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Epidermal stem cells

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1. ABSTRACT

Our understanding of adult epidermal stem cells has increased substantially over the last two decades especially; however even more detailed knowledge is imperative for realizing potential medical therapies. We need to know how to identify these cells and what regulates their function. In this review, we describe the literature to date and elucidate the clinical relevance of these findings.

2. INTRODUCTION

The importance of skin integrity and the consequences associated with having a skin deficit are readily apparent in day to day clinical practice, particularly in plastic and reconstructive surgery. For example, patients with large burns incur life threatening metabolic complications and have a short supply of donor tissue, whilst chronic leg ulcer patients suffer pain and risk infective complications resulting from their open wounds. Thus, managing disease states without resorting to creating large donor sites is a common goal of tissue engineering and basic science efforts alike. Additionally, more information about skin biology may help to treat other skin disorders like the congenital blistering disorder epidermolysis bullosa (1-4), and hyperproliferative dermatoses such as psoriasis. In particular, stem cells hold promise with regard to future gene therapies, and are thought to play a role in cutaneous malignancy (5,6).

Consisting of a variably thick epidermis, and an underlying dermis; the skin is the largest organ of the body (7). It functions to protect the inner parts of the body against physical damage and desiccation, and assists in thermoregulation and the maintenance of homeostasis. Additionally, the skin's sensory receptors harness pain, pressure, light, touch and temperature information from the environment.

The epidermis is largely comprised of keratinocytes in a stratified squamous keratinizing epithelium (8). Also contained within this superficial layer are immunological, pigment and mechanoreceptor units in the form of melanocytes, Langerhans and Merkel cells. As the keratinocytes mature, they move from the basal proliferative layer to the suprabasal layers and are eventually shed as squames, which are fused dead cells without nuclei, but full of keratin filaments providing structural support. As keratinocytes differentiate and move up through the layers of the epidermis, they gain keratin granules and lipid-laden lamellar granules. Keratins contribute to filament formation, and thus to maintenance of cell integrity and strength, and lipid is secreted to achieve water-proofing of the skin (9). This differentiation process has been measured in mice to take 8-14 days (10-13). In humans it is thought to take more like 14-75 days (14-17).

Separated from the epidermis by a basement membrane, the dermis is a thick layer of vascular connective tissue containing predominantly fibroblasts in an extracellular matrix, as well as epidermal skin appendages that assist in thermoregulation and homeostasis. The basement membrane is a specialized extracellular matrix rich in various proteins and proteoglycans including collagen type IV and laminin. These components act as ligands to bind the keratinocytes via specialized adhesive receptors on their surface, adding to the development of a tight skin barrier. One example of these surface receptors is the family of integrins which are found on stem cells and their progeny.

Unlike many other organs, self renewal processes are constantly occurring to maintain the skin's integrity, and are accelerated when the skin is injured.

Epidermal stem cells are a specific population of keratinocytes that have characteristic properties including the ability to divide to produce both daughter stem cells and cells that go on to differentiate, and thus the ability of lifelong tissue regeneration of the epidermis (18).. They are important in homeostasis and wound repair, and show great promise in their ability to be incorporated into tissue engineering constructs, as well as to be therapeutically targeted for oncological treatments and gene therapy for congenital disorders.

3. STEM CELL BIOLOGY

3.1. Structural organization of the epidermis and location of stem cells

Generally, it is accepted that stem cells reside in a stable microenvironment that supports and controls the behaviour of the stem cell. This environment, termed the niche (19), is believed to strongly influence the self-renewal and progeny production that stem cells are vital for. These niches are thought to be maintained even when the stem cell is removed from it, and it is possible that they may even be capable of inducing 'stemness' or rather, stem cell behaviour, in cells that come to reside within the specialised microenvironment (20).

