TECHNOLOGY UPDATE III

CLINUVEL

STEM CELLS AND REPIGMENTATION IN VITILIGO

CELLULAR MESSAGING: RECEPTORS, LIGANDS AND SIGNALING PATHWAYS

Each cell within the body has one or more receptors; molecules on or within the cell to which other molecules, called ligands (such as peptides), bind. This binding causes changes within the cell which normally follow set 'signaling' pathways. Signaling pathways are responsible for cellular responses, allowing other cells and external elements to alter how a cell functions. There are two types of ligands which bind to receptors: agonists, which then promote a response in the pathway, and antagonists, which lead to an alternate response.

Much of modern drug development is based on a simple concept: whether a pharmaceutical, when correctly administered, can activate or inhibit a pathway within cells to achieve a therapeutic benefit.

THE SKIN AND ITS PIGMENT PRODUCING CELLS

The skin is the largest organ in the human body and is exceedingly complex. Multiple cells and layers which comprise the skin serve different functions to help protect and regulate the body. By viewing a cross-section of the top layers of skin (the epidermis and dermis, see Figure 1), one can gain an understanding of the interactions between skin cells and how environmental factors affect the skin. While the complete function of the skin is discussed in greater depth on our website, a brief overview is necessary here.

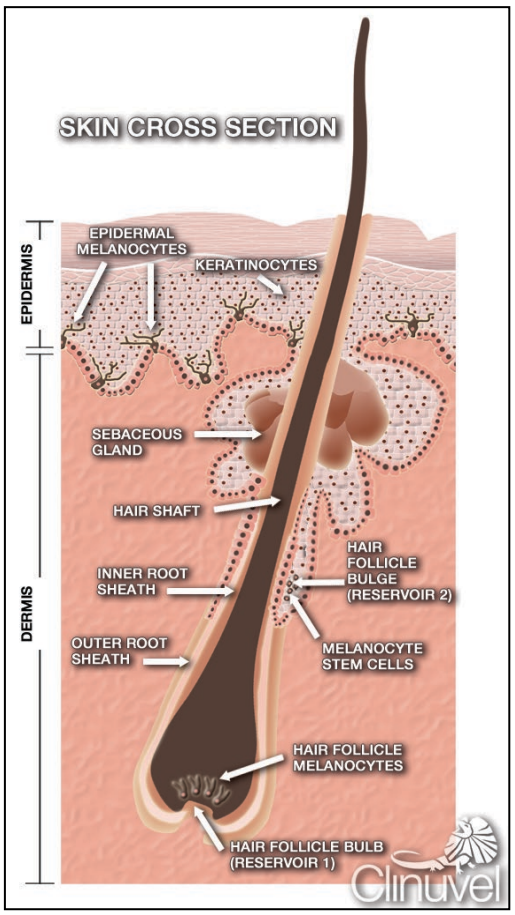


Figure 1. A cross section of human skin

Figure 1 shows a cross section of skin including a hair follicle, approximately 1.2 mm in depth, representing the approximate thickness of skin on an adult's arm or leg skin. Here the main types of skin cells are highlighted, with keratinocytes and melanocytes being the most relevant.

Epidermal melanocytes, cells which produce pigment in the skin, lie at the base of the epidermis, while keratinocytes – both squamous cells and basal cells – make up the majority of the top layer of the skin. The ratio of melanocytes to keratinocytes in healthy skin is 1:36. Keratinocytes turn over quickly in the skin, migrating from the base to the top of the epidermis as they age and 'sloughing off' at the surface; generally this turnover takes 28 days in healthy adults. Melanocytes, by contrast, live for many years but are significantly less able to multiple and renew themselves compared to keratinocytes.

Melanocytes also exist at the root of the hair follicle, in the matrix of the inner root sheath or shaft, and are responsible for giving the hair its colour. The dermis, the layer of the skin beneath the epidermis, consists mostly of collagen, elastic tissue and reticulum fibres, along with some

specialised nerves and glands. The base of the hair follicle is also embedded in this layer. Part of the outer root sheath of the hair follicle forms a bulge, a region called the 'niche'. Contained within the niche are partially differentiated stem cells: immature cells which, given the right conditions, can be activated to develop into several cell types.

