Repigmentation through Melanocyte Regeneration in Vitiligo

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KEYWORDS

• Vitiligo • Repigmentation • Melanocyte stem cell • Bulge • Hair follicle • Proliferation • Migration • Differentiation

KEY POINTS

- Repigmentation is an active process during epidermal crisis reversing the loss of epidermal melanocytes.
- It usually develops in hair-bearing areas.
- The most common clinical presentation is the perifollicular pattern.
- The initiating event is the activation of melanocyte precursors from the hair follicle and of immature melanocytes from the basal epidermis.
- It is induced by different stimuli: UV light, drugs (steroids, calcineurin inhibitors).

INTRODUCTION

The loss of epidermal mature melanocytes in vitiligo depends on melanocyte-specific CD8+ cytotoxic T lymphocytes. It is reversed by halting the immune attack and by activating melanocyte precursors in the bulge and hair follicle infundibulum, to proliferate, migrate, and differentiate through the process called repigmentation.[1–4] Although repigmentation refers to the replenishment of pigment cells only, keratinocytes in vitiligo skin demonstrate architectural abnormalities and are also likely to be directly involved in repigmentation. Changes of the keratinocytes architecture seem to appear in the absence of basal melanocytes, in the sun-exposed skin. Therefore, significant increase in thickness of both stratum corneum and viable epidermis in vitiligo-depigmented skin, as compared with the adjacent, normal-appearing skin, was reported.[5] This increase likely occurs as an adaptive response to lack of melanin that can minimize and counteract the harmful UV effects on the skin.

Based on current knowledge, vitiligo repigmentation depends on available melanocytes from 2 sources:

- The hair follicle, which is the main source of pigment cells and is often unaffected by the T cell–mediated attack, likely because the hair follicle bulge is an immune privileged location[6]
- The epidermis at the lesional borders, which contains a pool of functional melanocytes and represents a secondary source for repigmentation

Melanocyte activation, followed by migration, proliferation, and differentiation is triggered by several stimuli, such as UV radiation (delivered as treatment or by natural sunlight) and drugs

Disclosure Statement: The authors do not have any commercial or financial conflicts of interest.
This work was supported by an American Skin Association Research Scholar Award in Vitiligo/Pigment Cell Disorders to Stanca Birlea.
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Dermatol Clin 35 (2017) 205–218
http://dx.doi.org/10.1016/j.det.2016.11.015
0733-8635/17/Published by Elsevier Inc.

(systemic and topical steroids and topical calcineurin inhibitors).

CLINICAL PATTERNS OF REPIGMENTATION AND THE REPIGMENTATION SOURCES

There are 4 classic repigmentation patterns observed on clinical examination[7–9]: perifollicular (most common) (**Fig. 1**A-i), marginal (see **Fig. 1**A-ii),[9] diffuse (see **Fig. 1**A-iv),[7] and combined, which includes more than one pattern (an example of marginal combined with perifollicular pattern is provided in **Fig. 1**A-v).[9] A fifth newly described repigmentation pattern, the medium-spotted patern,[10] is presented in **Fig. 1**A-vi.[11]

The perifollicular pattern presents as small, round, pigmented macules around the hair follicles (see **Fig. 1**A-i). This clinical observation was confirmed by numerous previous *in vivo* studies[12–16] that identified amelanotic, inactive, 3,4-dihydroxy-L-phenylalanine (DOPA)(−) melanocytes in the infundibulum outer root sheath of hair follicles collected from healthy individuals[12,13,16,17] or from vitiligo patients.[14,15,18] The origin of DOPA(−) melanocytes was later identified as the hair follicle bulge (both in the transgenic mouse model[19] and then in human skin).[20] From this location melanocyte precursors ascend to repopulate the depigmented epidermis (**Fig. 1**B, right side) in the ultraviolet radiation (UVR)-treated vitiligo. Activation by UV treatment or ionizing radiation induces these precursors to migrate, proliferate, and differentiate, finally expressing the full pigmentation pathway in the interfollicular