This unique environment is created and maintained by secreted factors, integrins, cell to cell interactions and the extracellular matrix (21). These regulatory factors and other, as yet, unknown genes and regulatory factors hold great promise for the future prospects of modulating stem cell behavior (8,17). It is not surprising to find that stem cells exist in special locations as they are after all, an important reservoir of the cells that can replicate throughout an individual's lifetime and are responsible for the vital self-renewal capacity of the epidermis.

The exact location of stem cells in human skin has been the subject for debate over the years that now has been clarified to some extent. There is a large amount of evidence including label retaining data that confirms stem cells to be within the bulge region of the hair follicle (22). This specialized region is in the upper portion of the hair follicle within the dermis and has been found to contain cells with the greatest *in vitro* growth capacity when compared with cells from other regions of the follicle and epidermis (23-25). Some authors have not localized these follicular cells to the human hair bulge, but rather a sub-bulge region (25,26). This variation in findings could be due to the lack of a distinct bulge region in humans compared with mice (27,28). There is also some evidence for a reservoir of stem cells within the bulb of the hair follicle (24,25,29). Many authors discount the latter region as a possible stem cell reservoir because follicular regeneration is still observed after removal of the lower hair follicle as long as there is a dermal papilla close by (30-35). However, it is possible that in homeostatic circumstances these deeply protected cells play a role in at least follicular maintenance.

In addition to these follicular stem cells, there are interfollicular (IF) stem cells. The precise location of these cells is not yet universally agreed upon. They are known to be within the basal layer, and it makes sense that they would be in a protected environment (Figure 1).

In murine epidermis, there is good evidence that the location of IF stem cells is at the centre of epidermal proliferative units (EPUs) (36-39). The EPU, a phrase coined by Potten (36), described the epidermis as being comprised of multiple microscopic hexagonally shaped regions. Each region contains a quiescent stem cell within the basal layer, adjacent proliferating transit amplifying (TA) cells, and superficial committed keratinocytes more peripherally. Structural units like EPUs have also been found in human relatively thin skin including the limbs, abdomen and buttocks (17). In other studies, columnar organization lacking the precise alignment found in rodent and monkey epidermis was found in various human epidermis specimens, but the evaluation concentrated on the suprabasal layers and did not focus on stem cells per se (40). Recent studies with genetic tagging with Lac Z also support the existence of EPUs with a stem cell at its base (41,42). These EPUs have not been found in human volar (palm and sole) skin (43).

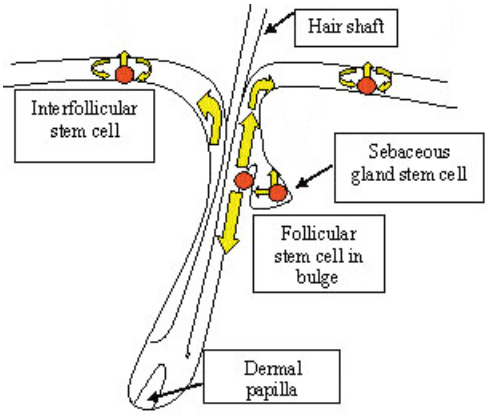

Figure 1. A schematic representation of the location of the epidermal stem cells. The more frequently cycling interfollicular stem cells are found in the basal layer of the epidermis, whilst the quiescent follicular cells are mostly located in the bulge region of the hair follicle (Adapted with permission from Blackwell-Synergy).

Another theory that explains the structural organization of IF epidermis in volar skin was postulated by Lavker and Sun, and based on observations of DNA label retention cell cycling studies and cell morphology in monkey and human palm epithelium (44,45). Two different populations of basal cells were identified and found to differ not only in their location in relation to the rete ridges, but also in their dermal-epidermal junction properties. The cells in the shallow rete ridges had highly convoluted ("serrated") dermal-epidermal junctions which were postulated to be involved with anchoring the epidermis to the dermis. The other non-serrated population had flatter dermal-epidermal junctions, and was found at the tips of the deep rete ridges. This second population was thought to represent stem cells that produce TA and differentiated cells. These serrated and non-serrated populations have also been identified in other human epidermal regions, including the back, abdomen and limbs (45).

