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Stem Cells and Tissue-Engineered Skin

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Abstract

Advances in tissue engineering of skin are needed for clinical applications (as in wound healing and gene therapy) for cutaneous and systemic diseases. In this paper we review the use of epidermal stem cells as a source of cells to improve tissue-engineered skin. We discuss the importance and limitations of epidermal stem cell isolation using biomarkers, in quest of a pure stem cell preparation, as well as the culture conditions necessary to maintain this purity as required for a qualitatively superior and long-lasting engineered skin. Finally, we review the advantages of using additional multipotent stem cell sources to functionally and cosmetically optimize the engineered tissue.

Keywords

Epidermis; Stem cell; Tissue regeneration; Gene therapy; Stem cell isolation

Introduction

The use of stem cells as the basic material for skin engineering has the potential to improve clinical outcomes both in wound healing and in gene therapy for cutaneous and systemic diseases. Studies of tissue-engineered skin have shown that epidermal stem cells may provide a superior source of multipotent stem cells for tissue engineering. Here we focus on epidermal stem cells as the basis for tissue engineered epidermis and also discuss the potential use of different adult stem cell types in tissue-engineered skin.

Epidermal Stem Cells and the Limitations of Tissue Engineering

The use of tissue-engineered skin has resulted in less than satisfactory clinical results, and in most cases there is loss of the cultured autograft even after successful grafting initially [1]. Previous work suggests that the success of tissue engineering depends on the choice of suitable seed cells in culture to assure the persistence and function of the regenerated tissue for the patient's lifetime [2]. Indeed, failure of tissue-engineered skin to satisfactorily replace tissue lost in full-thickness burns is usually due to poor long-term functionality of the tissue. The long-term function of the graft is presumably limited by the maintenance of the epidermal stem cell compartment during in vitro culture. A solution to the issue of stem cell depletion during autograft preparation would be to start with a pure population of stem cells and maintain this population during autograft preparation.

Several studies have demonstrated the superiority of progenitor cells over more differentiated keratinocytes in the generation of tissue-engineered skin [3,4]. Keratinocytes sorted based on BrdU label-retaining capacity reveal 2 distinct basal cell populations, stem cells and transit-amplifying cells. Both stem cell and transit-amplifying cell populations were used to bioengineer an epidermis in combination with a collagen type I gel containing neonatal mouse dermal fibroblasts. Both populations formed an epidermis by gross examination, but after 2 months only the stem cell-derived skin maintained a normal epidermis, while the epidermis formed from transit-amplifying cells had completely differentiated [3]. In order to isolate a pure epidermal stem cell population, the identification of epidermal stem cell surface markers is crucial [2]. Dunnwald et al. [3] used a Hoechst and propidium iodide cell-sorting strategy with improved sorting conditions to isolate an epidermal stem cell population. This population, when used as a source for tissue-engineered skin, was able to maintain an epidermis and expression of an integrated recombinant gene [3]. The above studies suggest that there may be an advantage in using primitive tissue progenitors for tissue engineering.

The relative contributions of follicular and interfollicular stem cells in epidermal maintenance and wound healing have been studied. Ito et al. [5] addressed this question using the ablation of bulge cells with a suicide gene encoding herpes simplex virus thymidine kinase under a keratin 15 promoter. In this model, they observed a total loss of hair follicles but a normal epidermis. In addition, they performed fate-mapping analysis using Tg(Kr1-15-cre/PGR) 22Cot mice crossed with R26R reporter mice that express LacZ. After epidermal injury, they observed the migration of LacZ-positive bulge cells centripetally into the wound and found that $26 \pm 11\%$ of cells in the reepithelialized wound were LacZ positive 8 days after injury [5]. Recent work from Langton et al. [6] addressed the role of hair follicle bulge-derived stem cells and interfollicular stem cells in skin wound healing. This study utilized an animal model that entirely lacks the hair follicle bulge on the tail and trunk and does not form primary hair placodes. In this model there was a delay in wound reepithelialization compared to wild-type animals, and an expanded area of interfollicular epidermis was shown to be recruited to achieve closure and reformation of the epidermal barrier, suggesting an important role for both follicular and interfollicular stem cells in wound healing [6]. Levy et al. [7] studied the contribution of keratinocytes derived from the follicle to the wound epithelium. This was evaluated by lineage tracing in ShhGFPcre;R26R mice [mice harboring an inducible form of cre recombinase (cre-ERT2) inserted into the Shh locus (ShhcreERT2)]. Shh expression in the skin is restricted to keratinocytes of follicular origin even after epidermal injury. In wound-healing experiments, $23 \pm 12.5\%$ of labeled cells in the basal layer of wound epidermis were from follicular origin but were phenotypically indistinguishable from adjacent unlabeled basal cells of interfollicular origin. The authors concluded that follicular epithelium contributes to the initial resurfacing of the wound and follicular cells remain resident in the basal layer of the epidermis months later [7]. From these different independent studies, it appears that follicular stem cells respond first and rapidly to epidermal wounding and contribute to the regenerated epidermis along with interfollicular keratinocytes.

