Pigmented Skin Models: Understand the Mechanisms of Melanocytes

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Skin and melanocytes

Skin is composed of three layers: epidermis, dermis and hypodermis. The epidermis is organized into five layers in which there are different cell types. The most important cell type in the epidermis is the keratinocytes which constitute approximately 95 % of the total epidermal cells. Melanocytes, Langerhans cells, Merkel cells and inflammatory cells form the remaining 5 % [1]. Among these cells, the melanocytes, which are dendritic cells, are the second most important cell type in the epidermis. They have the capacity to synthesize melanin, a skin pigment. Melanocytes are not only found in the skin, but can also be observed in hair, eyes, ears and central nervous system (Table 1) [2-3]. Their different localization gives them different functions in the organism, but they all keep a common function: melanogenesis [1].

1.1.1. Skin melanocytes

Skin melanocytes are localized in the basal layer of the epidermis, at the junction of the dermis, and their dendrites expand between keratinocytes of the next layer. These dendrites allow melanocytes to make contact with the keratinocytes for the melanin transfer. This cell-to-cell contact stimulates proliferation and differentiation of melanocytes due to growth factors produced by the keratinocytes [4]. Thus, the melanosome, organelle containing melanin pigments, can be transferred to the adjacent keratinocytes, which store the pigments, and degrade them when they move to the skin surface [5]. When the pigments are in keratinocytes, they give a color to the skin with a mix of other pigments such as carotenoids and hemoglobin derivatives [6-7]. However, melanin is the principal pigment present in the skin [7] and can be

Body localization	Functions
Skin	Skin pigmentation
	Photoprotection
Hair	Hair pigmentation
Eyes	Vision
	Photoprotection
	Eye pigmentation
Ears	Hearing
	Protection against high intensity noise
Central nervous system	Scavenge toxic cations

Table 1. Melanocyte functions dependent on the body localization

found in two different colors: yellow/red (pheomelanin) and brown/black (eumelanin) [8]. These two types of pigment are one of the explanations for different ethnic skin color in the world. The other causes will be covered in the next section. However, ultraviolet radiations (UVR) are the main factor causing pigmentation variation in the skin. They can produce photodamage, erythema, mutations, vitamin D synthesis and tanning of the skin. Current research on the mechanism of UV-induced pigmentation (tanning) suggests that UVR induced DNA damage, and the mechanism for repair of these damages by a specialized endonuclease increases the production of melanin [6, 9]. This quantity of produced melanin acts as a skin protective barrier against the UVR by absorbing the UV photons [10]. Some research demonstrates that people who have more pigmented skin have less risk of developing skin cancer or sunburn. Moreover, eumelanins, which are presented in high quantity in dark skin, are more photoprotective than pheomelanins. They absorb free radicals generated in the cells by UVR, thus preventing DNA damage [11].

1.1.1.1. Ethnic skin types

The different ethnic skin color worldwide depends on various factors such as UV exposition, genetics, environmental factors and skin pigments [12]. Melanin is the principal pigment which can affect skin color in several ways. The number of melanocytes, the melanogenic activity, the melanin type, the size and the number of melanosomes and the distribution in the epidermis can also affect the skin pigmentation [6]. In 2002, Alaluf *et al*. demonstrated that Causasian skin was characterized by a low number of melanocytes (1 for 36 keratinocytes), small melanosomes and light pigments such as pheomelanins while black skin was characterized by the presence of a higher number of melanocytes, larger melanosomes, a higher quantity of melanin and more eumelanins [13]. It seems that the size of melanosomes is important in the skin pigmentation because large melanosomes are found in the epidermis as single particles, while small melanosomes tend to aggregate them. It could have the effect that the large melanosomes are transferred to keratinocytes individually and thus, can absorb light better than melanosomes transferred in complex [14].Their distribution in the epidermis is equally very important and research demonstrated that in white skin, melanosomes are

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completely degraded before the *stratum corneum* while in dark skin, melanosomes are found in this layer [15]. Therefore, all these characteristics contribute to ethnic groups their specific skin color.

1.1.2. Other melanocytes

1.1.2.1. Hair and eye melanocytes

Melanocytes have a predominant role in skin color, but they have equally the same function in hair and in eyes. In hair, they are localized in the bulb, at the bottom of the hair follicles, to give the hair's color. The same pigments found in skin, such as pheomelanin and eumelanin, are also responsible for the different colors. Counter to skin melanocytes that rarely proliferate, hair melanocytes can proliferate and differentiate in each hair cycle. This cycle includes the growing phase (anagen) in which there is melanogenic activity and hair is pigmented. At the end of this phase, the hair follicle regresses, the melanogenic activity decreases and the melanocytes retract their dendrites to finally stop the pigmentation. These non-active melanocytes are replaced by new melanocytes, which are recruited from the pigment cell reservoir, to restart a new hair cycle [16]. In eyes, there are two types of melanocytes: conjunctival and uveal. The first type is localized in the conjunctiva and transfers melanin to conjuctival epithelium while the second is localized in the iris and produces as well as stores melanin, but it does not transfer melanin to any other cells [17]. The quantity or the type of melanin in uveal melanocytes characterizes different eye colors. Such as in the skin, melanin contains in uveal melanocytes may have a protective role for the eyes against UVR. However, few studies have been conducted on this subject and uveal melanocytes remain unknown.