Some authors proposed that stem cells could be found in clusters at the tips of dermal papillae (46-51) (. However, in more recent studies also in human tissue, the site of the IF keratinocyte stem cells (KSCs) was specifically investigated and cells from the tips of the deep rete ridges were found to have greater clonogenic capacity and proliferative output (52). The intracellular protein keratin 15 (K15) is a proliferative cell marker and was found to highlight cells at the tips of the deep rete ridges on immunostains of adult human skin, and when these cells were dual stained with α_6 integrin, they were also positive (53). Cells at the tops of the shallow rete ridges were negative for K15 as were most α_6^{dim} cells. This work supports the theory that stem cells and their proliferative progeny are found in the deep rete ridges in adult human skin where they exist in a relatively protected environment.

3.2. Label retaining cells

Long-term labelling of cells with a nucleotide analogue such as bromodeoxyuridine (BrdU) or tritiated thymidine [^{3}H]Tdr is one method of identifying slow-cycling cells, which is a feature of stem cells in their normal state. The qualification of 'normal' is required because stem cells appear to cycle much faster in response to injury (54). These nucleotide analogues are incorporated into synthesized DNA and will remain within cells that divide only rarely. Using this technique, IF label-retaining cells are seen scattered throughout the epidermis (39,54,55). Applying this method to follicular stem cells, the well localized bulge region can be readily identified (22).

The mechanism of label retention is not necessarily due to quiescence alone, as one would expect depletion of label even in stem cells eventually as they replicate, but this is not observed in young mice. These murine skin cells replicate more than 10 times in the first 2 to 3 months of life and yet label persists in the putative IF stem cell population (17). Thus it is possible that these specialized cells have another mechanism of retaining label. One alternative explanation proposed is that stem cells are able to protect their original or parental template DNA strand and only replicated DNA is transferred to new daughter cells (56). The daughter cells thus carry the risk of replication errors occurring, whilst protecting the more permanent stem cell compartment. Elegant double labelling studies in the small intestine support this theory of DNA protection (57). Potten and co-workers found retention of [^{3}H]Tdr which had been supplied during development or tissue regeneration, but depletion of BrdU which had been given after the neonatal period of expansion, and thus provided further evidence of conservation of the parental DNA strand.

3.3. Stem cell characteristics

Investigators have used plasticity, that is, the ability to give rise to different cell types from those usually found in the organ in which the stem cells normally reside (58), as a stem cell characteristic. The two populations of epidermal stem cells, follicular and IF, were both originally considered to be lacking in plasticity and multipotency. IF stem cells seemed to be committed to one lineage only, and hair follicle stem cells were thought to only contribute to the different hair follicle components. However, in recent years it seems that the micro-environment of the cells can influence their potential, and certainly this factor is known to stimulate plasticity in tissues other than skin.

Follicular epithelial stem cells are multipotent, having the capacity to give rise to all the cell types of the hair, as well as the interfollicular epidermis and the sebaceous gland in murine studies (59-63).

IF stem cells are thought to have some pluripotency in that many studies have shown that these keratinocytes can give rise to pilosebaceous and sweat gland structures (64-67). In particular, human foreskin keratinocytes have been able to establish cutaneous appendages in animal models (64) and experiments with rat dermal papilla cells and foot pad epidermis have demonstrated that hair follicle formation can be induced in plantar skin (68), thus supporting the notion that hair follicle induction may be common to all adult papilla cells.

A possible example of plasticity in epidermal tissues involving putative epidermal stem cells from neonatal mice found evidence of these cells persisting in many different tissues after they were injected into developing embryonic mice. Ectodermal, mesenchymal and neural-crest-derived tissues showed these injected cells to be present, implying that somatic epidermal stem cells may have some plasticity that can be harnessed under certain environmental conditions (69), or at the very least, that there is potential for contribution of epidermal stem cells to different tissues and lineages during development. However, this study used Hoechst efflux and size criteria to identify putative stem cells, and thus may well have included follicular and IF stem cells as well as neural precursors, fibroblasts, haematopoietic and other lineage cells in their isolation technique. Therefore, this study does not give us more information about the multipotency of the IF KSCs. This property of plasticity has been more convincingly demonstrated in adult corneal epithelium where central putative transit amplifying (TA) cells under the influence of embryonic hair-forming dermis could form an epidermis and hair follicles (62).