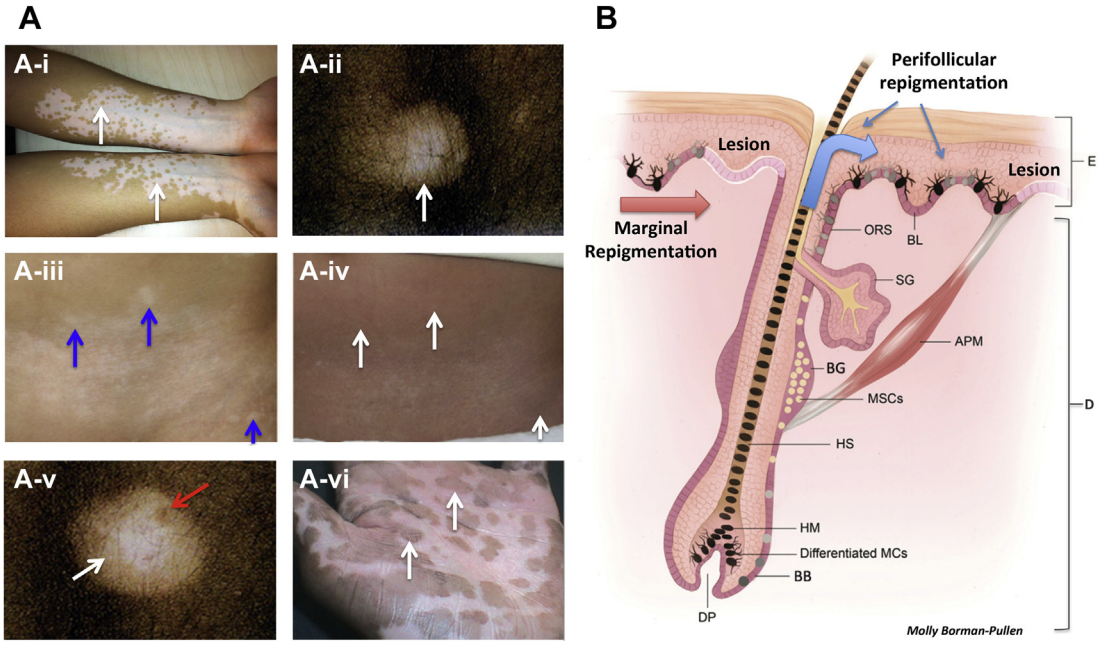


Fig. 1. (*A*) Clinical patterns of repigmentation. (*A-i*) Perifollicular pattern-multiple black dots of pigment are seen around the hair follicles (*white arrows*) in a patient with vitiligo treated with narrow band UVB (NBUVB). (*A-ii*) Marginal repigmentation pattern presented as a pigmented rim at the borders of the lesions (*white arrow*). (*A-iii*) Depigmented spots (*blue arrows*) treated 12 weeks with NBUVB repigment the skin following a diffuse repigmentation pattern, as indicated in (*A-iv*) by white arrows. (*A-v*) Combined repigmentation pattern including marginal pattern (*white arrow*) and perifollicular pattern (*red arrow*). (*A-vi*) Medium-spotted repigmentation pattern[10] in a patient who underwent psoralen plus UVA treatment. Repigmentation of palmar surface presents as round brown macules (*white arrows*). (*B*) Cellular mechanism of perifollicular repigmentation (*right side, blue arrows*) and marginal repigmentation (*left side, red arrow*) in human vitiligo. APM, arrector pili muscle; BB, bulb; BG, bulge; BL, basal layer; D, dermis; DP, dermal papilla; E, epidermis; HM, hair matrix; HS, hair shaft; MCs, melanocytes; MSCs, melanocyte stem cells; ORS, outer root sheath; SG, sebaceous gland. ([*A-i, ii, v*] *From* Gan EY, Gahat T, Cario-André M, et al. Clinical repigmentation patterns in paediatric vitiligo. Br J Dermatol 2016;175:555–60, with permission; and [*A-iii, iv*] Yang YS, Cho HR, Ryou JH, et al. Clinical study of repigmentation patterns with either narrow-band ultraviolet B (NBUVB) or 308 nm excimer laser treatment in Korean vitiligo patients. Int J Dermatol 2010;49(3):317–23, with permission; and [*A-vi*] Davids LM, du Toit E, Kidson SH, et al. A rare repigmentation pattern in a vitiligo patient: a clue to an epidermal stem-cell reservoir of melanocytes? Clin Exp Dermatol 2009;34(2):246–8, with permission; and [*B*] This cartoon was drawn by Molly Borman-Pullen, biomedical illustrator, Fort Collins, CO.)

epidermis.[4,17] Data confirming the follicular reservoir also came from animal and human models of pigmentation, which is revisited in this review.

The marginal pattern is observed as a repigmentation rim at the borders of the lesions[7] (see **Fig. 1**A-ii).[9] It is the result of activation of functional epidermal melanocytes in the lesional borders.[3] Some investigators have proposed an epidermal repigmentation reservoir,[21,22] consisting of more immature melanocyte precursors with supposed migratory, differentiation, and perhaps proliferative abilities (see **Fig. 1**B, left side).

The diffuse repigmentation pattern appears as generalized darkening across the patches of vitiligo[3] (see **Fig. 1**A-iv),[7] whereas, in the combined pattern (see **Fig. 1**A-v),[9] the repigmentation does not fit into any single type or where more than one pattern contributes to the repigmentation process.[7] The diffuse pattern suggests that repigmentation can arise from interfollicular melanocyte precursors either in the dermis or interfollicular epidermis (by reactivation of DOPA(−) melanocytes, which are hypothesized to persist in the center of the lesions).[3]

A very recent study reported in pediatric vitiligo patients a medium-spotted repigmentation pattern, located in non–hair-bearing to minimal hair-bearing sites, such as the palms, soles, lips, ankles, and anterior wrists.[10] This uncommon pattern begins as larger spots that are not centered on any particular hair follicle. Consistent with these findings, a previous study[11] reported a rare pattern of repigmentation on the palms of a patient, consisting of irregular brown macules developed after a course of psoralen plus UVA (PUVA) therapy (see **Fig. 1**A-vi).[11] Tyrosinase (TYR)(+) melanocytes were found along the basement membrane of the repigmenting lesions, in contrast to the adjacent vitiliginous skin, which lacked these cells. Based on observation of repigmentation that occurred in the center of an initially fully depigmented lesion on the palm (a region devoid of hair follicles), the investigators hypothesized that the melanocyte precursors/stem cells can remain in vitiliginous lesions serving as repigmentation reservoir. The pattern seen in this case seems to support medium-spotted repigmentation, recently reported in children with vitiligo.[10]

Other reports identified melanocytes within the depigmented vitiligo,[23–25] and one of them claimed that melanocytes in depigmented epidermis were "never completely absent."[24] The investigators hypothesized that these melanocytes can recover their functionality *in vivo* and *in vitro* on the removal of hydrogen peroxide,[24] which they propose is the major mediator of cytotoxicity.

Interestingly, in some of the frozen epidermal sections of depigmented untreated skin collected from vitiligo patients of skin types II and III, the authors' group observed large cells, with fragmented bodies, and diminished and abnormal, fragmented dendrites, expressing a Premelanosome protein (PMEL)(+)/Tyrosine kinase receptor (C-KIT)(−) phenotype (**Fig. 2**A). The authors noticed similar aspects in paraffin sections of depigmented skin, in which epidermal cells carried a dopachrome tautomerase (DCT)(+)/C-KIT(−) phenotype (see **Fig. 2**B). These rare cells were amelanotic (Fontana-Masson(−) staining, see **Fig. 2**B) and located not only close to lesional borders but also within the lesions. It was previously proposed[26] that these cells might be residual nonfunctional, senescent cells (progressing toward apoptosis)[26] rather than poorly differentiated melanocyte precursors with potential to form functional melanocytes. Loss of C-KIT expression is an interesting and expected finding, knowing the major implication of stem cell factor (SCF)/c-kit in melanocyte survival and migration.[27] The authors think that acquisition of C-KIT expression is essential in the repigmentation process associated with the migratory, proliferative, and differentiating phenotype of post–stem cell melanoblasts.[4]

Based on indirect evidence of stem cell markers, tissue culture studies, and repigmentation patterns observed in patients with vitiligo, another previous report hypothesized[28] the existence of a pool of extrafollicular melanocyte stem cells in a well-protected area of the dermis with the ability to replace any damaged melanocytes in the basal layer of the epidermis. More recently, a dermal source of melanoblasts (DCT(+)) was identified in the secretory portion of the eccrine sweat glands after skin exposure to ionizing radiation.[29] It seems that the precursors of these cells colonize sweat glands during development, being maintained in an immature, slow-cycling state; they were shown to renew themselves in response to genomic stress (eg, ionizing radiation) having the capacity to provide their differentiating progeny to the epidermis. It has been hypothesized that these melanoblasts can provide an anatomic niche for melanocyte-melanoma precursor cells[29]; however, their implication in regeneration of vitiligo epidermis awaits investigation.