1.1.2.2. Ear and central nervous system melanocytes

Melanocytes have a well-known pigmentation function, but they also have an important role in ears and in the central nervous system. Melanocytes in ears are located in the stria vascularis of the cochlea, which is formed of three cell types: marginal cells, basal cells and intermediate cells [18]. Intermediate cells are composed of two types of melanocytes, such as light cells that are able to synthesize melanin, and dark cells that are incapable. They are necessary to the normal function of ears, and damages or loss of these cells can cause the hearing loss [19]. Some studies demonstrated that albino guinea pigs are more sensitive to high intensity of noise than pigmented guinea pigs because of their low quantity of melanocytes [20]. In the nervous central system, melanocytes are distributed on the meninges, particularly on the leptomeninges that cover the brain. Their role in the organism is not yet well-determined, but it is known that leptomeninge melanocytes have the capacity to capture toxic cations and free radical species from the blood circulation [19].

1.1.3. Melanogenesis

Melanocytes originate in neural crest precursor cells: the melanoblasts. Melanoblasts migrate, proliferate and differentiate into melanocytes to reach their destination, such as skin, eyes, hair, meninges and ears [3]. Melanin is synthesized in these melanocytes in specialized lysosome-

like organelles named melanosomes. These organelles have four stages of maturation in which the melanosome begins unpigmented and ends pigmented [5]. The process of melanin synthesis is called melanogenesis and it needs three enzymes to assure its good working: tyroninase, tyrosinase-related protein 1 (TRP1) and tyrosinase-related protein 2 (TRP2). These three enzymes are necessary for the regulation of the melanogenesis, but tyrosinase is the limiting factor of this pathway. It catalyzes the first two reactions of the biosynthesis of melanin that are necessary for producing eumelanin and pheomelanin [21] while TRP1 and TRP2 are only involved in the pathway of eumelanin [1]. Tyrosinase uses tyrosine, DOPA and 5,6-dihydroxyindole (DHI) as substrate to produce respectively DOPA, DOPA-quinone and DHI-melanins (Figure 1). Tyrosinase activity is regulated by some factors such as the pH, which needs to be optimal at 6.8 in melanosomes [22], and the melanocyte-stimulating hormone (α-MSH) secreted by melanocytes. The α-MSH binds melanocortin receptor-1 (MC1R), which is expressed by melanocytes, and triggers eumelanin pigments production whereas when α-MSH does not recognize MC1R, pheomelanin pigments are generated [9]. Following this production of melanin, the melanosomes which have pigments are transported towards the end of the melanocyte dendrite by actin and tubulin filaments. Then, melanosomes are transferred to keratinocytes (when it is skin melanocytes). The mechanism of transfer is still unknown, but there exist some hypothesis about this mechanism such as cytophagocytose [23], discharge melanin in the intercellular space [24], or transfer by filopodia [25]. Once in keratinocyte, melanosomes are distributed around the nuclei and, in response to UVR, they form a supranuclear melanin cap on the sun-exposed side of the nuclei to protect DNA against the UVR damages [26]. Melanin pigments are degraded with the keratinocytes when they move to the epidermal surface for their differentiation [11].

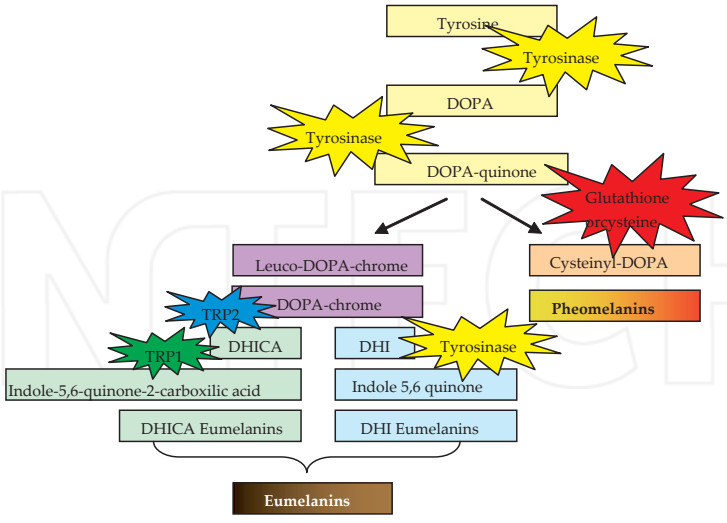

Figure 1. Melanogenesis mechanism. TRP1: Tyrosinase-related protein 1; TRP2: Tyrosinase-related protein 2; DHI: 5,6-dihydroxyindole; DHICA: 5,6-dihydroxyindole-2-carboxylic acid.

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1.2. Pigmentation diseases

Disorders in melanogenesis or in melanocytes can lead to different skin pigmentation pathologies. Some disorders are characterized by a loss of skin pigmentation (hypopigmentation) while others are recognized by the presence of dark plaques on the skin formed by an increase of pigmentation (hyperpigmentation).

1.2.1. Hypopigmentation disorders

Hypopigmentation disorders affect skin pigmentation by the destruction of melanocytes, by preventing development of melanocytes and by inhibiting or retarding melanin production. Vitiligo is characterized by the first mechanism, piebaldism by the second, while oculocutaneous albinism and tinea versicolor are characterized by the third mechanism [27]. These three mechanisms lead to white macules/plaques on the skin because of the lack of melanin pigments.