Vitiligo is a skin disorder in which the melanocytes within the basal layer of the skin are damaged or completely destroyed. It is this loss of melanocyte function that causes depigmentation of the skin, which generally occurs in patches or 'lesions'.

SIGNALING PATHWAYS IN THE MELANOCYTE

There are four significant signaling pathways that affect melanocyte function within the skin. Each pathway involves a receptor – melanocortin 1 (MC1R), Kit, Frizzled and EdnrB – to which specific ligands bind. When the appropriate ligand binds to a receptor is creates a series of physical and chemical changes within the cell known as a 'signaling cascade' which affects change within the melanocyte and alters its function (see Figure 2). MC1R is the key receptor involved in the signaling pathway which leads to the production of the brown pigment, eumelanin, in the skin (henceforth all references to melanin refer specifically to eumelanin unless otherwise stated).

Receptor	MC1R	Frizzled	EDNRB	Kit (c-Kit)
Ligand	α-MSH	Wnt1/3a	EDN3	Kitl (SCF)
Signal intermediary	cAMP/PKA	β-catenin	PKC	MAPK (RAS RAF MEK 1/2 ERK 1/2)
Transcription factor	CREB	TCF/LEF	PAX3 SOX 10	MITF
	MITF			
Target genes	Differentiation - MITF, Tyr, Tyrp1, Tyrp2, Mart-1, Aim-1, MC1R, Silver, Dct Proliferation - Tbx2, p16, p21, CDK2 Survival/maintenance - Bcl2, Met *PAX3 represses Dct expression			

Figure 2: Melanocyte signaling pathways - Each of these signaling pathways leads to changes in, or growth of, the melanocyte. (Adapted from Hocker, Singh & Tsao 2008 and Hou & Pavan 2008)

THE PRODUCTION OF MELANIN PIGMENT: AN ESSENTIAL ROLE OF ALPHA-MELANOCYTE STIMULATING HORMONE

Melanin is not cohesive, nor is its presentation (shape, size and grouping) across different skin types consistent, yet its role in the skin and method of production has been better understood in recent years.

Melanin is a photoprotective to skin cells (protecting them from light/UV radiation) and the greater its density in the epidermis, the more protection is given to the layers of skin below. It does this by absorbing, reflecting and refracting light (particularly UVR) and preventing it from penetrating to the nucleus of keratinocytes or to lower levels of the skin. Melanin is also believed to play a role in scavenging free radicals, which can injure skin cells, and in facilitating UV induced apoptosis (programmed cell death), which removes damages cells. Thus, melanin's protective role goes beyond providing a physical barrier. It has been suggested that the 2-3 fold melanin levels seen in darker skin types, compared to lighter skin types, convey up to 100-fold difference in sensitivity to ultraviolet radiation due to these protective functions (see Rees 2004).

To produce melanin naturally, the MC1R pathway must be activated by the ligand alpha-Melanocyte Stimulating Hormone (α-MSH) binding to the MC1R on the outside surface of the melanocyte. Approximately 1000 of these receptors exist on each healthy melanocyte.

In the skin α-MSH is expressed by keratinocytes and, less commonly, melanocytes and Langerhans cells as a protective response to damage caused by ultraviolet radiation (UVR, see below). α-MSH molecules then bind with the MC1R on the melanocyte to activate the MC1R pathway and produce melanin. Following this process, melanin granules are deposited in packages called melanosomes which are then transported to the ends of the melanocyte projections, called dendrites (you can see a magnified example of this here). The tips of these dendrites are then enveloped by nearby keratinocytes into which the melanin granules are released. These spread out to form a pigmented, protective barrier over the keratinocyte's nucleus.

In addition to activating melanin, α-MSH is known to have several other roles in the skin, although the exact mechanisms are not fully understood. Recent research has shown that α-MSH enhances the repair of the DNA damage (such as cyclobutane pyrimidine dimers or CPDs) – a process known as nucleotide excision repair or NER – and reduces the generation of free radicals (particularly hydrogen peroxide) following UVR impact. Both of these factors reduce the overall damage caused by UVR; thus reducing the risk factors for certain skin cancers. One mitigating factor, however, is that α-MSH must be able to bind to the MC1R to achieve this function. In some fairer skin types, and individuals with mutations or damage to the MC1R (approximately 70% of the Caucasian population have MC1R mutations), the normal response when α-MSH binds to the MC1R is significantly impaired or absent, reducing the protective response of these individuals. Alpha-MSH is also known to play a role in inhibiting both the expression and activity of pro-inflammatory molecules (cytokines) in skin, meaning that it acts in an anti-inflammatory capacity.