CLINICAL PARTICULARITIES OF REPIGMENTATION AND THEIR CORRELATION WITH CELLULAR AND MOLECULAR CHANGES

The Lag Time Between Initiation of Stimulus (Ultraviolet) and Visible Repigmentation

Repigmentation is unpredictable, not proportional to the magnitude of the lesions, and often cosmetically insufficient.[30] Curiously, in the same patient,

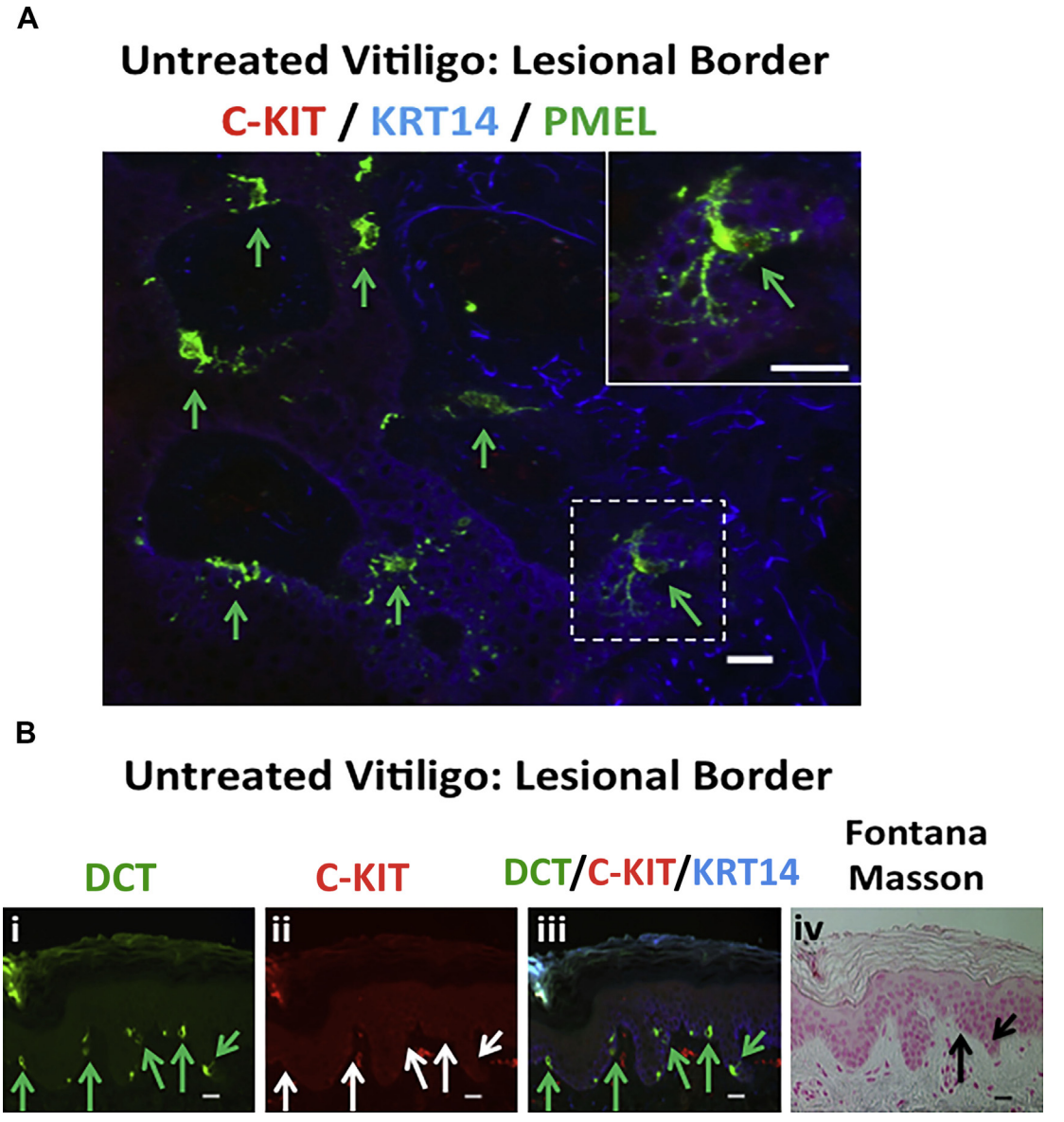


Fig. 2. (*A*) Longitudinal paraffin sections of interfollicular epidermis of depigmented vitiligo (immunostained with combination of anti-human C-KIT [*red*], anti-human K14 [*blue*], and anti-human PMEL [NKI-beteb antibody] [*green*]). NKI-beteb(+)/C-KIT(−) melanocytes (*green arrows*) can be seen with abnormal morphology, consisting of fragmented and distorted bodies, diminished dendricity, and cell debris. White dotted lines indicate area of higher magnification (*inset*). Scale bars = 20 μm. (*B*) Transverse paraffin sections of interfollicular epidermis of depigmented vitiligo (immunostained with combination of [*i*] anti-human DCT [*green*], [*ii*] anti-human C-KIT [*red*], and [*iii*] anti-human K14 [*blue*]). DCT(+) (*green arrows*) and C-KIT(−) (*white arrows*) melanocytes can be seen with abnormal morphology, consisting of fragmented and distorted bodies and diminished dendricity. None of the melanocytes exhibit pigmentation in Fontana-Masson staining (*iv, black arrows*). Scale bars = 20 μm.

vitiligo repigmentation in some regions can commonly parallel active depigmentation of other regions.[1] Neither sex nor skin type are associated with differences in onset or pace of repigmentation following UVR treatment of vitiligo.[4] Patients' clinical response to narrow band UVB (NBUVB) exposure, applied twice weekly, has a lag time ranging between 4 weeks and 4 months, variable between body areas and from patient to patient. This lag time may depend on the integrity of the bulge stem cell reservoir, on the melanocyte precursors susceptibility to activation, and on their migratory and proliferative abilities. If there is a lack of any visible repigmentation after 6 months of treatment,

further therapy is discouraged, in both adult and pediatric patients,[31] and consideration of a different alternative can be advised.