During homeostasis, follicular stem cells can give rise to the epidermis found adjacent to the hair follicle (38). However, elegant labelling studies showed that IF stem cells were capable of long term epidermal renewal without the need for follicular stem cells (38). In wound healing, the follicular stem cells are capable of regenerating all the epidermis (60,70). Some authors have mooted that in hairy skin, the IF stem cells may in fact be TA cells derived from these follicular cells (60,71). However, label retaining studies have difficulty highlighting single cells like the IF stem cells compared to the clusters which are found in the bulge region, thus this method of stem cell identification is less useful. Additionally, the IF stem cells would be expected to cycle more frequently than the bulge cells as the latter, hidden in deep recesses, are more likely to act as an emergency reservoir of epidermal stem cells rather than the cells necessary for day to day maintenance of the epidermis (25). Label retention is a dynamic property and a function of the amount of proliferation required of labeled cells. Therefore, the more frequently cycling IF cells will deplete their label more quickly when compared with the more quiescent bulge cells (72).

3.4. Identification of KSCs

Irrespective of where the IF stem cells are located and what their exact differentiation potential is, we need to establish ways of identifying them. Human keratinocytes have been grouped into three sub-populations based on their clonal expansion in culture (73). Holoclones were found to have the greatest proliferative potential with subsequent passaging of these cells and were therefore thought to be stem cells. Meroclones had less proliferative capacity, whilst paraclones created aborted colonies and underwent differentiation. Paraclones seemed to be like TA cells in their behavior, and meroclones seemed to be intermediate between TA cells and KSCs (73). This classification was based on colony forming ability *in vitro* and assumed that TAs would proliferate less extensively than KSCs *in vitro.* The difficulty with this classification is that it is retrospective: it relies on the observed functional behavior of cells to be able to classify them. Ideally, we need to be able to identify stem cells without having to test them functionally.

Various techniques have been used to identify the epidermal stem cell population including the aforementioned label-retaining studies. Whilst these methods have been fruitful in mice and *in vitro* human tissue, their usefulness in humans is limited *in vivo* because of ethical considerations.

Stem cell markers that identify and thus allow isolation of sub-populations of keratinocytes including KSCs, are required to avoid relying on clonogenicity and label retaining assays. Integrins represent a good candidate marker as they are found on the cell surface and are important for anchoring cells to the basement membrane. As stem cells are found in a basal location, integrins are particularly important to these and other basal cells compared to more differentiated suprabasal sub-populations. The only caveat being: integrins also play a role in cell-cell adhesion, and thus the presence or absence of integrins in a population is not as helpful as their relative proportions. Integrins consist of a heterodimer of one α and one β subunit and these can be labeled with fluorochrome tagged antibodies for analysis by fluorescence activated cell sorting (FACS).

β_1 integrin was first suggested as a prospective stem cell marker for human neonatal foreskin epidermis on the basis of the colony forming efficiency of β_1 integrin positive cells over 2 weeks (74), but found to be located both basally and suprabasally and thus on too great a percentage of cells to be identifying stem cells alone (46).

α_6 integrin, when combined with the transferrin receptor, CD71, (a marker of proliferating cells) has been found to help isolate a population with a higher long term proliferative potential, low incidence and relative cell cycle quiescence in neonatal human skin (75). This KSC fraction was found to be integrin α_6 positive and transferrin receptor negative (α_6^{bri} CD71 ^{dim}) (75,76).