Appropriate culture conditions are presumed to be important to prevent stem cell loss in the preparation of autografts. Pellegrini et al. [4] could maintain the normal relative percentages of holoclones, meroclones and paraclones using fibrin-cultured autografts (produced by mixing fibrinogen and thrombin, inducing a rapid polymerization of fibrinogen). These cultures maintained the clonogenic ability, growth rate and long-term proliferation potential of the graft. Using these fibrin-cultured autografts on massive full-thickness burns resulted in permanent (up to 20 months of follow-up), reproducible grafts with mostly over 70% of a graft surviving. Demonstration of stem cell population maintenance with individual culture methods is necessary to ensure that stem cells are not depleted during construction of the tissue engineered skin.

A further issue with tissue engineering is the unnatural appearance of tissue-engineered skin even with successful grafting. Skin is composed of many more types of cells than just surface keratinocytes and fibroblasts. There are nerves, sebaceous glands, sweat glands, melanocytes and Merkel cells to name a few. A more complex reconstruction with hair follicles, stimulated by addition of dermal papillae cells, and perhaps addition of melanocyte and/or sebocyte stem cells could conceivably result in a more cosmetically and functionally normal skin. In this review, we first discuss epidermal stem cells but also discuss the use of multipotent stem cells from other sources that might contribute to the production of a more 'complete' skin.

Isolation of Epidermal Stem Cells

Epidermal stem cells represent a promising source of stem cells as discussed above. However, despite their self-renewal and multipotency, more work is needed to define markers for effective isolation of epidermal stem cells. Indeed, while the hematopoietic stem cell compartment has been molecularly defined at a single-cell level [8], epidermal stem cell markers capable of isolating stem cells at the single-cell level have not yet been found.

Given the knowledge in the hematopoietic stem cell field, it is to be expected that multiple markers will be needed to isolate epidermal stem cells at a single-cell level. Different methods to isolate epidermal stem cells have been proposed, including (1) $\alpha 6$ -bright/CD71-dim human keratinocytes [9,10], (2) rapid adhesion of cells to collagen IV [11], (3) DNA label-retaining cells representing slow-cycling cells [13–15] and (4) side population cells that efflux Hoechst 33342 fluorescent dye [16,17].

The combination of high $\alpha 6$ integrin expression ($\alpha 6^{\text{bri}}$) and low expression of the transferrin receptor (CD71^{dim}) are perhaps the most accepted epidermal stem cell markers to date [9,18]. $\alpha 6$ integrin is present on the inferolateral surfaces of basal cells, through which the cells adhere to the basement membrane. The $\alpha 6^{\text{bri}}$ CD71^{dim} cells are relatively quiescent in vivo and populations of these cells have very high long-term proliferative capacity. Tani et al. [18] characterized $\alpha 6^{\text{bri}}$ CD71^{dim} murine dorsal keratinocytes and observed that these cells, when compared to the $\alpha 6^{\text{bri}}$ CD71^{bri} population, were a quiescent population of small cells, with a high nuclear to cytoplasmic ratio, consistent with primitive cells. In addition, they observed that 1.4% of total isolated keratinocytes are both $\alpha 6^{\text{bri}}$ CD71^{dim} and label-retaining cells [18]. In human skin, the $\alpha 6^{\text{bri}}$ CD71^{dim} population has also been shown to contain smaller cells with a high nuclear to cytoplasmic ratio, capable of producing a high number of large colonies after 10 days of culture. The authors also tested the regenerative ability of $\alpha 6^{\text{bri}}$ CD71^{dim} keratinocytes and found that skin equivalents generated from those cells had a stratified and thick epidermis, while skin equivalents from $\alpha 6^{\text{bri}}$ CD71^{bri} cells produced a thin and less well-differentiated epidermis [19].