1.2.1.1. Vitiligo

Vitiligo is an autoimmune hypopigmentation disorder that affects approximately 1-2 % of the population worldwide. This disease is characterized by the presence of white macules or patches on the skin caused by the loss of functioning epidermal melanocytes [28]. Studies suggest three principal hypotheses on the mechanisms of the melanocyte destruction: autoimmunity, neural and toxic hypothesis [29-30]. Autoimmune diseases such as thyroid diseases [31] and diabetes mellitus [32] are often associated with vitiligo. These diseases cause defects in the immune system, which can cause destruction of melanocytes and the loss of pigmentation [29]. In addition, antibodies against melanocytes were found in serum of patient, and these can engage the apoptosis of melanocytes when they are present [33]. T cells were also found in perilesional vitiligo plaque biopsies and they are enriched with cytotoxicity against melanocyte antigens [34]. The neural hypothesis is based on the contact of the melanocytes with nerve endings in depigmented skin [35]. Neuropeptides and nerve growth factors such as tumor necrosis factor-α, intercellular adhesion molecule-1 and interferon-γ were found in perilesional skin, which suggest that nerves can have a role in destruction of melanocytes [30]. The toxic hypothesis suggests that the mechanism of natural protection of melanocytes is defective. The melanocytes are unable to eliminate toxic molecules, and these are accumulated in the cells [36]. More than these three mechanisms, the loss of melanocytes can be induced by environmental factors, genetic predispositions [37], apoptosis or metabolic dysfunctions [38]. All these hypotheses are a good way to understand the pathology, but the one single mechanism of the melanocyte destruction in vitiligo is still unknown.

1.2.1.2. Piebaldism

Piebaldism is an uncommon hypopigmented disorder characterized by the presence of a congenital white forelock and white macules on the extremities, forehead, frontal scalp and ventral trunk [39]. This pathology is caused by mutations of loss-of-function in the *KIT* gene that encodes for the stem cell growth factor receptor expressed in mastocytes and in melano-

cytes. When activated, *KIT* is essential to the development of melanocytes and stimulates their proliferation [40]. These mutations prevent the development and the proliferation of melanocytes, thereby causing white macules without melanocytes in skin and in hair.

1.2.1.3. Oculocutaneous albinism

Oculocutaneous albinism (OCA) is characterized by a disorder in the melanin synthesis in the melanosome due to mutations in specific genes. Tyrosinase or other enzymes essential to the melanogenesis are absent or dysfunctional, resulting in an incapacity in the melanosome to synthesize melanin [41]. People affected by this disease have a complete or a partial loss of pigmentation of the skin, hair and eyes, with the reduced pigmentation in eyes causing a lowering of visual acuity. Four types of albinism exist, all characterized by mutation in a different gene (Table 2). The most severe type is OCA1A, which is characterized by a complete loss of hair and skin pigmentation, while eyes are light blue almost pink. People affected by OCA1B can develop pigmentation on skin, hair and eyes, but they have a characteristic temperature-sensitive pigmentation. Hairs on hands and feet can be pigmented, while body hairs stay depigmented. The most common type worldwide is the OCA2 characterized by various amount of cutaneous pigment and better vision than OCA1. OCA2 and OCA4 have the same clinical characteristics, but differ in the responsible gene. People affected by OCA3 have characteristic red hair and reddish brown skin [42].

1.2.1.4. Tinea versicolor

Tinea versicolor is a common pigmentation disorder that can be characterized by round shaped hypopigmented and hyperpigmented macules on the face, trunk and arms. These macules are caused by the fungal infection, *Malassezia*, which is a genus normally found in the skin flora [43]. Several factors play a role in the transformation of the benign form of the fungi to the parasitic form. Fatty skin, exposure to sunlight, genetic predispositions, malnutrition and corticosteroids can lead to development of lesions [44]. This lipophilic fungus metabolizes various fatty acids and releases, as one of the metabolites, azelaic acid. This acid acts as an inhibitor of tyrosinase, blocking the transformation of tyrosine in melanin pigment, resulting in hypopigmented macules on the skin. Tinea versicolor is often found in young adults because their sebaceous glands are very active due to the action for sex hormones [45]. The mechanism of hyperpigmented macules is not as well-known as the previous mechanism. Some studies demonstrate that melanosomes are larger in hyperpigmented macules than melanosomes in normal skin and in white macules, but the cause of these enlarged melanosomes is still unknown [14, 45-46].

1.2.2. Hyperpigmentation disorders

Hyperpigmentation disorders are characterized by darker skin that can be caused by an increase of melanin synthesis or an increase of the melanocytes in the epidermis. “Café-au-lait” macules, Addison’s disease and postinflammatory hyperpigmentation are characterized by an increased production of melanin, while melanoma is the result of the two causes.

OCA type	Responsible gene
OCA1	Tyrosinase
OCA2	*P* gene
OCA3	*TRP1*
OCA4	*SLC45A2*

Table 2. Gene responsible of oculocutaneous albinism (OCA) different types. TRP1: Tyrosinase-related protein 1

1.2.2.1. "Café-au-lait" macules

"Café-au-lait" macules are characterized by light to dark brown spots of 1 to 20 cm on the skin that can be of a congenital origin, or appear during life [47]. These spots are often the first sign for the diagnosis of type 1 neurofribromatosis, a disorder caused by mutations in the *NF1* gene, and which can lead to benign and malignant tumors of the peripheral nerve sheath [48]. "Café-au-lait" macules are caused by an increase of melanin in melanocytes and the presence of larger melanosomes in keratinocytes, but the mechanism is still not well-known [49].