ULTRAVIOLET RADIATION AND ITS EFFECTS ON THE SKIN

Ultraviolet (UV) radiation forms part of the electromagnetic spectrum between visible light and X-rays; it is invisible to the human eye. UV light can be separated into three bands, UVA (400-320nm), UVB (320-290nm) and UVC (290-100nm). Of the UV radiation that reaches the Earth's surface from the sun, approximately 6% is UVB and 94% UVA. Skin maintains a curious balance with UV radiation. When UV impacts upon skin, the various wavelengths of light penetrate to different levels and thus have a number of effects.

The longer wavelength, UVA, penetrates deeply into the skin. Commonly known as the 'aging' ray, UVA breaks down collagen and indirectly contributes to the risk of skin cancer by causing the production of free radicals, which can damage DNA. The shorter wavelength, UVB, is known to directly damage the DNA within skin cells, as well as causing sunburn. UVB also initiates the synthesis of vitamin D in the skin by exciting a precursor molecule. Finally, it is predominantly UVB wavelengths which are responsible for activating the protective melanin response (i.e. the 'tanning response') in the skin; this helps to reduce the impact of subsequent UV exposure. Figure 4, below, summarises the effects of UVA and UVB on skin.

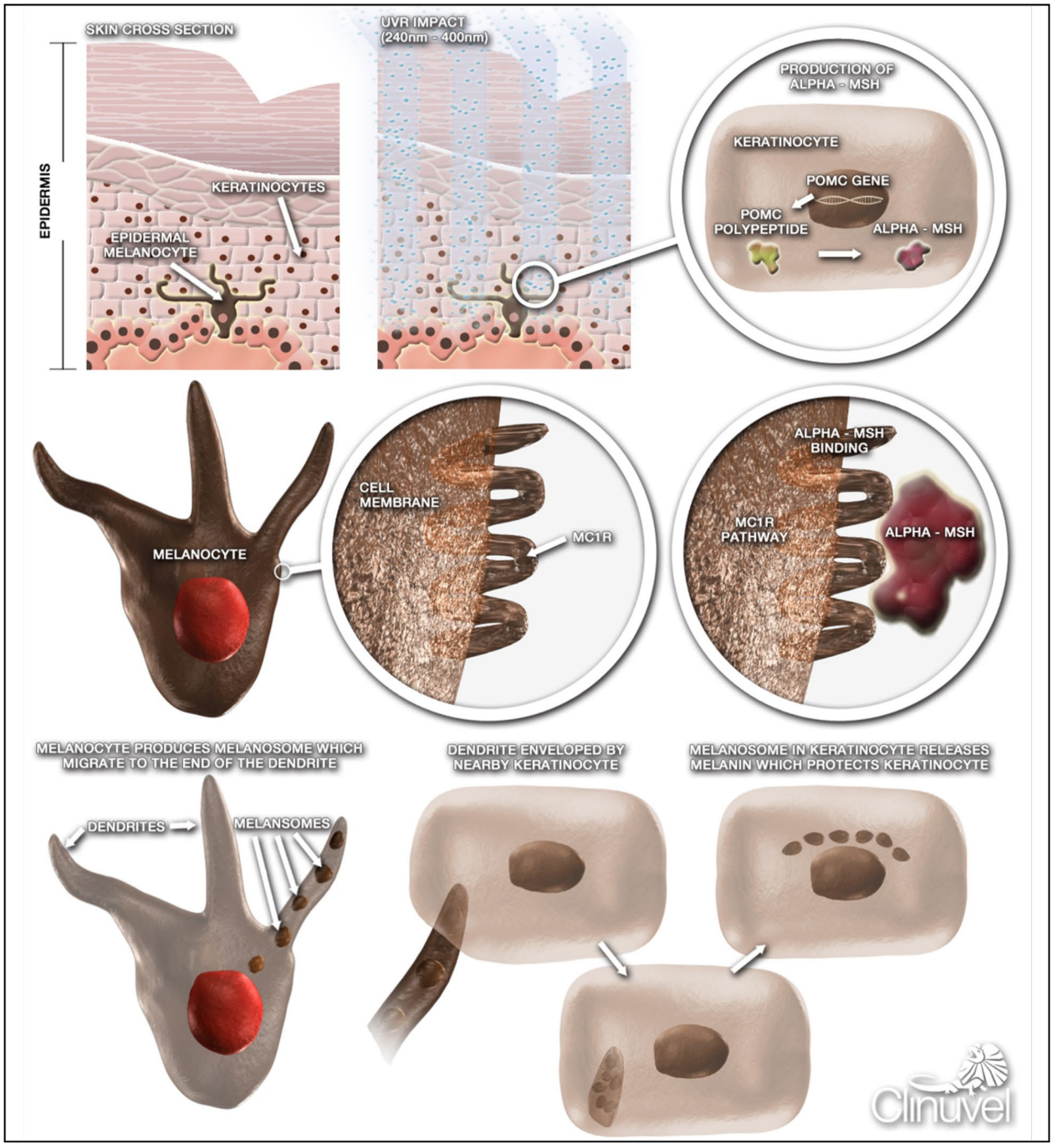


Figure 3. The process of α-MSH mediated melanin production in skin activated by ultraviolet light.

Response	UVA	UVB
Immediate (seconds-minutes)	Targets epidermal and dermal cells, penetrating 1-4mm deep Oxidative damage, inducing Reactive Oxygen Species (ROS)/Free Radicals leading to DNA damage (8-OHdG) Activates fibroblasts to synthesize collegenase I: enzyme responsible for degrading collagen Immediate pigmentary darkening (IPD)* - visible darkening of the skin after minutes of exposure; believed to be the result of redistribution of, and changes to, existing melanin in skin	Targets epidermal cells, penetrating 75-150µm deep DNA damage in melanocytes: • cyclobutane (5-5) • cyclobutane pyrimidine dimers (CPD) • pyrimidine pyrimidinone dimers (6-4 PDs) Localised immunosuppression (direct and indirect): • Expression of immunosuppressive cytokines (IL-4, IL-10) • Converts trans-urocanic acid into cis-urocanic acid (an immunosuppressive) • Inhibits pathways of certain immunomodulatory cytokines (IFN-gamma, IL-2) Excites vitamin D precursor (7-dehydrocholesterol) leading to vitamin D synthesis