$\beta 1$ integrin is expressed in all basal keratinocytes and as they leave the basal layer, basal keratinocytes down-regulate the expression of $\beta 1$ integrin [20]. Human keratinocytes have been analyzed on the basis of whether they are rapidly or slowly adherent to a $\beta 1$ integrin ligand, type IV collagen. Rapidly adhering cells were found to have a high proliferative potential in vitro, whereas cells that adhere slowly divide only a few times before all of their progeny undergo terminal differentiation [21]. Furthermore, rapidly adherent cells form a robust stratified epidermis [19]. High $\beta 1$ integrin expression marks 20–40% of the basal cells, which is in great excess of the proportion of basal cells that are estimated to be stem cells in vivo and thus it is likely that high $\beta 1$ integrin expression selects for transit-amplifying cells as well [22]. In addition, $\beta 1$ -deficient cells from transgenic mice have some alterations in proliferation and differentiation but not a complete block, suggesting that $\beta 1$ integrin is not essential for the proliferation of basal keratinocytes in vivo [23,24].

Studies of human and mouse interfollicular epidermis revealed that the proliferation compartment in the basal layer is heterogeneous in regard to proliferation state [25,26]. Monitoring of skin labeled either continuously or pulse with tritiated thymidine revealed the existence of at least 2 distinct cell populations with different cell cycle times [27]. Based on the concept that infrequent cell division correlates to stemness label-retaining cells that retain their label due to lack of cell division were considered stem cells [13]. In a recent review, Braun et al. [28] examined other stem cell properties of label-retaining cells. Label-retaining cells had higher colony-forming potential in vitro, expressed higher integrin levels than other basal cells, and retained carcinogen [28]. One of the issues with BrdU retention to mark stem cells is the low probability of labeling a slowly cycling cell at the outset. Furthermore, if label retention is simply a marker of the proliferative history of a cell, one cannot assume that label-retaining cell is synonymous with stem cell and label-retaining cells may represent a subset of epidermal stem cells [for review, see 28].

Markers that may be useful for isolating epidermal stem cells have been found in various other tissues. Hematopoietic and muscle stem cells can be identified by looking at the ability of cells to exclude Hoechst 33342 dye [29,30]. A multidrug resistance P-glycoprotein pump shown to be present in stem cells mediates this exclusion. This multidrug resistance pump is also associated with resistance to some anticancer drugs and is overexpressed in several cancer cell lines [31]. Basal epidermal cells have also been shown to express this P-glycoprotein [32]. A recent study characterizing side population and non-side population cells in mouse epidermis showed that $\alpha 6$ integrin, $\beta 1$ integrin, Sca-1, keratin 14 and keratin 19 were all highly expressed by side population cells, and that CD34, CD71 and E-cadherin were more weakly expressed by side population cells than by non-side population cells. These results indicate that side population cells express previously established stem cell-associated proteins and are not differentiated cells [33]. However, Terunuma et al. [16] found that side population keratinocytes are distinct from the label-retaining cell population, since side population cells and label-retaining cells showed distinctly different and nonoverlapping expression profiles of $\beta 1$ and $\alpha 6$ integrins.

In addition to the examples discussed above, other potential markers such as p63, a homologue of p53 tumor suppressor gene, have been studied. p63^{-/-} mice lack all stratified squamous epithelia [34,35]. Keratin 19 [36], keratin 15 [37] and also elevated levels of β catenin [38] have been reported as putative stem cell markers.

To date, we appear to have found techniques for enriching populations of keratinocytes for early progenitors, but not at the single-cell level. Given the difficulty in finding one specific epidermal stem cell marker, further investigation is needed to determine combinations of markers that can enrich for epidermal stem cells at a single-cell level. Markers found in stem cells from other tissues, embryonic stem cell markers or even cancer stem cell markers found in tumorigenic tissues may provide useful strategies for the isolation of normal epidermal stem cells at a single-cell level.

Gene Therapy

Treating hereditary human diseases by providing functional copies of relevant genes is a concept of gene therapy that can be applied to tissue engineering [39]. Genetically engineered epidermal stem cells represent an attractive approach for gene therapy [40]. Two basic approaches to cutaneous gene transfer have been pioneered. One of the strategies for cutaneous gene transfer is an ex vivo approach in which keratinocytes are grown from skin biopsies and genetically engineered in vitro prior to grafting [39]. An in vivo approach involves direct administration of genetic material to intact epidermis, avoiding skin grafting and all the complications caused by it.

In a renewing tissue such as epidermis, cells are continuously shed into the environment, and this represents a major obstacle to designing a long-lasting gene therapy. In human epidermis, most cells are replaced every 26–28 days and therefore any persistent genetic defect needs to be present in the stem cells, with expression passed to daughter cells at each cell division [41]. For this reason, any permanent genetic correction must be aimed at the stem cell population [42].