1.2.2.2. Malignant melanoma

Malignant melanoma is one of the most severe skin cancers affecting both men and women. It begins in melanocytes which have been mutated and proliferates to form an irregular naevus, a dark pigmented spot on the skin. In several cases, melanoma begins by forming a normal mole, which is grows increasingly and becomes a metastatic melanoma [50]. Some factors can affect the proliferation of the melanocytes such as genetic factors [51], environmental factors [50], spontaneous mutations [52] and endocrine factors [53]. The most important factor playing a role in this cancer is ultraviolet radiation exposure. UVR cause damages to DNA, the cell usually being able to repair this by the transcription of one of the tumor-suppressor genes, for instance, *p53*. The protein produced by *p53* genes stops the cycle cell, preventing the reproduction of DNA mutations. When there are mutations in the *p53* gene, the cell cycle can not be stopped and mutations in DNA are reproduced and accumulated in the cell that will lead to a hyperproliferation of melanocytes and a melanoma [54]. Other genes such *GADD45*, *PTCH*, *p16*, and oncogenes such as *Bcl-2*, *ras*, *c-fos* can be involved in the pathway of UV-induced melanoma [55].

1.2.2.3. Postinflammatory hyperpigmentation

Postinflammatory hyperpigmentation occurs after cutaneous inflammation or injury and causes dark plaques on skin. It can affect all types of skin, but dark-skinned people are mostly affected, especially after acne [56]. Hyperpigmented plaques are caused by an overproduction of melanin or an irregular distribution of melanin in the epidermis [57]. The exact mechanism is not well-known, but it has been shown that cytokines, chemokines, inflammatory mediators such as leukotrienes, prostaglandins E2 and D2, thromboxane-2, interleukin IL-1 and IL-6, TNF-α and some others can stimulate melanocyte activity and promote the production of melanin [58].

1.2.2.4. Addison's disease

Addison's disease, also called adrenal cortical insufficiency, is a rare endocrinal disorder that in more than 50 % of cases is caused by an autoimmune disease. The adrenal glands can not produce enough steroid hormones, resulting in a deficiency in corticosteroids [59]. One of the first indicator symptoms of this disorder is the development of a diffuse hyperpigmentation of the skin (DHP). DHP is caused by the increase of adrenal corticotrophic hormone (ACTH) in the circulation, which has the same precursor molecule to α-MSH, the melanocytes-stimulate hormone. Considering that ACTH has approximately the same composition as α-MSH, they will have the same function in the organism: stimulate melanin production [60]. This production of melanin gives people affected by Addison's disease a brown skin, intensified at the sites that are exposed to light and pressure, in the skin folds, lines of the hands, nipples and areas of scarring [59].

1.3. Available treatments

1.3.1. Cosmetic treatments

Make up, topical dyes and tanning cream are frequently used to cover up undesirable plaques caused by different pigmentation diseases. Cosmetics treatments are practiced to improve the quality of live for young people that can not undergo surgeries because it is not recommended before adulthood. In vitiligo and in piebaldism, dihydroxyacetone (DHA), the browning ingredient in tanning formulations, can be used to camouflage depigmented lesions. DHA polymerizes amino acid of the skin creating pigmentation similar to UV tanning and covering the depigmented plaques of vitiligo and piebaldism patients [61]. However, no melanin pigment is produced and DHA is much less protective against UV than melanin [62]. Some other tanning products can be useful for people affected by a hypopigmentation disorder, while for the hyperpigmentation disorders, patients may use cosmetics such as make-up to cover up their lesions. In postinflammatory hyperpigmentation, scars of acne are frequently concealed with makeup and allow affected people to have a better quality of life [56].

1.3.2. Therapeutic treatment

1.3.2.1. Hypopigmentation disorders

Therapeutic treatments, unlike cosmetic treatments, are durable and usually treat the disease. For hypopigmentation disorders, treatment objectives are to increase the production of melanin and the quantity of melanocytes. In vitiligo, the most popular treatment is psoralen-UVA phototherapy (PUVA). This treatment uses the extract of plants such as 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and a synthetic compound, trisoralen (TMP). These compounds can be used orally or in topical agents by patients, after which, they have to be exposed to sunlight or UVA radiation [36]. PUVA affects skin by increasing the number and the activity of melanocytes in the epidermis resulting in an augmentation of the pigmentation [63]. Another attractive treatment for vitiligo is immunomodulators. It is suggested that, in this disease, T cells play a role in destruction of melanocytes, and researchers are trying to

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find immunomodulators that will inhibit them. Cyclosporine is one of them, and prevents the activation of T cells by inactivation of the calcineurin, which is a regulate transcription factor of T cells [64]. Levamisole is another immunomodulators that has been studied for vitiligo treatment, and its sound effectiveness has been demonstrated [65]. The mechanism of action of this compound is not well-known, but no side effect was reported. Vitiligo and piebaldism can be treated with surgeries such as suction blister grafting [36, 66], noncultured melanocytes transplantation [67], cultured epidermis grafting [68] and autologous minigrafting [30]. In tinea versicolor, considering that the disease is caused by a fungus, the principal treatments are topical or oral antifungal agents. Nonspecific topical antifungal agents exist that do not directly affect the fungus, and specific topical antifungal agents that specifically affect the fungus. The most popular nonspecific agents are selenium sulfide and benzol peroxide which chemically remove the infected tissue and prevent a recurrence. Azoles, terbinafine and ciclopiroxolamine are some groups of drugs that are frequently used as specific topical agents to directly attack the fungus [69]. Most of these agents can be taken orally as specific topical agents, and are more effective and simpler for the patient [43]. For oculocutaneous albinism, unfortunately, no treatments are reported to be effective for repigmentation of affected people.

