melanocytes, and this can be explained by i) redundancy of action of the factors used; or ii) different cell origins. In any case, a slightly different combination of factors could potentially give rise to melanocytes from adult fibroblasts. However, the need for viral vector transduction remains a barrier to medical use, as discussed above for iPSCs, and this strategy remains limited to obtaining experimental models.

Conclusions

The cell reprogramming strategy allows to obtain a large number of cells, but the ADSC pool remains the most promising, with rapid differentiation, better accessibility and, above all, minimal risk of use. A recent study demonstrated that undifferentiated ADSCs constitutively expressed significant levels of several melanocyte markers such as MITF and HMB45 (human melanoma black 45), showing that ADSCs are probably quite prone to easily differentiate into melanocytes [19].

Direct reprogramming could also be used in translational medicine, as it provides an autologous source of melanocytes. However, there are still i) the risks associated with the use of retroviruses, and ii) the ethical issues involved. Other biochemical or mechanical stimuli to induce differentiation to replace viral vectors could be investigated for possible clinical application.

With respect to transdifferentiation, cells pass through various intermediate stages that may or may not be identical to those that occur during development, as nicely illustrated by the reprogramming trajectory from fibroblasts to cardiomyocytes [20]. Understanding these mechanisms is therefore crucial to avoid uncontrolled conversion or anarchic proliferation. This is especially true since the initial cell type, the target cell type, the environment, and probably also sex, age, and other physiological conditions can influence this mechanism. The main risk is activating the wrong differentiation pathway or failing to inactivate the old pathway, resulting in a cell between two states, as reviewed elsewhere [21].

To date, no clinical trials have yet been conducted using a source of autologous cells reprogrammed or differentiated *in vitro*. These cell therapy products are recognized as Advanced Therapy Medicinal Products (ATMPs), for which legislation has set extremely high-quality requirements, which is understandable. Nevertheless, progress in differentiation protocols is accelerating, and it is highly likely that these products will be validated in the next few years. For now, ATMPs validated for the repigmentation of vitiligo only concern non-cultured autologous epidermal cells in suspension [22-25]. Previous clinical trials were also

conducted using autologous cultured keratinocytes. This surgical technique started in the 90's using Green's protocol to form epidermal sheets that are further grafted on the lesion site, coupled to phototherapy to induce pigmentation. The results indicate that this technique is however much more effective for SV than generalized vitiligo [26]. Interestingly, a very recent study conducted using non-cultured keratinocytes-melanocytes transplantation indicated good outcomes in refractory and stable vitiligo in different NSV subtypes [27]. Considering the importance of melanocyte transplantation in NSV, autologous differentiated stem cells would find a particular interest in this type of vitiligo.

Finally, whatever the strategy, it is important to consider the overall cost of cell therapy: unless new techniques are developed to reduce this cost, it is unlikely to be used to treat a non-lethal disease such as vitiligo. The best alternative to date therefore remains immunomodulation to limit the development of the disease. Nevertheless, the general principle of these different techniques could be applied to other more severe and/or more prevalent diseases.

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