Interestingly, in animal models, the cellular response to UVB seems to appear shortly after exposure. Therefore, in the C57BL/6 mouse, an increase of Tyrosinase protein 1 (TRP1)(+) epidermal melanocytes was first identified on the 5th day after UVB (one exposure of 0.18 J/cm^2 of energy, corresponding to 1.5 minimal erythema dose for the C57BL mice) and reached a cellularity 4 times as great as that of the normal control on the 14th day.[32]

Repigmenting Versus Treatment-Resistant Lesions

- Repigmentation develops best in the hair-bearing regions. Areas with a higher density of hair follicles (face, arms, forearms, thighs, legs, abdomen, back) respond more rapidly to treatment, and those with lower density (dorsum of hands, fingers, feet, and toes) respond more slowly.[1]
- Depigmented areas where hair follicles are absent or in low density (palms, soles, volar wrists, genital sites, mucosal or semimucosal surfaces) rarely respond to treatment; the response, if present, is slow and incomplete. The potential treatment response relies on the epidermal source of melanocytes from the lesional borders (that can migrate ~ 4–5 mm into the depigmented area),[2] on epidermal melanocytes that are hypothesized to persist in the center of the depigmented lesions,[10] or on melanocytes with extrafollicular dermal origin.[28]
- Depigmented areas with white terminal hairs (leukotrichia) are poor responders to medical treatment[33] with minimal chances of repigmentation. Leukotrichia is perhaps an indicator of more severe, long-lasting, and active CD8+ T-cell immune-mediated attack on melanocytes, which progresses gradually downward, from the epidermis to the bulge. Depletion of the bulge melanocyte stem cells leads to exhaustion of secondary hair germ in the bulb, which is composed of melanocytes precursors more committed to melanocyte differentiation.

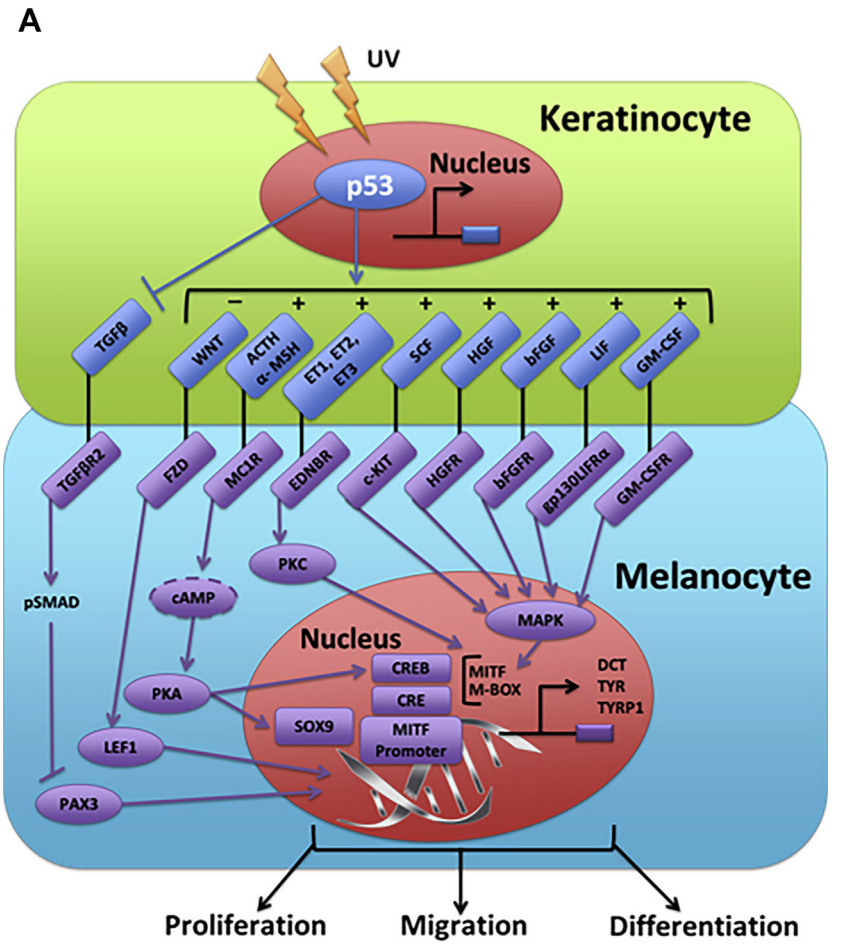
Cellular and Molecular Changes of Repigmented Skin

The sequences of repigmentation process

There has been surprisingly limited research addressing the repigmentation process in vitiligo, despite impressive progress studying the mechanisms of vitiligo immunology[34,35] and genetics.[36] It is known that the UV-induced repigmentation process includes keratinocyte stimulation and melanocyte activation by UV light (**Fig. 3**A)[37,38] melanocyte migration, proliferation, and differentiation (differentiation implies cell melanization). Melanocyte migration involves the following steps: melanocyte decoupling from the basement membrane and from keratinocytes, cell movement, and recoupling to the basement membrane and to keratinocytes. The intimate melanocyte-keratinocyte anatomic and functional interactions in the hair follicle and epidermis are essential for epidermal repopulation in vitiligo. However, how these processes develop during repigmentation and the triggers that initiate one process in favor of the other have not yet been identified.

There are 2 major pathways, p53 and Wnt/β-catenin, that strongly activate the pigmentation process. It has been clearly shown that cellular and DNA damage[39,40] by UVR induces p53 activation. In normal skin repeatedly exposed to UVR, p53 orchestrates melanocyte activation by coordinating the release of keratinocyte-paracrine/growth factors with melanogenic activity, which induces microphthalmia-associated transcription factor (MITF) expression in melanocytes[37,41] (see **Fig. 3**A).[37,38] Similarly, in vitiligo, repigmentation occurs following melanocyte activation by NBUVB orchestrated by p53 and its downstream effector, α-melanocyte stimulating hormone, which is a potent inducer of MITF.