1.3.2.2. Hyperpigmentation disorders

In hyperpigmentation disorders, therapeutic treatments have to diminish the melanin production and the quantity of melanocytes. "Café-au-lait" macules are mainly treated with lasers. These lasers must emit a specific wavelength that will be well-absorbed by the chromophore being treated. In this disease, the referred chromophore is the melanin that has a well-absorbance at 694 nm [70]. Melanin pigment absorption decreases when the wavelength of the laser increases, and thus in this treatment, it is important to choose the appropriate wavelength [71]. For malignant melanoma, several treatments are available, but research for more effective treatments still continue. Currently, the main treatment is the excision of the malignant melanoma, with excision of a large region around the site to make sure that no cancer cell remains. If there are metastases in many other organs, surgery can rarely treat it. In these cases, patients resort to chemotherapy, using drugs which will kill cancer cells by passing into the bloodstream. If the melanoma is recurrent, patient will undergo radiation therapy, high energy rays that cause damage to cancer cells and inhibit their growth, preventing the spread of other malignant melanomas. Immunotherapy is also used for the treatment of this cancer. This therapy consists of strengthening the immune system of the patient so that he can fight against cancer cells. Cytokines such as interpheron-α, interleukin-2 and TNF can be used to stimulate the patient's immunity [72-73]. Unlike the previous two diseases, postinflammatory hyperpigmentation is principally treated with the utilisation of medications such as hydroquinone, mequinol, retinoids, azelaic acid and ascorbic acid [56]. Hydroquinone, azelaic acid and mequinol all affect tyrosinase to inhibit the melanin production, but not in the same way. Hydroquinone and azelaic acid will interfere with tyrosinase, while mequinol acts like a competitive substrate of tyrosinase [74]. Retinoids such as tretinoin and tazatorene help the penetration of other medications through the skin barrier by causing skin irritation and inducing the apoptosis of mature melanocytes [75]. Ascorbic acid suppresses the melanin production by reducing the formation of quinones, creating a lack in the melanogenesis process

[76]. However, postinflammatory hyperpigmentation can be treated by a peeling surgery, a technique that uses chemical products for destruction of a part of the dermis and/or the epidermis [77]. Finally, diffuse hyperpigmentation in Addison's disease is usually treated with mineralocorticoid and glucocorticoid [78]. These two corticosteroids will compensate for the lack of corticosteroids, reduce the production of ACTH, and thus, reduce the production of melanin pigments. Considering that it is a rare disease, treatments are not very abundant. The development of pigmented skin models could be useful for studying unknown mechanisms involved in these disorders, and for developing more relevant treatments with few side effects.

2. Challenges for the development of pigmented skin models

2.1. Tissue engineering

It is known that there is a real need for human organs available for transplantations [79]. Each year, people die while they are waiting for a compatible organ. Just in Canada, in 2011, a person needing an organ had a 30 to 40% probability of not receiving it [80]. Moreover, there are some problems with allogeneic grafts, such as problems of incompatibility and reject preoccupations. To get rid of these problems, a new approach emerged at the end of the 1980s [81]. This approach, called tissue engineering, is a science that combines both biology and engineering expertise. Its goal is to develop biological substitutes for maintaining, repairing or regenerating human organs or tissue, such as skin [82-83].

2.2. Skin substitutes

Skin substitutes are useful in different fields. They can be grafted onto patients suffering from severe burns or chronic wounds such as skin ulcer [84-85]. They can also be used for fundamental research to analyze skin functional mechanisms. Moreover, skin substitutes can be used for cosmetic testing to replace animal testing [86]. While skin is the interface tissue between human body and exterior environment, it is an organ particularly exposed to chemical and mechanical wounds and to pathological agents. Furthermore, it is an important organ in area and complexity both in structural and functional ways [87]. Consequently, to regenerate a human skin with all its functionality is a big challenge.

2.2.1. Skin substitute characteristics

In recent years, many skin substitutes have been reported, becoming more and more similar to natural human skin, but different from each others, and still not perfectly simulating skin functionality. Those substitutes can be characterized by different factors. Provenance of cells used to produce the substitute can be either allogeneic, xenogeneic or autologous. Presence of autologous cells allows reduction of incompatibility and graft reject problems [88]. The purpose of substitutes can also vary between a permanent, semi-permanent or temporary use [83]. Depending on the use wanted, complexity of substitutes will change and can be either monolayered or bilayered. As there is no perfect substitute that has been developed to this

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day, research and development on skin substitutes are still very current. Many aspects must be considered in the elaboration of a skin substitute so that it will be as close as possible to human skin. First, histological properties must be evaluated. Indeed, a perfect skin substitute must have both dermis and epidermis, with an epidermis well-differentiated and the presence of all its four principal layers: *stratum basale, stratum spinosum, stratum granulosum* and *stratum corneum*. Moreover, skin structure is important and can be evaluated by the expression or not of different markers and their localization. Furthermore, skin plays an important role as a protective barrier against chemical, biological and mechanical aggression. Consequently, the presence of this barrier functionality is important. A method to evaluate the barrier efficacy is to perform permeability analysis. Finally, *stratum corneum* lipid organization is also important in skin barrier functionality. This organization must be well-structured and features can be analyzed by ATR-FTIR [88]. One of the lacks in the currently available skin substitutes is the absence of melanocytes that lead to a hypopigmentation of the skin where the substitute is grafted. While melanocytes are part of the skin protection against UV irradiations, the lack of this molecule in skin substitutes can become problematic for the patient, beyond the cosmetic preoccupations [89]. Therefore, there is a need for developing skin substitutes with melanocyte cells. Moreover, incorporation of ill melanocytes in skin models allows the study of melanocyte skin diseases such as melanoma. While tests on animal skin are not always representative of human skin and can be ethically problematic, the development of pigmented skin models becomes a real need.