To identify the more committed progeny of the stem cells, α_6 integrin and CD71 were also used. TA cells can be distinguished by their basal position and highly proliferative nature giving a bright α_6 integrin and CD71 signal ($\alpha_6^{bri}CD71^{bri}$), whilst early differentiating cells (PMDs) are seen to be α_6^{dim} (75,77). These findings could be extended to murine epidermis, where the $\alpha_6^{bri}CD71^{dim}$ fraction was enriched for label retaining cells. The bulge region was also found to express low to negative CD71 consistent with this stem cell phenotype (77). Thus populations with *in vivo* and *in vitro* characteristics of stem cells can be identified using cell surface markers via this FACS technique. This work has resulted in the description of phenotypically defined KSCs which probably represent the best characterised epidermal stem cell population to date (17,71). The limitation of these markers is that both are upregulated in wound healing and culture (72).

To correlate these sub-populations with a clonal classification is difficult; however, the $\alpha_6^{bri}CD71^{dim}$ (KSC) sub-population may give rise to the holoclones described by Barrandon (73), but it is possible that the TA fraction may contribute too. However, the FACS technique used to separate these cells does involve significant manipulation and therefore, could affect the true colony forming efficiency.

Flow cytometry has also been combined with label retaining studies to validate the use of CD34 in identifying murine follicular stem cells (78). In this investigation, CD34 positivity also correlated with the presence of the K15 protein which has been found to highlight human cells with a phenotype consistent with follicular stem cells (79). The usefulness of CD34 for the identification of IF stem cells is yet to be ascertained.

In immunostaining of human skin, K15 is seen to localize to cells of the bulge region (79), and in human hair follicles grafted onto immunodeficient mice, K15 was found to be associated with cells of a slow-cycling nature (79). Additionally, these cells were found to express other markers found on stem cells including β_1 integrin and K19 (79). The limitation of use of the K15 protein as a stem cell marker is its wide expression in the basal layer (53). Interestingly, in neonatal tissue, K15 immunostaining revealed all basal cells to be homogenously positive, in contrast to adult tissue where it localized to cells at the tips of the deep rete ridges (53). It is likely that K15 protein is retained in the highly proliferative stem cell progeny thus limiting the usefulness of K15 as a pure stem cell marker. However, the K15 promoter-driven GFP or β-galactoside expression is restricted to the hair follicle bulge region and thus is a putative follicular stem cell marker (67).

Another keratin, K19 is found in the bulge region of the hair follicle and along the basal layer of the epidermis (80). It is also present with keratin 18 on Merkel cells (81), and thus in experiments to explore the usefulness of K19 as a stem cell marker, keratin 18 was confirmed to be negative to exclude Merkel cells. These investigations found K19 correlated with label-retention, was absent in IF epidermis at hairy skin sites but present in deep rete ridges in glabrous skin sites and was proportionally reduced in expression from older donors (80). The limitation in using keratins for the identification of stem cells arises from the fact that keratins are intracellular, thus they are useful for identifying putative KSCs in tissue sections, but not for sorting cells which is potentially more clinically relevant.

Desmosomal proteins which assist in cell adhesion, in particular, desmoglein 3, have also been put forward as useful negative markers for epidermal stem cells (52). The low level of desmoglein in human adult skin correlated with high β_1 integrin expression, and to high colony forming efficiency. Furthermore, these β_1^{bri} cells with low desmosomal proteins correlated well with our work on localizing the stem and progenitor cells in adult skin to the depths of rete ridges with α6 integrin and K15 (53).

Another putative stem cell marker is Melanoma-Associated Chondroitin Sulfate which seems to correlate with α_6 and β_1 integrin expression, as well as the presence of stem cell-associated keratins 15 and 19 in human adult skin. In addition, it follows the expected distribution of stem cells to some extent, although it is perhaps a little more widespread and thus may be a progenitor cell marker rather than a stem cell marker alone (82). Additionally, it co-localizes with β_1^{bri} cells at the tips of dermal papillae, in contrast to the aforementioned work on desmosomal proteins and other markers pointing to the depths of the rete ridges being the location of stem and progenitor cells. Hence, there is more work to be done in clarifying the role of Melanoma-Associated Chondroitin Sulfate in stem cell identification.