The Wnt/β-catenin signaling pathway has also been implicated in the pigmentation process. UVB irradiation of the mouse F1 of HR-1 × HR/De induced robust expression of Wnt7a and subsequent β-catenin translocation into the nucleus in the melanocyte stem cells[42] (the model is presented in the *Transgenic mouse models* section). In the same model, intradermal injection of inhibitor of Wnt response 1(IWR-1) (a chemical inhibitor of β-catenin activation), and small interfering RNA against Wnt7a inhibited the proliferation of epidermal melanocytes. It was demonstrated that Wnt7a triggered melanocyte stem cell differentiation through β-catenin activation.[42] The Wnt/β-catenin pathway seemed to be modulated by the UVB treatment in the mouse model, but the effects of UVB on the Wnt/β-catenin pathway on melanocytes and keratinocytes in the bulge of human vitiligo skin await to be studied. Wnt signaling is essentially required for melanocyte development; its activation results in stabilization of β-catenin/lymphoid enhancer factor (Lef) complex, which leads to transactivation of downstream target genes, such as *Mitf*, to promote melanocyte-fate specification and melanocyte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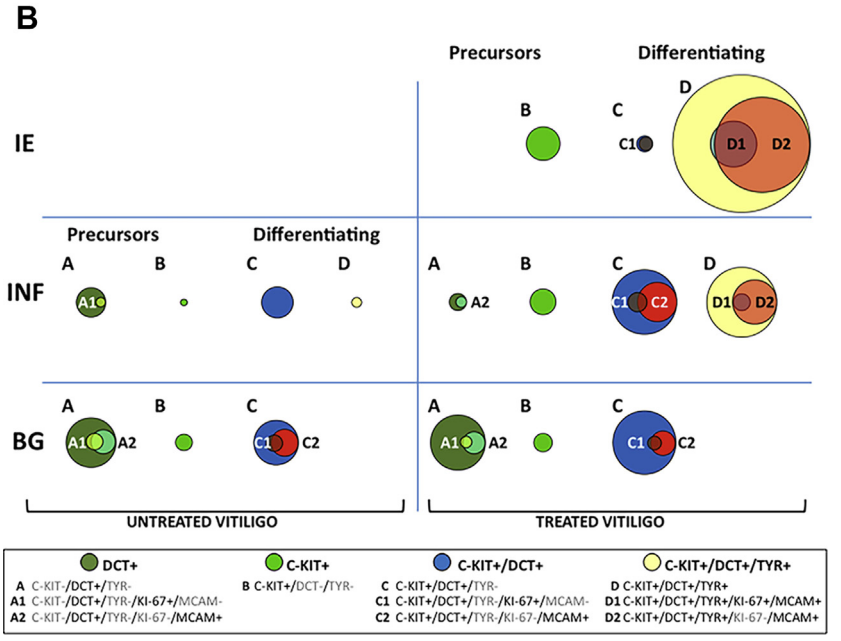
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differentiation.[43] Moreover, epidermal Wnt controls hair follicle induction by orchestrating dynamic signaling crosstalk between the epidermis and dermis.[44] Epithelial Wnt/β-catenin signaling is required for hair matrix cell proliferation, and β-catenin is required in bulge stem cells and also for epidermal proliferation.[45]

More recently, transcriptome analysis on lesional, perilesional, and nondepigmented skin from patients with vitiligo and from matched skin of healthy subjects identified decreased Wnt activation in depigmented vitiligo skin.[46] In addition, using *ex vivo* explants of depigmented human vitiligo skin, the authors found that treatment with Wnt agonists or glycogen synthase kinase 3 β (GSK3β) inhibitors induce increased expression of melanocyte-specific markers, triggering the differentiation of resident melanocyte stem cells in pre-melanocytes expressing paired box protein-3 (PAX3) and DCT. All these findings raise the possibility that Wnt normalization is important for the repigmentation process.

p53 seems to crosstalk with β-catenin in several cell lines, including the hair follicle stem cells. It has been reported that β-catenin and pygopus homolog 2 (Pygo2) (the latter with regulatory roles on Wnt/β-catenin target genes) converge to induce p53 in cultured keratinocytes and in cycling hair follicles. These findings identify Pygo2 as an important regulator of Wnt/β-catenin function in skin epithelia and p53 activation as a prominent downstream event of β-catenin/Pygo2 action in stem cell activation.[47] Whether p53-β-catenin crosstalk intervenes during the repigmentation process awaits further study.

Human and animal models to study repigmentation

There are several mouse models and few human models used to study repigmentation, which the authors summarize later. They have revealed valuable information about the sequence of melanocyte proliferation, migration and differentiation.

Transgenic mouse models

SLFTg1-1 mice injected with anti-c-Kit monoclonal antibody ACK2 Investigation using this model has revealed the melanocyte precursors in the hair follicle, their migratory capacity, and participation to the repigmentation process.[48] This model is a non-vitiligo repigmentation model, using the *SLFTg1-1* humanized mouse, expressing steel factor (SLF) in the basal layer, under control of the Keratin *(Krt)14* promoter. Treatment with the anti–c-Kit (ACK2) antibody eliminated the c-Kit(+) melanoblasts in the hair follicle and induced complete skin and hair depigmentation. In the next hair cycles, perifollicular repigmentation occurred, based on a population of residual melanocyte stem cells Dct(+)/c-Kit(−) maintained in the hair follicle, which showed migratory abilities.

K14-SLF/+; Dct-lacZ/+ transgenic mouse model The experiments done on this model identified the melanocyte precursors in the bulge, their

Fig. 3. Summary of the UV effects in normal and vitiligo skin. (*A*) UV-induced pigmentation pathways in normal skin. p53 is stimulated by UV light and induces the release of melanogenic paracrine growth factors and cytokines by keratinocytes. The keratinocyte factors further interact with their corresponding receptors on melanocytes and induce melanocyte activation, with subsequent stimulation of microphthalmia-associated transcription factor (MITF) and its downstream targets, the melanogenic enzymes TYR, tyrosinase-related protein 1 (TYRP1), and DCT, leading to synthesis of melanin and melanocyte differentiation. (*B*) Melanocyte precursors and more differentiated phenotypes in treated and untreated vitiligo. The precursors and more differentiated phenotypes are represented with different colors in the bulge (BG), infundibulum (INF), and interfollicular epidermis (IE). The relative diameter of each circle in each study region represents the estimated percent of melanocytes exhibiting particular phenotypes in each study region, normalized to the average number of melanocytes in each region. ACTH, adrenocorticotropic hormone; Alpha-MSH, alpha-melanocyte-stimulating hormone; bFGF, basic fibroblastic growth factor; bFGFR, basic fibroblastic growth factor receptor; cAMP, cyclic adenosine monophosphate; c-KIT, tyrosine kinase receptor; CREB, cAMP response element-binding protein; CRE, cAMP response element; EDNBR, endothelin receptor beta; ET, Endothelin; FZD, frizzled; GM-CSF, granulocyte-macrophage colony-stimulating factor; gp130 LIFR-alpha, leukocyte inhibitory factor receptor-alpha; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; HGF, hepatocyte growth factor; HGFR, hepatocyte growth factor receptor; LEF1, lymphoid enhancer binding factor 1; LIF, leukocyte inhibitory factor; MAPK, mitogen activated protein kinase; MC1R, melanocortin receptor 1; MSH, melanocyte-stimulating hormone; PAX3, paired box 3; PKC, protein kinase C; SCF, stem cell factor; SOX9, SRY-Box9; TGFb, transforming growth factor; TGFbR2, transforming growth factor b receptor 2; TGF-β, transforming growth factor-β. ([*A*] *Adapted from* Costin GE, Hearing VJ. Human skin pigmentation: melanocytes modulate skin color in response to stress. FASEB J 2007;21(4):976–94; and Hirobe T. How are proliferation and differentiation of melanocytes regulated? Pigment Cell Melanoma Res 2011;24(3):462–78, with permission; and [*B*] *From* Goldstein NB, Koster MI, Hoaglin LG, et al. Narrow band ultraviolet B treatment for human vitiligo is associated with proliferation, migration, and differentiation of melanocyte precursors. J Invest Dermatol 2015;135(8):2068–76, with permission.)

migratory capacity under SLF stimulation, and their differentiation ability.[19] This model is a non-vitiligo repigmentation model that, in addition to the *SLFTg1-1* mutation,[48] expresses the lacZ reporter gene under the control of the Dct promoter. Histologic examination of this mouse skin demonstrated that the progeny of surviving melanoblasts in the bulge could migrate upward to the epidermis in the presence of SLF, indicating that bulge stem cells are the source of melanocytes in the epidermis. Increasing numbers of lacZ+ cells appeared in the epidermis as pigmented spots in a concentric pattern around the terminal hairs.