	Production of Reactive Oxygen Species (ROS)/Free Radicals leading to DNA damage Activates the p53 protein** in keratinocytes which enhances the expression of POMC, the precursor molecule to α-MSH (minutes-1 hour) Alpha-MSH expression by keratinocytes, melanocytes and Langerhan's cells ACTH expression by keratinocytes Expression of other melanocortins (ACTH) and pro-inflammatory cytokines (IL-1, IL-6, IL-8 TNF-alpha) in the skin by various cells	
Delayed (hours-days)	Persistent pigmentary darkening (PPD)* - darkening of the skin after several hours	Sunburn (2+ hours-7 days): • Appearance of necrotic cells called "sunburn cells" (keratinocytes undergoing apoptosis) • erythema (reddening due to 'superficial vasodilation') • pain • skin flaking and blistering • dryness Apoptosis of melanocytes Delayed pigmentation: the 'tanning response' (2-3 days) – new melanin activation and migration to keratinocytes; visible skin darkening lasting several weeks Thickening of the stratum corneum following a 'hyperproliferation' of keratinocytes
Long term (months-years)	Skin wrinkling (photoaging)	Activation of stem cells in the hair follicle (repeat dose, see below)
	Long term hyperpigmentation or mottled colouring of the skin, commonly known as 'age spots' Accumulated damage leading to precancerous and cancerous skin lesions (actinic keratoses, squamous and basal cell carcinomas) Significant causal link to melanoma skin cancer	
*** These processes are not well understood** ****The p53 protein is a key regulator of the tanning response as well as a tumour suppressor gene (see Cui 2007)**		

Figure 4: The effects of ultraviolet light on skin

STEM CELLS: POTENTIAL FOR REGENERATION

Stem cells are immature, unspecialised cells which have the ability to develop into other adult cells with a specific function, i.e. skin cells, red blood cells, nerve cells (neurons), etc.