Another marker with a controversial degree of usefulness is the fluorescent vital dye Hoechst 33342. Drawing on the enormous reservoir of knowledge about haematopoietic stem cell biology, this dye has been employed to try to identify a side population within the epidermis. In the hematopoietic system, this dye identifies a primitive subset of stem cells on the basis of their ability to efflux this dye (83). A side population has been identified in the epidermis, but some investigators have found that this population has a different cell surface phenotype (low β_1 integrin and low α_6 integrin) from other putative stem cell populations that retain label (84). Other laboratories have purportedly found it a useful way of defining a stem cell population on the basis of label retention, clonal expansion, high proliferative potential and epidermal regenerative capacity of cells assayed after Hoechst efflux and size sorting (69,85). In our own laboratory, extensive work with murine tissue supports the existence of this side population as a subset of epidermal cells although functional data is still being gathered to verify its validity as a stem cell population (Redvers and Kaur, unpublished data).

There are some markers that are thought to be bulge-preferred including the Keratin 15 promoter (67), K15, K19, α_6 integrin, β_1 integrin, and CD34 (86). These markers have been investigated in association with label retaining methods and green fluorescent protein marking in a mouse microarray analysis to further define upregulated messenger RNAs that contribute to the stem cell niche. It is likely that these products are important for guiding the stem cell's ability to signal and respond to its environment and thus allowing for the stem cell's unique properties (86).

The importance of transcription factors in stem cell regulation including the Tcf/Lef family is becoming apparent. Lef1 knockout mice have deficient hair and whisker formation (87), while stabilized β catenin which activates Tcf/Lef transcription causes new hair follicle, sebaceous gland and dermal papilla development when controlled by an epidermal promoter (88). This process of new follicle and gland formation is normally only possible during embryonic development. Tcf was found to be upregulated in label retaining cells of the bulge region (86). β catenin is also required for Wnt (wingless) signaling, which in turn is known to be important for hair-follicle

formation (88,89). β catenin knock-out mice have impaired hair follicle stem cell differentiation, but the epidermis is maintained. Thus, the IF stem cells have the ability to renew the epidermis without the requirement of hair follicle stem cells (89).

With the recent increasing knowledge about molecular signaling pathways such as Wnt and β catenin, Sonic hedgehog (Shh) and the trans-membrane proteins Delta and Notch, it is not all that surprising that these rather ubiquitous pathways have been implicated in control of the stem cell compartment (50,90,91). Interestingly, in studies in transgenic mice, Shh was shown to cause epidermal hyperplasia by increasing the number of basal cells, but over expression caused the depletion of the basal cells and consequently a thin epidermis (92).

Other factors that seem to be involved in stem cell regulation are c-myc (myelocytomatosis oncogene) and p63. When absent in mice, the gene transcription factor p63 causes a lack of stratified squamous epithelia; thus implicating a lack of stem cells and or progenitors in this phenotype (23). p63 has also been shown to be abundantly expressed in epidermal holoclones and to be barely detectable in meroclones. This implies that p63 may be of value as a stem cell marker (93); however it is also possible that it is a marker of TA cells as these basal cells also have high colony forming efficiency [8]. The cell cycle regulator c-myc has been implicated in epidermal hyperproliferation in mice that have an elevated expression of this gene (94,95) and has been identified in the bulge region (96). Again, its specificity for stem cells over TAs is not yet defined.

3.5. Stem cell activity as a function of microenvironment

The difficulty in identifying stem cells hails from the removal of these cells from their *in vivo* micro-environment, and thus from the control mechanisms which influence stem cell behaviour. For example, stem cells cycle slowly *in vivo* but are highly proliferative *in vitro*. Furthermore, different cell populations can have improved regenerative capacity depending on their micro-environment (97). Additionally, stem cell markers can change in culture.