F1 hairless HR-1 x HR/De mouse model The experiments done on this model reveal the differentiation ability of melanocyte precursors in the bulge, their ability to migrate along the hair follicle outer root sheath, and also to repigment the interfollicular epidermis following UVB stimulation.[42] This model is also a non-vitiligo model, in which homozygous mutants (Hr^{hr}/Hr^{hr}) show normal development of the first hair cycle. They become completely hairless at 3 weeks of age; at 4 weeks of age they become depigmented. In this model, delayed pigmented spots are induced long after UV irradiation. Following UVB exposure, the melanocyte precursors were observed to proliferate in the bulge and differentiate to melanoblasts that migrated to the epidermis and became melanotic cells.

Dct-LacZ+ mouse model The experiments done on this model revealed the migratory ability of bulge melanocyte precursors along the hair follicle outer root sheath, achieving proliferative and differentiation ability only after they reached interfollicular epidermis.[49] This model is a non-vitiligo transgenic mouse model, expressing the lacZ reporter gene under the control of the *Dct* promoter. Following exposure to UVB or on induction of a wound on the back of the mouse, melanocyte stem cells were shown to exit the bulge and migrate along the outer root sheath infundibulum without proliferation; they proliferated and differentiated in the epidermis.

$Mc1r^{e/e}$ transgenic mouse model The experiments done on this model revealed the migratory ability of melanocyte precursors along the hair follicle outer root sheath under melanocortin 1 receptor (Mc1r) stimulation.[49] This mouse is a non-vitiligo, non-humanized mouse expressing a non-functional Mc1r. After wounding on the back of $Mc1r^{e/e}$ mice, a lower number of epidermal melanocytes were noticed as compared with their control littermate, $Mc1r^{+/+}$ mice; the difference in melanocyte number in the epidermis was caused by impaired melanocyte migration from the bulge to the interfollicular epidermis, which was attributed to the lack of Mc1r function in the mouse expressing the defective gene.

Human models of repigmentation

Human vitiligo model using punch grafts Punch grafts were performed on depigmented vitiligo lesions, and then they were exposed to khellin and UV light.[50] Immunostaining experiments revealed the migratory capacity of melanocytes (horizontal migration to depigmented areas).

Human vitiligo model using punch biopsies The experiments done on this model revealed the proliferative, migratory, and differentiation ability of melanocyte precursors in both hair follicle and interfollicular epidermis.[4] Skin biopsies taken from patients with untreated vitiligo and from patients treated with NBUVB for 3 and 6 months were immunostained with melanocyte markers (DCT, C-KIT, TYR, or PAX3), markers of proliferation (KI-67), and/or of migration (melanoma cell adhesion molecule [MCAM]), and a keratinocyte specific marker (K14). NBUVB was associated with a significant increase in the number of melanocytes in the infundibulum and with restoration of the normal melanocyte population in the epidermis (see **Fig. 3**B).[4]

Repigmented narrow band ultraviolet treated–skin versus normal skin and untreated vitiligo skin

Using immunostaininig techniques coupled with collection of skin biopsies from patients with vitiligo, the authors showed that in the hair follicle bulge, NBUVB treatment stimulated a slight increase of 2 populations of immature melanocytes, a stem cell population C-KIT(−)/DCT(+), and a melanoblast population C-KIT(+)/DCT(+) (see **Fig. 3**B).[4] The targeted immature melanocyte in the hair follicle bulge and infundibulum of untreated vitiligo contained only amelanotic melanocytes (ie, they expressed the melanocyte markers DCT and/or C-KIT and/or PAX3, but they were TYR(−) and Fontana-Masson(−)); these immature populations remained amelanotic in the bulge after 3 to 6 months of NBUVB treatment. Fontana-Masson(+) cells and TYR(+) cells were seen only in the upper infundibulum and epidermis after treatment. NBUVB treatment was associated with significantly increased expression of melanocyte markers in the vitiligo treated skin, the most striking contrast being observed between the untreated depigmented epidermis (devoid of melanocytes) and the treated pigmented epidermis, which was heavily DCT(+), C-KIT(+), PAX3(+), TYR(+), and strongly Fontana-Masson(+).

Melanocyte proliferation after NBUVB was indirectly supported by the observation of increased melanocyte numbers in all regions tested and was directly quantified by KI-67 coexpression with C-KIT, DCT, and TYR. The authors identified a presumed migratory population of melanocytes (DCT(+)/MCAM(+)) that was minimally expressed in the bulge but showed increased expression in the infundibulum and epidermis. The melanocyte precursors showed differentiation abilities in the upper infundibulum by gradually exhibiting TYR expression, a process that paralleled proliferation and migration and that continued in the epidermis (population TYR(+)/MCAM(+)/KI-67(+)). The authors found no significant difference in melanocyte marker expression between NBUVB-treated vitiligo skin and normal skin, which suggested that NBUVB exposure for 3 to 6 months returns depigmented skin to a normal status in respect to pigmentation.

REPIGMENTATION INDUCED BY ULTRAVIOLET LIGHT AND BY DRUGS

The key principle of vitiligo therapy is to stabilize depigmentation (by halting the immune response) and to stimulate the melanocyte precursors to repigment the skin.[1] Most treatment alternatives (UV, steroids, calcineurin inhibitors) seem to act on both steps, although at present, it is not clearly understood to what extent these alternatives must suppress the autoimmune process versus stimulate melanocyte repopulation of the epidermis to provide maximum efficacy.