Studies of direct gene transfer into skin have not resulted in consistent gene expression. Direct transfer of genes into the skin through both viral and nonviral vectors leads to gene expression, but often only transiently and at low levels [for review, see 43,44]. Using plasmid DNA in various liposomal spray formulations, or a gene transfer method based on biphasic lipid vesicles, resulted in gene expression at low levels [45,46]. Higher levels of expression were obtained using intracutaneous injection of DNA, liposomes, gene gun or electroporation [for review, see 47].

The *ex vivo* approach allows the possibility of using methods that increase the percentage of stem cells in the population and thus increasing the possibility of transfecting stem cells [for review, see 42]. A recent study reported success in long-term human skin regeneration from a single genetically modified stem cell. In this work, human keratinocytes were transduced by retroviral or lentiviral GFP vectors. Holoclones were then selected by their high clonogenicity, growth rate and cell number/area ratio characteristic. Skin equivalents generated from these holoclones showed normal epidermal architecture similar to that of native human skin [48], suggesting that this may be a useful approach for tissue engineered skin.

Gene therapy is a promising therapy for several inherited skin diseases, such as junctional epidermolysis bullosa, recessive dystrophic epidermolysis bullosa and xeroderma pigmentosum [44]. Junctional epidermolysis bullosa is a blistering disorder caused by mutations in genes encoding the basement membrane protein laminin 5, and resulting in defective cellular adhesion. This pathology affects about 500,000 people worldwide and is characterized by hard-to-heal blisters and infected crusts. A recent study reported successful complete epidermal regeneration on both legs of a patient throughout a 1-year follow-up after transducing primary keratinocytes with laminin B3 cDNA. Successful transduction of stem cells in the regenerated epidermis were thought to be responsible for the long-term success of the gene correction [49,50].

Another form of this disease, recessive dystrophic epidermolysis bullosa, has also been studied. This form of epidermolysis bullosa is caused by mutations in the COL7A1 gene that codes for the epidermal adhesion protein collagen VII. Φ C31 bacteriophage integrase was used to stably integrate large DNA sequences containing COL7A1 into the genome of primary epidermal cells from 4 unrelated patients with this disease [51]. Skin regenerated using these cells showed a stable correction of recessive dystrophic epidermolysis bullosa disease features, including collagen type VII expression.

Xeroderma pigmentosum is an inherited skin disease, caused by impaired nucleotide excision repair leading to UV-induced hypermutagenesis and high predisposition to cancer. Most cases of xeroderma pigmentosum arise from inactivating mutations in any of 7 genes designated from XPA to XPG that are required for nucleotide excision repair of DNA damage caused by exposure to sunlight [52]. In tissue culture, the phenotype of XP keratinocytes was normalized after retroviral transduction of wild-type XPC gene into XPC keratinocytes [53]. In another study, the subcutaneous injection of a recombinant adenovirus carrying the human XPA gene led to expression of the XPA protein in basal keratinocytes of XP mutant mice, and prevented deleterious effects of UV radiation in the skin such as squamous cell carcinoma [39].

While we steadily progress towards using gene therapy for multiple genetic skin disorders, there is little doubt that a stem cell-targeted approach could improve our chances for clinical success. Stem cell maintenance will be important both during gene correction procedures as well as during engineering the tissue construct.

Other Multipotent Stem Cell Sources

Recently, multiple new sources of multipotent cells have become available that might be used either to generate the epidermal component of tissue-engineered skin or to improve the functionality of the dermal component.

A promising source of multipotent epidermal stem cells could be provided by the reprogramming of somatic cells into multipotent epidermal stem cells. Takahashi et al. [54] recently successfully generated induced pluripotent stem cells from adult human dermal fibroblasts by transduction of Oct3/4, Sox2, Klf4 and c-Myc. These induced pluripotent stem cells were shown to be indistinguishable from embryonic stem cells in morphology, proliferation, surface antigens, gene expression, pluripotency and telomerase activity, and were also capable of producing germ line-competent chimeras. The same group modified this protocol to reprogram dermal fibroblasts into induced pluripotent stem cells without the presence of Myc retrovirus, which was responsible for teratomas in previous chimeric progeny as well as NODSCID mice transplanted subcutaneously with those cells [55]. Other work also demonstrated reprogramming of human fibroblasts into embryonic stem cells using the same factors [56], or Oct4, Sox2, Nanog and Lin28 [57]. These cells could potentially be used in the construction of tissue-engineered skin.