Stem cells exist in the specialized microenvironment of the niche and are subject to various factors that influence their proliferation and differentiation like the integrins and secreted Wnt. Epithelial - mesenchymal interactions are also known to control epidermal growth and differentiation. Such interactions can occur through cell to cell contact, cell and matrix interactions, and diffusion of soluble factors. The original Rheinwald and Green *in vitro* culture system illustrates the dependence of keratinocytes upon fibroblasts for effective growth (98). Organotypic culture systems requiring the inclusion of human fibroblasts also exemplify this principle.

In other organotypic cultures investigating the role of basement membrane components and dermal fibroblasts, keratinocyte differentiation was found to be independent of the presence of fibroblasts and the basement membrane, but sustained growth required both mesenchymal components to be present (99).

An example of the importance of the dermal micro-environmental influence is in short-term organotypic cultures where our laboratory has shown that epidermal tissue formation is possible from the TA and PMD fractions when minimally passaged fibroblasts are used. Passage seven fibroblasts in organotypic culture yield less optimal growth of the differentiated cell population in comparison to passage one fibroblasts (97).

Interestingly, it is not just the dermal cell populations that can be useful in improving the keratinocyte microenvironment, but also other mesenchymal cells. Bone marrow stromal cells and pre-adipocytes have both been shown to accelerate epidermal regeneration using rat keratinocytes in a co-culture system (100). Simple electrolytes are also known to influence keratinocyte behaviour, as shown *in vitro* by the failure of keratinocytes to stratify in low calcium media (101).

3.6. Stem cells and skin aging

Aged skin is known to have a thinner epidermal layer, a flattened dermal-epidermal junction with retraction of the dermal papillae and blunting of the rete ridges, and elastosis within the dermal layer. These properties make the skin of older people less resistant to trauma, especially shear.

The process of skin aging is thought to be due to a combination of photo-aging and intrinsic factors that lead to replicative senescence (102,103). Ultra-violet light induced photo-aging is caused by cellular damage, whereas the replicative senescence (that is, the inability of a cell to divide and undergo DNA replication) results from chronological aging.

In vitro differences are also observed between early passage neonatal and adult human keratinocytes. These differences have been well known since Rheinwald and Green's landmark article on serial cultivation of keratinocytes in 1975, but earlier work had been done on chick fibroblasts inversely correlating growth potential with age (104,105). Plating efficiency and culture lifetime in serial cultivation of keratinocytes was found to be lower for older people (98). Gilchrest reported that younger keratinocytes proliferate more rapidly than their older counterparts, and are also more responsive to mitogenic factors, such as keratinocyte growth factor (KGF) and epidermal growth factor (EGF) (106). Most recently, a study of oral keratinocytes demonstrated slower growth and poorer plating efficiency after the fourth passage in cells from donors over 60 years old compared to those under 60 years old (107).

Barrandon and Green hypothesized that the decline in the cultured lifespan of keratinocytes might be related to the lower proportion of holoclones (cells with high proliferative potential). They found fewer holoclones and more paraclones (cells with lower proliferative

potential) in older donors (73). In other words, with increasing age, it is possible that the poorer growth *in vitro* is due to a lower proportion of stem cells and or progenitors and a higher proportion of post-mitotic differentiated cells.

Interestingly, age related changes are thought by some investigators not to be due to the ageing process of stem cells themselves. In a murine model using blastocysts combined with TAs and KSCs sorted on the basis of Hoechst dye exclusion and small size, the putative stem cells were found to have differentiation abilities beyond the TAs irrespective of their age (108). The authors concluded that aged murine epidermal stem cells show similar plasticity to neonatal stem cells when placed in a developmental environment, but extrapolation of this developmental model to the normal physiological state of stem cells is probably not justified without further supportive evidence. As discussed earlier, the purity of the putative epidermal population was not unequivocally demonstrated.

Possible explanations for poorer growth *in vitro* of adult keratinocytes could include reduced potency of the older KSCs or more fastidious requirements of growth factors.