The clinical observation that pigmented terminal hairs are present within depigmented spots of most patients with vitiligo suggested that the human bulge melanocyte precursors are preserved in the depigmented skin. These bulge precursors constitute a source for bulb secondary hair germ to provide immature melanocytes for normal hair shaft pigmentation but also for epidermal regeneration (perifollicular repigmentation). The authors' immunostaininig study of vitiligo depigmented skin showed that the melanocyte precursors in the hair follicle bulge are present (**Fig. 4**A), in similar proportions with the normal skin[4] ready to be activated by UV or drugs. These precursors consist of melanocyte stem cells (DCT(−)/C-KIT(−)) and melanoblasts (DCT(−)/C-KIT(−)).[4]

The authors discuss later the repigmentation outcome of few meta-analyses of vitiligo treatment presented in the literature and of reviews of literature data. The most recent meta-analysis, including 4512 participants,[51] reflected the need of new methodology to assess permanence of repigmentation as well as the need for better designed studies: high-quality randomized trials using standardized measures and also addressing quality of life. None of the studies were able to demonstrate long-term benefits.

Repigmentation and Ultraviolet Light

The strongest stimulator of melanocyte precursors is UVR (delivered mainly as NBUVB light, broad band UVB [BBUVB] light, or PUVA). The deep penetration of NBUVB to the level of the human hair follicle bulge was indirectly suggested by the authors' immunostaining studies.[52] The authors found increased expression of DNA damage markers in the bulge keratinocyte stem cells of skin exposed for 3 months to twice-weekly NBUVB treatment (**Fig. 4**B-i), as compared with the bulge of skin unexposed to UV (see **Fig. 4**B-ii).

An earlier meta-analysis of all vitiligo therapies showed that the highest mean repigmentation rate was achieved with NBUVB, BBUVB, and PUVA.[53] NBUVB therapy was considered the most effective and safest alternative for generalized and localized vitiligo. The most recent meta-analysis of vitiligo therapies showed that marked repigmentation (of >75%) appeared more often in the NBUVB-treated patients as compared PUVA-treated patients.[51] The repigmentation induced by NBUVB was also reported to be more stable than that induced by PUVA.[54]

In a review of published clinical studies about excimer laser in vitiligo, the repigmentation induced by the UVB-excimer laser was described as marked and relatively fast ($\leq$15 weeks in most studies analyzed).[55]

Repigmentation and Steroids

The role of steroids on pigmentation is mainly informed by clinical studies. Melasma, commonly seen on the face during pregnancy (a state accompanied by high levels of the sex steroid hormones like estrogens and progesterone often coexist with increased pigmentation in other areas [areolae, linea alba, and perineal skin]. Pigmentation of all these areas fades following parturition.[56] This clinical observation suggests that steroids stimulate melanocyte differentiation. Oral contraceptives containing estrogens can also result in hyperpigmentation of the face; ointments containing estrogens can produce intense pigmentation of the genitals, mammary areola, and linea alba of the abdomen in male and female infants.[56] Moreover, experimental data support the hypothesis that a decrease in the antibody-mediated cytotoxicity against melanocytes in patients with vitiligo treated with systemic steroids improves depigmentation.[57] The most recent meta-analysis of

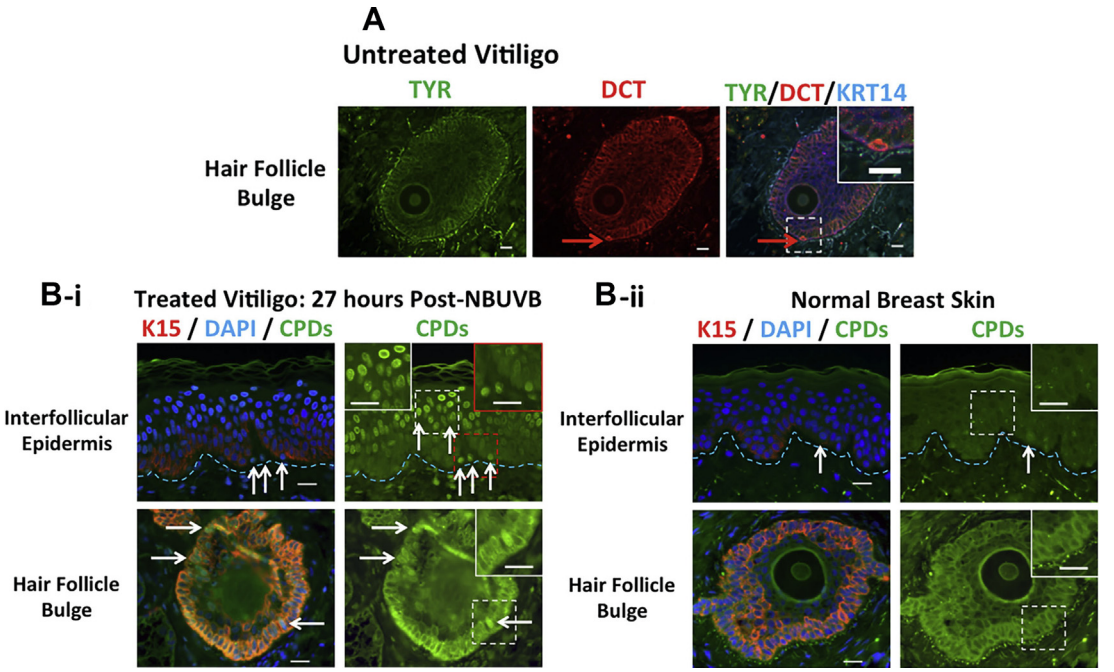

Fig. 4. (*A*) Triple fluorescent immunostaining of untreated vitiligo skin, showing absence of TYR expression in the hair follicle bulge (TYR being a marker of differentiated melanocytes, *green channel*). An immature, DCT(+) melanocyte (*red arrows*) with very low TYR expression can be seen in the hair follicle bulge outer root sheath. (*B-i*) Transverse paraffin sections of bulge of NBUVB-treated human vitiligo (immunostained with combination of anti-chicken K15 [*red*], anti-human cyclobutan pyrimidine dimers (CPDs) [*green*], and DAPI ([*blue*]). CPDs staining showed that CPD(+) cells were mainly located in the interfollicular epidermis (*green cells*, *white arrows*) 27 hours after UV exposure in an Ultralite Phototherapy Chamber source (NBUVB phototherapy lamps 311 nm). Sporadic CPD(+) cells, both K15(+) or K15(−) cells, are also seen in the hair follicle bulge (*white arrows*). (*B-ii*) Normal breast skin showing very few CPD(+) cells in the interfollicular epidermis (*green cells*, *white arrow*) and no visible staining in the hair follicle bulge. Blue dotted lines highlight epidermal boundary. White dotted lines indicate area of higher magnification (*inset*). The bulge regions were mapped by K15 staining in consecutive sections (*not shown*). Scale bars = 20 μm.

vitiligo therapy[51] showed that repigmentation induced by:

- Topical corticosteroids were better than that induced by PUVA sol.
- Topical hydrocortisone plus laser light was better than that induced by laser light alone.
- Oral mini-pulse of prednisolone (OMP) plus NBUVB was better than that induced by OMP alone.
- Topical clobetasol propionate was better than that induced by PUVA sol.