3. *In vivo* and *in vitro* pigmented skin models

Human melanocytes have been cultured selectively for two decades when, in 1982, Eisinger and Marko published their work on selective proliferation of human melanocytes. Their selective culture was based on the proprieties of phorbol ester. Indeed, at a certain concentration, this compound is toxic for keratinocytes, but not for melanocytes. By adding it to an epidermal cell solution, it allows the selective proliferation of melanocytes only [90]. In 1986, Topol *et al.* went further and reported the first pigmented human skin equivalent. This equivalent consisted of human neonatal melanocytes plating onto a dermal skin substitute with keratinocytes. They were added before those cells overgrew the dermal equivalent [91]. Since that year, many other models have been developed.

3.1. *In vivo* models

3.1.1. Spontaneous and induced mutations

Spontaneous mutation models can be use to study some diseases when the mutations have similarity with human diseases. In melanocyte skin diseases, there are not many spontaneous mutation models available. One of them was developed in 1981 by Smyth which proposed a chicken model to study human vitiligo [92]. Indeed, he developed a mutant line of chickens characterized by a higher than normal spontaneous postnatal cutaneous amelanosis. This affection has similarity to human vitiligo. They are both a consequence of melanocyte de-

struction. Those chickens are called "chickens of the autoimmune delayed-amelanotic" (DAM) or "Smyth chickens".

In melanomas studies, spontaneous mutations on mice are rare because, even if some chemical agents can induce them, it takes a long time, and the melanomas are not very representative of human ones, with the frequent absence of metastases that are frequent in human [93]. Most of the models found in the literature use other animals. An example is Millikan *et al.* which proposed in 1974 a Sinclair swine model to study pigment tumors [94]. Lesions developed by those swine are similar histologically and clinically to some different human tumors. Indeed, they show flat lesions that can be compared to human junctional neavus and elevated lesions similar to human compound neavus. Some other lesions found in swine are raided blue tumors, the counterpart of human blue neavus, peripheral depigmentation, the counterpart of vitiligo, and ulcerative tumors, the counterpart of melanoma. Previously, in 1966, Chernozemski has proposed a Syrian hamster model which presented spontaneous or induced by DMBA melanomas [95]. DMBA was also used to induce melanomas on Guinea pigs by Clark *et al.* ten years later [96]. Clark *et al.* have reproduced Edgcomb's and Mitchelich's work of 1963 and had shown that tumors in guinea pigs have some similarities with those in humans, but are not histogenetically the same. In 1989, Setlow *et al.* reported a platyfish-swordtail hybrid model susceptible to melanoma when exposed to UV radiations [97]. This fish had already been used by other authors in the past such as Anders *et al.* who used it in 1984 for melanoma research [98]. Setlow, in his work, studied different strains of this fish and their response to some UV wavelengths to find two that were susceptible to melanomas under UV irradiations. Fish of those two strains developed melanomas that were quite similar to the human ones. The principal difference was the presence of melanophores in fish melanomas. In vertebrates, melanophores represented the last stage of pigmented cells differentiation. The same year, Ley *et al.* also reported an animal model of melanomas induced by UV radiations using South American opossum [99]. In their study, they also used the concept of photoreactivation repair pathway for DNA damages to investigate pyrimidine dimer's implication in melanoma induction. Their research allowed them to make two principal conclusions: first, they concluded that UV radiations can be used to induce malignant melanoma; second, they came to the conclusion that pyrimidine dimer is involved in melanoma formation, and that the radiations induced DNA damages. Reported spontaneous models of melanoma have been less and less frequent in the recent years probably as a result of the improvement of science in different fields such as gene modifications and *in vitro* models.

3.1.2. Transgenic models

Transgenic animals can be useful for mimicking some human diseases such as melanoma. In 1991, Bradl *et al.* reported a transgenic mouse with the simian virus 40 (SV-40) controlled by a tyrosinase promoter [93] that promotes ocular and cutaneous melanomas. The melanomas reported were histopathologically the same as their human counterparts. One year later, Iwamoto *et al.* also reported a transgenic mouse model for studying melanocyte tumors. Their model consisted of a mouse metallothionein promoter enhancer coupled to a *ret* oncogene inductor [100]. They developed four independent mouse lines:

three of those lines were well-predisposed to developing melanocytic tumors, and the other one reported an acceleration of melanogenesis with no clear proliferative disorders. In 1994, Klein-Szanto *et al.* also used the SV40 driven by tyrosinase promoter to develop a transgenic mouse model. Their model allowed them to study the induction of malignant skin melanomas by short ultraviolet radiation exposure and without chemical carcinogen application [101]. Inbred line choice and other factors such as the age of the mice and the intensity of the UV treatment can be modified in the protocol to improve melanoma induction. In 1997, Takayama *et al.* proposed a new transgenic mouse model, also using metallathionein promoter driving, this time an hepatocyte growth factor/scatter factor (HGF/SF) [102]. They wanted to study the oncogenic role of those factors. Their transgenic mice developed a large variety of tumors, including melanoma. Some tumors, including this one, overexpressed HGF/SF. The same year, Chin *et al.* reported another transgenic mouse model using a different gene. Indeed, their model consisted of H-ras driven by tyrosinase promoter with INK4a knockout mice [103]. Their studies allowed them to conclude that development of melanoma can be accelerated by both the loss of INK4a allele and the activation of Ras. In 2009, Goel *et al.* reported a BRAFV600E transgenic mouse [104]. Indeed, in more than half melanoma cases, there is presence of a mutation that affects BRAF, a protein activated by Ras. Transgenic mice presented benign melanocytic hyperplasia of which progression to the melanoma stage depended on BRAF expression. In 2011, Meyer *et al.* used *ret* transgenic mice to study melanoma evolution. They also studied inflammatory tumor microenvironment when there is enrichment of myeloid-derived suppressor cells (MDSCs). They concluded from their studies that, before starting an immunological treatment for melanoma, the immune status should be controlled and the immunosuppressive microenvironment should be neutralized [105]. However, transgenic models are not the best for mimicking some cancers such as melanoma because, such as spontaneous mutations, they present a lack of metastase production. Indeed, it is known that the importance or not of metastases in large amount is crucial for the patient's survival. By consequence, it is often a target in treatment development and so their presence is important to have a valuable model.