Other age related changes of skin include: reduced laminin 10/11 in the basement membrane (109) reduced collagen VII (important in anchoring fibrils) production (110), and increased sensitivity to growth inhibitory effects of interferon (111).

4. PERSPECTIVE: CLINICAL RELEVANCE OF EPIDERMAL STEM CELL RESEARCH

The clinical need for improvements in skin 'deficit' management is enormous. Burns, ulcers and various genetic diseases affect the integrity of the skin, and thus impair the homeostasis of the patient as a whole. Various skin substitutes are commercially available, but skin grafts are still the mainstay of burn, and often ulcer, surgery.

Skin grafts are shavings of the patient's own skin in unaffected areas that are made thick enough to heal the injured part, yet not so thick that the patient cannot heal the donor area. Thinner grafts allow faster healing and often take more easily, but lack the skin appendages that are important in thermoregulation and for cosmesis, namely the hair follicles, sweat and sebaceous glands. ('Take' refers to the revascularization or blood vessel ingrowth of these grafts which have no inherent blood supply, and is necessary for their re-incorporation into living tissue) Obviously, the limitations are the healing and scarring of the donor site as well as the size of the unaffected and therefore potential donor area, compared to the injured skin. The most prevalent example of this is the situation of a major burn where more than 60 percent of the patient's skin is affected.

Basic research into stem cell biology offers the hope that eventually patient donor sites will be minimized by laboratory expansion of host cells. This technique is currently available in the form of cultured epithelial autografts, but the expansion *in vitro* takes some weeks (usually around 3), the sheets are incredibly fragile making handling difficult, and the take of these sheets is variable. Studies have shown good long term maintenance of these grafts (112). An aerosol or spray on form of cultured epidermis is also available to reduce the handling difficulties, but the expansion time is the same. The problem with the period of expansion is that the patients are at high risk of infection and are also metabolically volatile until they get adequate skin coverage. Thus early coverage with whatever is feasible, including the use of cadaver skin, is imperative for saving the life of large percentage burns patients.

Most skin deficits requiring treatment are not usually purely epidermal, so there needs to be a concomitant improvement in dermal substitute technology to truly address the treatment issues. Whilst there are acellular allogeneic and synthetic-animal combinations of dermal substitutes currently available, they are all prohibitively expensive and have technical limitations. It is hoped that in time, keratinocytes may be seeded into dermal substitutes and allow for revascularization and maturation of the dermis whilst the epidermis continues to expand.

In addition to skin replacement, culturing keratinocytes offers the possibility of a simple method of gene therapy. This has been successfully achieved in animal models for the correction of epidermolysis bullosa, but is not yet clinically available (2,113).

Another area of clinical relevance is cutaneous malignancy. In models of carcinogenesis, the carcinogen has been found to remain in cells that are quiescent, thus implicating stem cells (5,18,56), and to be retained in cells that have a high proliferative potential which again is suggestive of stem cells (6). However, it is also possible that the highly proliferative progeny of the KSCs, that is the TAs, are capable of being immortalized by a genetic mutation caused by a mutagen such as a viral oncogene like human papilloma virus for example. This well known carcinogen is associated with squamous cell cancer of the cervix and has been shown to transform cells (114). It has also been demonstrated to infect cells at locations that correlate with epithelial stem cells (115,116), but given that the precise location of individual epidermal stem cells has not been proven, it is possible that this transformation has occurred in non stem cells.

In summary, the hope is that through improvements in the basic science knowledge of epidermal stem cell biology, we may be able to better address the management of benign and malignant skin disorders.

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Abbreviations: KSC keratinocyte stem cell, TA transit amplifying cell, PMD early differentiating cell, IF interfollicular, EPU epidermal proliferative unit, FACS fluorescence activated cell sorting, K15 keratin 15, [^{3}H]Tdr tritiated thymidine, BrdU bromodeoxyuridine, K19 keratin 19

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