Neural crest stem cells also represent a multipotent population with potential use in tissue engineering. Neural crest cells originate from the dorsal margins of the neural folds during vertebrate development. The neural crest cell is a multipotent cell population that can give rise to a diversity of neural and nonneural cell types in the adult, such as melanocytes, neurons and glial cells of the sensory and autonomic ganglia of the peripheral nervous system and some endocrine cells [58]. Recently, evidence for the persistence of neural crest cells in postnatal and adult tissues suggests that these cells could be an excellent source of pluripotent stem cells [for review, see 58,59]. In mammalian skin, skin-derived neural progenitors were isolated and expanded from the dermis of rodent skin and adult human scalp and could differentiate into both neural and mesodermal progeny [60,61]. Skin-derived neural progenitor cells were isolated based on the sphere formation of floating cells after 3–7 days of culture in uncoated flasks with epidermal growth factor and fibroblast growth factor, and characterized by the production of nestin and fibronectin, markers of neural precursors. In addition, skin-derived neural progenitor cells were identified as neural crest derived by the use of Wnt1 promoter driving LacZ expression in the mouse. Some of the X-gal-positive cells were found in the skin of the face, as well as in the dermis and dermal papilla of murine whisker [62]. These skin-derived neural crest cells have already shown promising results in regenerative medicine such as the promotion of regenerative axonal growth after transplantation into injured adult mouse sciatic nerves [63] or spinal cord repair [64], resulting in the recovery of peripheral nerve function.

A mixture of hematopoietic and mesenchymal progenitors has been used in patients with nonhealing chronic skin wounds [65], as well as mesenchymal stem cells, or a subset of these cells with a vascular endothelial phenotype. Bone marrow-derived stem cells show an important plasticity and are capable of regenerating blood vessels, skeletal muscle and cardiac muscle [66–68]. Bone marrow-derived stem cells have been used in the treatment of chronic wounds [65; for review, see 2,69]. Badiavas and Falanga [65] published clinical results using autologous bone marrow cells directly applied on chronic cutaneous ulcerations from 3 patients

with wounds resistant to standard conventional treatment for more than 1 year. All patients showed improvement of their wounds within days following administration, characterized by a steady overall decrease in wound size and an increase in the vascularity of the dermis and the dermal thickness of the wound bed [65]. While new dermal cells observed did not stain with hematopoietic stem cell markers, some stained with endothelial progenitor markers, and it was thought that cells responsible for this plasticity were mostly mesenchymal stem cells. These cells are characterized by their ability to differentiate into multiple cell types including osteoblasts, chondrocytes, endothelial cells and even neuronal-like cells [70]. Recently, an important pluripotency has been attributed to mesenchymal stem cells, as they have been shown to differentiate into a number of nonmesoderm cell types, including ectodermal and endodermal cells [71]. Thus, bone marrow may be an important source of pluripotent stem cells for tissue-engineered skin.

Finally, circulating peripheral blood also contains cells derived from bone marrow, termed fibrocytes, that can migrate to a site of tissue injury in a wound chamber model [72]. These bone marrow-derived mesenchymal progenitors are found to be upregulated systemically in the peripheral blood of burn patients [73,74; for review, see 72]. The fibrocyte of peripheral blood and the mesenchymal stem cell populations found in bone marrow might overlap. However, either one or both could potentially be used in tissue-engineered skin.

Thus, sources of pluripotent cells with potential application for tissue-engineered skin have become abundant and these sources need to be tested for their ability to improve resultant constructs. These pluripotent cells may have applications for both the epidermal and dermal portions of tissue-engineered skin.

Conclusion

In conclusion, there is still much work to be done to attain a long-lasting and cosmetically acceptable tissue-engineered skin. The choice of suitable seed cells, the purity of epidermal stem cell populations used as well as adequate culture conditions are all factors important to the long-lasting engraftment of a regenerated skin. In addition, it is likely that tissue-engineered skin of the future will comprise a more complex reconstruction, utilizing first epidermal stem cells for generation of the epidermal component, along with the addition of stem cells from other relevant tissue lineages (for example, melanocytic). For the dermal construct, it is likely that endothelial, mesenchymal, neural and/or other primitive stem cells may help with generation of dermal components including a new vasculature. Such a construct should allow the skin to fulfill its many normal functions: barrier formation, pigmentary defense against UV irradiation, thermoregulation, as well as mechanical and aesthetic functions [69]. Furthermore, improving tissue-engineered skin and stem cell-targeted cutaneous gene transfer will be important for successful gene therapy. In the meantime, it is essential that we isolate epidermal stem cells at the single-cell level to better define them as well as learning to manipulate them for use in tissue engineering as well as many other therapeutic endeavors.

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