Stem cells have two important properties:

1. They are capable renewing themselves indefinitely, i.e. they can divide many times to produce lots of other stem cells.
2. They have the ability to differentiate. This means that they can grow and mature into a specialised cell. Differentiation may involve changes in the cell's size, shape, interaction with their surrounding environment and activity/function. This process requires external stimulation from chemical factors or other cells to activate and regulate the development of stem cells into specialised cells when they are needed.

There are two main types of stem cells; embryonic stem cells and adult stem cells (also known as 'tissue specific' or 'somatic' stem cells). Embryonic stem cells exist in an embryo at approximately 5-7 days and are 'pluripotent'; they have the potential to become any type of body cell, given the correct stimulation. In contrast, adult stem cells inhabit a particular area within adult tissues (i.e. muscle tissue, fat tissue, connective tissue) and can only differentiate into cells of that tissue, termed 'multipotent'. So while still immature and undifferentiated, to a certain extent, their fate is predetermined. A small number of tissue-specific, adult stem cells are positioned in different parts of the body, in regions called niches, early on in development. Adult stem cells can remain dormant, or quiescent (non-dividing), in the niche for many years. They serve as a continual source and origin of new cells to maintain and repair body tissues throughout one's lifetime.

Much recent scientific energy (and indeed media coverage) has focused on the potential of stem cells to regenerate or replace lost and damaged cells in the body. The use of stem cells in this way, to prevent, treat or cure various medical conditions and diseases, is called 'cell-based therapy'. Much controversy has arisen over the use of embryonic stem cells, which are often derived from excess embryos donated by IVF participants. By comparison, adult stem cells – which can often be sourced from different tissues – tend to be less contentious, with their use in the regenerative process proving most exciting and worthy of significant resource investment.

THE DEVELOPMENT OF MELANOCYTE STEM CELLS AND THE ROLE OF UV THERAPY

Based on modeling in animal studies, it is believed that melanocytes in the hair and skin develop early on in the lifecycle of a human embryo. As well as residing in the skin, melanocytes are also present at the base of the hair follicle where they produce the pigment responsible for hair colour. At a later stage, melanocyte stem cells are also formed and deposited within a specific region of the hair follicle. This 'reservoir' of stem cells (which sometimes contains only a single cell) is the aforementioned 'niche' or 'bulge' and is continuous with the epidermis.

Melanocyte stem cells can be activated and, given the correct stimulation, are able to mature into fully functioning melanocytes within the epidermis. Despite significant research efforts in recent years aimed at uncovering the pathways and interactions responsible for the activation and migration of melanocyte stem cells, this process is still not completely understood. What is known is that the expression of several genes, in response to ultraviolet light, leads to the production of various factors which play a critical role in activating stem cells. Initially, the stem cells divide and some begin to mature, forming melanoblasts; intermediate cells which are a precursor to adult

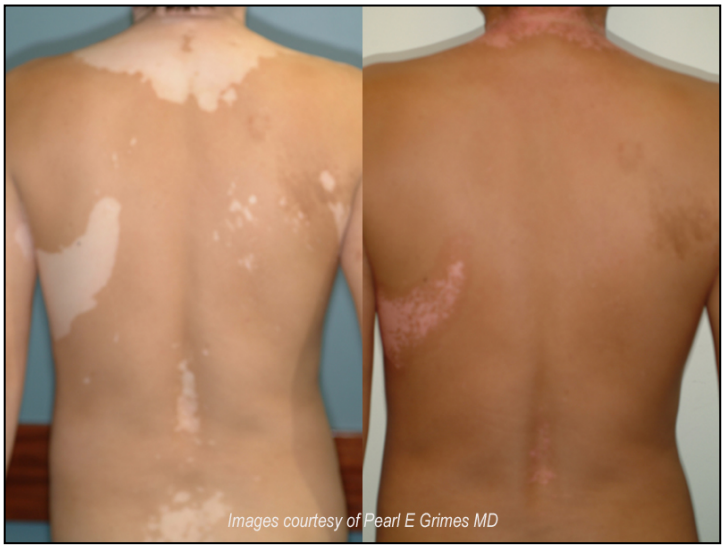
melanocytes. With continued stimulation, melanoblasts further develop and differentiate, migrating to the epidermis where they become fully functioning, pigment producing melanocytes.