3.1.3. Xenotransplantation models

Another type of model that can be used to study human melanoma consists of xenotransplantation of human skin onto an animal. For models on mice, three different types of this animal are mainly used: athymic nude mice, severe combined immunodeficient mice (SCID) and spontaneous AGR129 mice models. The immune systems of those mice differ from those of normal mice so that their immunological potential is decreased. Athymic nude mice do not have T cells because of the absence of a thymus. SCID mice do not have either T cells, and are also deficient of B cells. AGR129 mice do not have either T or B cells as SCID mice, and also have immature natural killer cells [106]. In 1993, Juhasz *et al.* proposed a xenotransplantation model of human skin grafted onto either nude or SCID mice. Beforehand, human melanomas were injected onto the human skin graft [107]. This

model can allow reproduction of human melanomas and their metastases because tumour cells invaded human vessels, and in more than half case, metastases were found in lungs. In 1998 Atillasoy *et al.* proposed another model for studying UV irradiations and chemical carcinogen implication in skin melanoma development. Their model consisted of human newborn foreskin grafted onto RAG-1 mice. Mice were separated into four groups and received different treatments. While the first group was the control group and received no treatment, the second received a treatment of DMBA, a chemical carcinogen. The third group received UVB irradiations and the last one, both DMBA and UVB irradiations [108]. Only DMBA treatment was not conclusive, as the only impact was the development of melanocytic hyperplasia in 16%. UVB only treatment was a little better as it caused solar lentigo in 23% and melanocytic hyperplasia in 68%. Combined treatment caused solar lentigo in 38% and melanocytic hyperplasia in 77% and was the only one that generated melanoma in 2.1% after 15 months. This is representative of melanoma incidence in Caucasian Americans that is 1.4%. In the last years, xenotransplantation models were also used to test different treatments. For example, in 2010, Schicher *et al.* used a xenotransplantation model of a SCID mouse with a human melanoma graft to test treatment of Erlotinib combined with some chemotherapeutics agents [109]. Erlotinib is a treatment already used for non small cell lung cancer. The melanomas treated with a combined treatment showed a higher reduction than those only treated with chemotherapeutic agents.

3.1.4. Other models

Other testing systems that can be used are injection of mouse melanoma into mice. For that, some mouse melanoma cell lines have been produced. One of these lines originated from one of the rare spontaneous melanomas in mice, an event that occurred on the ear of a C57BL/6 mouse in 1954. It was Fidler *et al.*, who at the beginning of the 1970s really set up the line known as B16 melanoma cell line [110]. Those murine melanoma cells can be used for studying melanoma treatments as reported by some authors. For example, in 2002, Lucas *et al.* injected B16 melanoma in C57BL/6 mice in order to test injection by electroporation of interleukin-12 [111]. They tested an intratumoral and an intramuscular treatment. While the first one was useful for treating tumour in 47% of the cases, the second one was not conclusive. They also tried those two treatments in a nude mouse model, but neither the intratumoral or the intramuscular treatment worked. Those results allowed them to conclude that T cells probably have a role in the melanoma regression, at least in this model. The same year, Garcia-Hernandez *et al.* investigated on the implication of interleukin-10 in the promotion of B-16 melanoma growth [112]. This study allowed them to conclude that IL-10 seems to have a role in this promotion in three fields: first, they simulate the proliferation of the tumour-cells; second, they have implications for the angiogenesis process and third, they are implicated in the immunosuppression. B16 melanoma cells can therefore be useful to many types of studies.

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3.2. *In vitro* models

3.2.1. Monolayers

Monolayer models are characterized by the culture of one cell type, which has previously been extracted from a skin biopsy. Cells can be extracted from normal or lesional skin such as cells of skin affected by hypo- and hyperpigmentation disorders. Monolayer models are useful for studying melanocyte properties and testing different conditions or drugs. In 2008, in a comparative study of melanocytes culture and melanocytes-keratinocytes co-culture, Liu *et al*. demonstrated the effect of melanogenic stimulators (α-MSH and L-tyrosine) and inhibitors (arbutin and hydroxybenzyl alcohols (HBA)) in the two conditions [113]. Results showed that α-MSH and L-tyrosine increased the melanin content of melanocytes, and that the increase was better in the co-culture with keratinocytes. 4HBA and arbutin inhibit the melanogenesis in the two conditions, but, in co-culture, the inhibition was much better than in melanocytes alone. These results suggest that cytokines released by keratinocytes can have an effect on the regulation of melanin synthesis, and that the co-culture model has interesting properties for testing drugs related to the treatment of pigmentation disorders. In the same vein as Liu *et al*.,Criton *et al*. tested 22 N-hydroxy-N-phenylthiourea and N-hydroxy-N-phenylurea analogues, which could inhibit tyrosinase activity and reduce melanin synthesis on melanocyte culture [114]. Results showed that compound 1 inhibits tyrosinase and reduces 78 % of melanin synthesis. It is a promising candidate for the treatment of hyperpigmentation disorders to replace whitening agents that have undesirable effects. These studies demonstrate that monolayer models allow testing of several conditions, and the possibility of observing melanocytes behavior in some pigmentation disorders.