An earlier meta-analysis of nonsurgical vitiligo therapies[53] showed that among randomized controlled trials on localized vitiligo, the repigmentation with topical class 2 corticosteroids was highly significant as compared with placebo; they were considered, together with NBUVB, the most effective and safest treatment of generalized and localized vitiligo. The highest mean success repigmentation rates in the patients' series were produced by topical classes 1 and 2 corticosteroids.

Repigmentation and Calcineurin Inhibitors

Topical calcineurin inhibitors can be effective in vitiligo therapy because of their ability to restore the altered cytokine network. Tacrolimus has been shown to inhibit T-cell activation by downregulating transcription of genes encoding pro-inflammatory cytokines interleukin (IL)-2, IL-3, IL-4, IL-5, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor in T cells.[58] In addition, direct effects of tacrolimus on melanocyte migration[59] and differentiation[60] during repigmentation have been reported, although the roles on cell growth/proliferation remain controversial.[59,61]

A recent meta-analysis of the effect of topical calcineurin inhibitors (tacrolimus, pimecrolimus) in vitiligo[62] showed that:

- Repigmentation with calcineurin inhibitors is significantly higher than with placebo.
- Repigmentation with calcineurin inhibitors is significantly higher when these compounds are combined with NBUVB as compared with their use as monotherapy.

In addition, an earlier review of literature data on calcineurin inhibitors in vitiligo[63] showed that:

- Repigmentation obtained in a double-blind study with combination tacrolimus 0.1% ointment and excimer laser is superior to placebo, especially for UV-resistant areas (eg, bony prominences).
- Repigmentation obtained with tacrolimus 0.1% ointment monotherapy is almost as effective as clobetasol propionate 0.05% ointment.

Repigmentation and Vitamin D Analogues

Topical vitamin D analogues could restore pigmentation in vitiligo by inducing skin immunosuppression, which halts the local autoimmune process, and via direct activation of melanocytic precursors and melanogenic pathways.[64] However, a comprehensive review of the literature[30] reported the lack of consistent evidence to support the stimulatory effect of calcipotriol/tacalcitol monotherapy on vitiligo repigmentation, although at both the cellular and molecular level experimental data suggest a stimulatory effect of vitamin D compounds on human and animal melanocyte pigmentation.[64] The therapeutic effect of vitamin D analogues seems to be obtained in combination with phototherapy[30,51,65] or with topical steroids.[65]

Repigmentation and JAK Inhibitors

JAK inhibitors can be a promising treatment of human vitiligo; besides their anti–IFN-γ effect, they also seem to activate the hair follicle melanocyte stem cells.[66]

However, their effect in inducing repigmentation was reported in a very limited number of studies, and their safety and efficacy need to be explored in depth in the future.

- Good repigmentation on all depigmented areas (more visible on the face) was observed in one case using oral ruxolitinib (given for alopecia areata), until the medication was discontinued.[67]
- Repigmentation of 5% body surface area, described as nearly complete on the forehead and hands, was observed in another vitiligo case (with generalized, progressive disease resistant to topicals or NBUVB), after oral intake of JAK 1/3 inhibitor tofacitinib citrate.[68]

Repigmentation and Afamelanotide

The synthetic analogue of α-melanocyte-stimulating hormone, afamelanotide, is a promising treatment alternative for vitiligo, currently in phase III clinical trials. Repigmentation generated by the combination of afamelanotide and NBUVB has been shown to be superior to that obtained with NBUVB monotherapy and being seen significantly earlier on the face and upper extremities in a significantly higher percent of patients, as compared with NBUVB monotherapy. Repigmentation with combination therapy was significantly superior at day 84 as compared with day 56 for patients of darker skin types (IV–VI), as measured using the Vitiligo Area Scoring Index.[69]

Repigmentation and Simvastatin

The 3-hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitor simvastatin, approved by the Food and Drug Administration for treatment of hypercholesterolemia, was shown to inhibit IFN-γ-induced signal transducer and activator of transcription 1 (STAT1) activation *in vitro*.[70] High-dose simvastatin administrated in a patients with vitiligo with hypercholesterolemia resulted in rapid repigmentation of the skin, supporting simvastatin as a potential therapy for vitiligo.[71] Simvastatin given 3 times per week for 5 weeks in therapeutic doses used for human patients with hypercholesterolemia prevented and reversed depigmentation in the Krt14-Kitl* transgenic mice and reduced the number of CD8+ T cells in the skin.[72] Studies of safety and efficacy of this drug should be explored in depth in the future in vitiligo clinical trials. Its effect in inducing repigmentation in humans, observed only at high doses, suggests its utility as an adjuvant therapeutic alternative.

Repigmentation and Biologics

The effects of anti-TNF-α agents on repigmentation have been studied on a limited number of patients and have shown inconsistent results. Repigmentation following disease stabilization was reported after treatment with etanercept[73,74] or infliximab[75] or with the anti-CD20 monoclonal antibody-rituximab.[76] Repigmentation was not observed in a small number of patients with greater than 5% body surface area affected taking infliximab, or etanercept, or adalimumab,[75] whereas other studies actually reported spreading/onset of vitiligo after taking adalimumab[77–80] or infliximab.[75–79]

SUMMARY

- Both hair follicle and epidermal melanocyte precursors have the ability to proliferate, migrate, and differentiate in NBUVB-treated vitiligo, serving as a source for repigmentation, most commonly resulting in perifollicular and marginal patterns.
- Clinical observation of repigmentation in the glabrous skin, recently described as a medium-spotted pattern, suggests that melanocyte precursors/stem cells can remain in vitiliginous lesions, serving as a repigmentation reservoir. Confirmatory experimental work at the cellular and molecular levels is necessary.
- A reservoir of immature melanocytes was identified in the sweat gland ducts. Nevertheless, the role of an extrafollicular dermal source in vitiligo repigmentation needs further studies for clarification.
- Two signaling pathways, Wnt/β-catenin and p53, have been implicated in NBUVB-induced pigmentation. Further research is essential to identify in detail the cellular and molecular pathways governing the complex repopulation process.
- The treatments summarized earlier can provide acceptable results in hair-bearing areas and are typically unsatisfactory in areas devoid of hair follicles. To improve treatment outcomes in vitiligo repigmentation, we need to design new pharmacologic compounds that provide more robust stimulation of melanocyte precursors in the hair follicle and epidermis.

ACKNOWLEDGMENTS

The authors thank the National Disease Research Institute for providing human skin samples.

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