Under normal physiologic conditions, the maturation of melanocytes from early stages of human embryonic development into the niche and to fully grown melanocytes in the epidermis is controlled by a variety of signaling pathways. These pathways, including Ednrb and Kit, as well as transcription factors PAX3, SOX10 and Mitf, play diverse roles in the stimulation, development, survival and migration of melanocyte stem cells.

Many of the processes described above are also influenced or enhanced when UV radiation impacts upon the skin. Therefore, in conditions where melanocytes are damaged, or completely lost from the skin, ultraviolet therapies can be employed to aid in their restoration. This is is believed to be the underlying mechanism of action of current ultraviolet treatments in vitiligo patients.

FOLLICULAR REPIGMENTATION IN VITILIGO USING NARROWBAND UVB PHOTOTHERAPY

Clinically, when vitiliginous lesions (sections of skin which have lost their pigment due to vitiligo) are repeatedly exposed to an intensive dose of narrowband UVB radiation (308 or 311-313nm), it is common to see small spots, or 'islands', of repigmentation forming within the lesion. This occurs because the new melanocytes producing the melanin have migrated to the skin surrounding the hair follicle. As the melanocytes continue to migrate and produce melanin, these 'islands' begin to spread and merge, eventually creating broader, but seldom perfect, repigmentation in the treated area (see Figures 5 and 6).



Figures 5 & 6. A vitiligo patient before and after NB-UVB treatment. On the large lesion and the neck of this patient you can clearly see the 'islands' of repigmentation. Images courtesy of Pearl E Grimes, MD.

The follicular repigmentation with NB-UVB therapy takes time (generally 2-3 weekly treatments for up to 18 months), but is believed to work through two distinct mechanisms within the skin. Firstly, UVB radiation activates the stem cells within the hair follicle bulge to mature and migrate into the epidermis. Secondly, UVB radiation impacts upon keratinocytes within the epidermis, stimulating them to produce α-MSH, which in turn binds to receptors on the melanocyte and activates melanin production (see Figure 7).

Unfortunately, this process is not guaranteed to repigment the skin of all patients; approximately 75% of patients see some degree of repigmentation, but the level is not consistent. There is no known time scale involved for repigmentation, nor is there a reliable method to evaluate which patients are suitable for treatment; frustrating factors given the time and financial investment

required to undergo NB-UVB therapy. Further, while the wavelengths of light used in NB-UVB are recognised as being less carcinogenic than broadband UVB radiation, and the risks are considered minimal, the long term effects of this treatment are yet to be fully investigated.

AFAMELANOTIDE: AN ANALOGUE OF A-MSH DESIGNED TO ASSIST IN VITILIGO REPIGMENTATION

Significant advances in the understanding of the factors which influence melanocytes and their stem cells have lead to improved clinical care for patients with vitiligo. The potential of α-MSH and its analogues to further stimulate these developing melanocytes following ultraviolet therapy, to aid in repigmentation of the vitiliginous skin, is an exciting prospect.

Patient responses to NB-UVB are hugely variable. It is impossible to predict whether vitiligo will improve as a result of the treatment, and if so, to what extent. Considerable time and resources are required to reach a clinical conclusion as to whether NB-UVB therapy is effective and there are potential long term risks associated with repeated exposure to UV radiation, a known carcinogen. Thus, there is a clear argument for the exploration of potential combination therapies with NB-UVB which could reduce the number of clinical visits required to achieve repigmentation.

Based on knowledge of the processes involved in repigmenting vitiliginous skin with NB-UVB, there are clear scientific grounds for the combined use of this light therapy with α-MSH analogues. Afamelanotide, the most clinically advanced α-MSH analogue, is thus a natural therapeutic candidate. It has a greater binding affinity with the MC1R on melanocytes than natural α-MSH and is therefore able to more readily activate melanin to repigment skin. It is hoped that treatment with afamelanotide, in conjunction with NB-UVB therapy, will produce faster, more consistent repigmentation of vitiliginous skin.