3.2.2. Collagen gels

Unlike monolayer models, which are composed of only one cell type, collagen gel models allow formation of a dermis on which more than one cell types can be seeded. Globally, for the construction of a pigmented equivalent with collagen gel, fibroblasts, keratinocytes and melanocytes are extracted from a skin biopsy and are cultured separately. Fibroblasts are seeded onto the collagen gel, then, after few weeks of culture, keratinocytes and melanocytes can be seeded onto fibroblasts and collagen matrixes [115]. These types of equivalent that contain various cell types are useful for studying the interactions between keratinocytes and melanocytes and understanding different mechanisms of pigmentation disorders. Recently, Duval *et al*. developed a pigmented skin model using collagen, such as a dermal matrix that is very representative of normal human skin with melanocytes [116]. They demonstrate that their model has a functional pigmentary system by the presence of melanocytes well-developed with melanosomes, the expression of tyrosinase, TRP1 and TRP2, the transfer of melanosomes containing melanin to keratinocytes and the stimulation of melanin synthesis by α-MSH. This model has most of the normal human skin melanocyte characteristics [9] and seems to be an interesting pigmented skin model for studying cell interactions of the pigmentary system. A study with this type of skin model has observed the response of melanocytes after UV radiation [117]. Archambault *et al*. demonstrated by a comparative study of monolayer

melanocyte culture and pigmented skin equivalent that melanocytes have a better capacity for surviving UVR than melanocytes in culture. These results suggest that keratinocytes and fibroblasts secrete factors that enhance melanocytes survival and migration, which could explain UVR-induced pigmentation by melanocytes. Unlike other teams, Freeman *et al*. elaborated another technique of culture with collagen gels [118]. They put a complete skin biopsy, which was affected by a melanoma, onto a collagen gel that contained fibroblasts. This technique allowed conservation of the *in vivo* properties of the melanoma and observation of the proliferation and other characteristics *in vitro*. All of these models composed with a collagen matrix are representative of human skin, and are effective for the study of pigmentation disorders. However, collagen models may not be useful for testing drugs, because they can be absorbed by the collagen and a higher quantity of drugs must be used [119].

3.2.3. De-Epidermized Dermis (DED)

Such as collagen gels, DED allowed the construction of pigmented skin equivalent that reproduced human skin for studying the pigmentary system. Unlike collagen gels, this method has a native extracellular matrix and a basal membrane that facilitates melanocyte adhesion. Principally, DED preparation is very similar. From a skin biopsy, the epidermis is removed and the dermis is incubated in saline solution or undergoes freezing-thawing cycles to kill cells [120]. After this treatment, the dermis remaining is called a dead de-epidermized dermis, and is ready to be seeded by keratinocytes and melanocytes. In 1993, Todd *et al*. used this model to demonstrate the effect of UV radiation on a pigmented equivalent [121]. They demonstrated that, after UV radiation, there is an increase of TRP1 activity, an increase of pigmentation and an increase of DOPA-positive melanocytes such as observed *in vivo*. In 2000, for a better understanding of melanoma invasion, Dekker *et al*. developed and characterized a DED skin equivalent with four types of melanoma cells [122]. They observed the expression of different integrins that play an important role in the behavior of melanoma cells. Their model is a useful tool for studying melanoma and other mechanisms involved in this cancer. In 2007, Cario-André *et al*. developed a DED skin model with normal and non-lesional vitiligo cells to understand if the loss of melanocytes in vitiligo is caused by a detachment of melanocytes due to stress factors [123]. The model with non-lesional vitiligo cells contains less melanocytes than the model with normal cells, and hydrogen peroxide and epinephrine could be the cause of the detachment of melanocytes. Todd, Dekker and Cario-André models demonstrated that de-epidermized dermis is an interesting model for studying different pigmentation disorders and, in comparison with collagen gels, DED produced an epidermis, which is closer to the native epidermis than collagen gels [124].

3.2.4. Commercial models

Commercial *in vitro* skin models have been developed to reduce tests on animals, replace animal models and refine methodologies. For the cosmetic industry, it allows testing of the toxicity of their products and their pharmaceutical effects on a complete human epidermis and dermis. Several companies produce skin models and some of them produce equally pigmented skin models that are useful for testing photoprotection, whitening agents and repigmentation

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products. The list of the main commercial pigmented skin models and their principal features is presented in Table 3.

Models	Type of models	Epidermis	Epidermis + dermis	Different degree of pigmentation	References
SkinEthic™	Cells seeded on a polycarbonate filter	x		x	[125]
MelanoDerm™	Cells seeded on a collagen gels	x		x	[126]
StratiCell™	Cells seeded on a polycarbonate filter	x		x (only 2)	
Melanoma skin model™	Cells seeded on a collagen gels		x		[127]

Table 3. Different commercial pigmented skin models

4. Conclusion

Melanocytes are underestimated cells that are more than pigmentation cells. They protect the skin against environmental factors and they are necessary for sight and hearing. However, melanocytes can also be the cause of some skin disorders and cancer. Even more mechanisms involved in pigmentation disorders remain unknown and need to be elucidated upon in order to give affected people a better quality of life. *In vitro* pigmented skin substitutes produced by tissue engineering and *in vivo* models are useful tools for understanding these mechanisms and developing appropriated treatments or drugs.

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