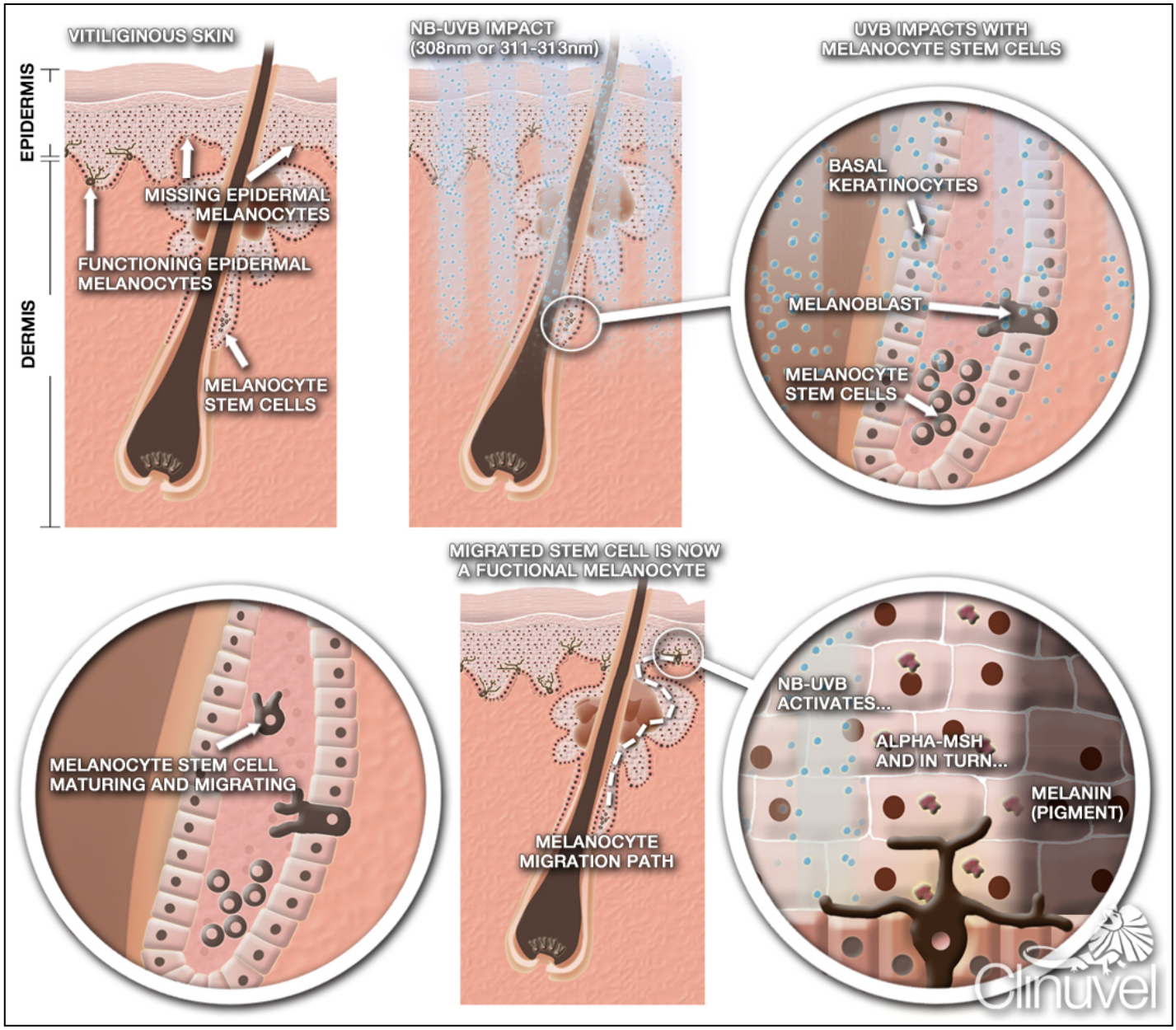


Figure 7. The process of melanocyte migration and repigmentation in vitiliginous skin following NB-UVB therapy.

FURTHER READING

- National Institutes of Health, *The Adult Stem Cell*
- Clinuvel, *Technology Update I: The physiological 'UV tanning response', original thoughts on UV and pigmentation*
- Clinuvel, *Technology Update II: Afamelanotide as an adjunct to phototherapy*
- Clinuvel, *UV damage and carcinogenesis*

REFERENCES

Abdel-Malek, Z.A & Kadekaro, A.L, 2006, 'Human Cutaneous Pigmentation: A Collaborative Act in the Skin, Directed by Paracrina, Autocrine, and Endocrine Factors and the Environment', in VJ Hearing & SPL Leong (eds), *From Melanocytes to Melanoma*, Humana Press Inc., New Jersey.

Abdel-Malek, Z.A et al., 2008, 'The melanocortin 1 receptor and the UV response of human melanocytes--a shift in paradigm', *Photochemistry and Photobiology*, **84**(2):501-508. (abstract)

Berneburg, M, Roecken, M & Benedix, R, 2005, 'Phototherapy with Narrowband UVB', *Acta Dermato-Venereologica,* **85**:1-11. (online)

Bickers, D.R, 2008, 'Photosensitivity and Other Reactions to Light', in AS Fauci et al. (eds), *Harrison's Principles of Internal Medicine, 17th* edn, McGraw-Hill Medical, New York.

Böhm, M et al., 2005, 'alpha-Melanocyte-stimulating hormone protects from ultraviolet radiation-induced apoptosis and DNA damage', *The Journal of Biological Chemistry*, **280**(7):5795-5802. (online)

Chakraborty, A.K, 1999, 'UV light and MSH receptors', *Annals of the New York Academy of Sciences,* **885**:100-116. (abstract)

Cui, R, et al., 2007, 'Central role of p53 in the suntan response and pathologic hyperpigmentation'. *Cell*, **128**(5):853-864. (abstract)

Falabella, R & Barona, M.I, 2008, 'Update on skin repigmentation therapies in vitiligo', *Pigment Cell & Melanoma Research,* **22**:42-65. (abstract)

Hocker, T.L, Singh, M.K & Tsao, H, 2008, 'Melanoma Genetics and Therapeutic Approaches in the 21st Century: Moving from the Benchside to the Bedside', *Journal of Investigative Dermatology*, **128**:2575-2595.

Hou, L & Pavan, W.J, 2008, 'Transcriptional and signaling regulation in neural crest stem cell-derived melanocyte development: do all roads lead to Mitf?', *Cell Research*, **18**:1163-1176.

Ko, M.S.H, 2008, 'Stem Cell Biology', in AS Fauci et al. (eds), *Harrison's Principles of Internal Medicine, 17th* edn, McGraw-Hill Medical, New York.

Miyamura, Y et al., 2010, 'The deceptive nature of UVA tanning versus the modest protective effects of UVB tanning on human skin', *Pigment Cell & Melanoma Research,* **24**: 136-147. (online)

Nishikawa, S & Osawa, M, 2007, 'Generating quiescent stem cells', *Pigment Cell Research*, **20**(4):263-270. (online)

Odland, G, 1991 'Structure of the skin', in LA Goldsmith (ed), *Physiology,*

biochemistry, and molecular biology of the skin, Oxford University Press, Oxford.

Osawa, M, 2009, 'Melanocyte stem cells', *StemBook,* Harvard Stem Cell Institute, retrieved 9 May 2011, <http://www.stembook.org/node/581>. (online)

Rees, J.L, 2004, 'The genetics of sun sensitivity in humans', *The American Journal of Human Genetics*, **75**(5):739-751. (online)

Rouzaud, F et al., 2005, 'MC1R and the response of melanocytes to ultraviolet radiation', *Mutation Research,* **571**(1-2):133-152. (abstract)

Suzuki, I et al., 1999, 'Participation of the melanocortin-1 receptor in the UV control of pigmentation', *Journal of Investigative Dermatology Smposium Proceedings*, **4**(1):29-34. (abstract)

Tobin, D.J, 2011, 'The cell biology of human hair follicle pigmentation, *Pigment Cell & Melanoma Research*, **24**(1):75-88. (online)

Weichenthal, M & Schwarz, T, 2005, 'Phototherapy: how does UV work?', *Photodermatology, Photoimmunology & Photomedicine,* **21**(5):260-266. (abstract)

Yamaguchi, Y et al., 2006, 'Human skin responses to UV radiation: pigment in the upper epidermis protects against DNA damage in the lower epidermis and facilitates apoptosis', *The FASEB Journal*, **20**(9):1486-1488. (online)

Yamaguchi, Y, Brenner, M & Hearing, V.J, 2007, 'The regulation of skin pigmentation', *The Journal of Biological Chemistry*, **282**(38):27557-27561. (online)