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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
Transduction of a Preselected Population of Human Epidermal Stem Cells: Consequences for Gene Therapy

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Continuously renewing tissues, such as the epidermis, are populated by a hierarchy of dividing transient amplifying cells, which are maintained by stem cells. Transient amplifying cells divide to maintain the tissue, but they are limited to a finite number of cell divisions before they differentiate and are sloughed. Only the stem cells remain for the life of the tissue. Thus, it is critical to target stem cells when designing gene therapy regimes for genetically inherited diseases, such as epidermolysis bullosa simplex (EBS). Unfortunately, isolating pure epithelial stem cells has been problematic. In this study, we used rapid adherence to collagen type IV to successfully enrich for epidermal stem cells from adult human skin. These preselected stem cells were slow to proliferate, but they ultimately formed large colonies. When recombined with the dermal substrate AlloDerm, the stem cells re-formed a stratified squamous epidermis within 1 week after raising the AlloDerm to the air-liquid interface. These organotypic cultures grew continuously and, even after 6 weeks in culture, they maintained a proliferative basal layer. When transduced with a retroviral LacZ vector, preselected stem cells formed β-galactosidase-positive clones in submerged and organotypic cultures. Transduced cells showed persistent expression through 12 weeks in organotypic culture, demonstrating the feasibility of using preselected stem cells for gene therapy. Currently, we are developing two models of EBS to test a gene therapy approach, which is based on the premise that EBS stem cells with a mutant keratin (K)14 gene corrected to wild type will have a growth advantage over noncorrected EBS stem cells.

Stratified squamous epithelia are continuously renewing tissues that are maintained by the division of cells in the proliferative basal layer to replace cells in the outer layer, which are sloughed into the environment. This mechanism of balancing the rate of cell division with the rate of cell loss is essential for epithelial homeostasis and must be maintained for life (1). Although the mechanisms for controlling the relationship between cell division and cell differentiation are not clear, it is believed that stratified squamous epithelia consist of a hierarchy of dividing cells maintained by a small subpopulation of slowly cycling stem cells (reviewed in Refs. 2 and 3). These stem cells rarely divide but, when they do, they produce both stem cells and daughter cells, called transient amplifying cells. The transient amplifying cells divide more often than the stem cells, but they are limited to a finite number of cell divisions before they differentiate, move out of the proliferative compartment, and ultimately are sloughed into the environment. Only the stem cells remain for life (4). Thus, in a renewing tissue, such as the epidermis of the skin, any gene therapy approach must be targeted toward the stem cell population.

Since the molecular basis for many of the blistering skin diseases is known (reviewed in Refs. 5 and 6), several could be possible target diseases for a gene therapy approach. It is known that a single gene mutation is the direct cause of the specific structural abnormalities seen in most of these diseases. For example, in epidermolysis bullosa simplex (EBS) a point mutation in either the basal keratin K5 or K14 genes results in an abnormal cytoskeleton, weakening the basal cells, which rupture upon exposure to mild mecami-
cal trauma (7). In epidermolytic hyperkeratosis, point mutations in the suprabasal K1 or K10 genes cause blisters seen only in the suprabasal spinous cells (8). Other blistering diseases show ruptures at the junction of the epidermis and dermis, either within (junctional EB) or just beneath (dystrophic EB) the basement membrane zone. The dominant and recessive types of dystrophic EB have mutations in collagen type VII (9,10), which makes up the anchoring fibrils that extend from the basement membrane to the upper papillary dermis. Junctional EB is caused by mutations in various other basement membrane proteins, including both the α6 and β4 integrins (11,12), the α3, β3, and γ2 chains of laminin 5 (10,13), and BP180 (14). Although gene therapy has yet to be tried in any of these blistering skin diseases, an ex vivo attempt has been made in the hyperkeratotic disorder lamellar ichthyosis, in which defects in the transglutaminase I enzyme have been shown to be causal (15). A retroviral transfer of the genetic information encoding wild type transglutaminase into cultured keratinocytes from these patients resulted in the formation of a normal human epidermis in a severe combined immunodeficiency (SCID) mouse model. However, the effect was short lived (16), suggesting that the stem cells had not been successfully transduced. Because human epidermal cells are replaced every 20 days (17), any persistent genetic treatment must be directed toward the stem cell genome. This is an important concept in the treatment of these genetically inherited skin diseases (18). Our study is based on the idea that preselecting the epidermal stem cells increases the chance of transfecting these cells, and once the recombinant gene is integrated, the transduced stem cells and all of their progeny will show persistent expression of this gene.

ENRICHMENT OF EPIDERMAL STEM CELLS

Previously, we were able to take advantage of two specific characteristics of epidermal stem cells: They rarely divide, but they do divide when necessary, for example, during a growth phase. After injecting young growing mice with four pulses of 3H-thymidine, then letting the mice grow (without more injections), we found a small percentage of the basal cell population in all epithelia that retained the label for at least 90 days (19). We called these cells label-retaining cells (LRCs) and determined that they showed many of the characteristics associated with stem cells in other tissues (19,20). Other information about integrins suggested that epidermal stem cells might adhere to the basement membrane more than the transient amplifying cells would, and this adhesion might be mediated through differential expression of specific integrins (21,22). We used this chemotactic property to separate stem cells from the basal cell population in label-retaining mice and found that while the total number of adherent basal cells increased as time for adherence increased, all the LRCs had adhered by 10 min (23). Thus, rapid adherence yielded a population enriched for stem cells. In this study, we used rapid adherence to collagen type IV to preselect successfully epidermal stem cells from adult human skin. Pieces of skin were floated on 0.25% trypsin overnight at 4°C, then the epidermis was teased away from the dermis using fine forceps, and the basal cells were dissociated by gently shaking the epidermal sheets in the medium. Epidermal stem cells were preselected by plating the basal cells on collagen type IV–coated culture dishes for 10 min. Nonadherent cells were rinsed off the dishes and the adherent stem cells were used in further studies.

GROWTH OF HUMAN EPIDERMAL STEM CELLS IN CULTURE

One of the primary tests for stem cells from all tissues is in vitro clonogenicity (2,24). For epidermal cells, only the large colonies that can be propagated are believed to be derived from the stem cells (25). In our culture system, we saw very few large colonies when plating the total basal cells from adult human skin at clonal density. To determine whether the preselected epidermal cells formed large colonies, we plated the adherent cells at clonal density and counted the number of cells in the clones that formed. Although these preselected stem cells were slow to begin proliferation, they formed very large colonies on the collagen substrate (Fig. 1a). These cultures became confluent in approximately 3 weeks and were easily subcultured. At this time, cells have been passaged at least 12 times, producing viable cultures that have yet to reach a senescent stage, suggesting that although stem cells may be slow to enter a proliferative phase, they may have a great capacity for proliferation.

The main function of the skin is to provide a protective barrier for all the internal tissues of the body. When this barrier is broken, for example, when the skin is burned or the epidermis is ruptured because a genetic defect causes disruption in the structural integrity, it can be life-threatening as the body loses fluids and is exposed to harmful factors in the environment. Current technology for replacing large areas of damaged skin consists of placing either a temporary skin or a cultured graft from the patient onto the damaged area (26). Most skin substitutes rarely last longer than a month or two and require replacement several times before the patient's own skin grows back. How-
Figure 1. Growth of preselected human epidermal stem cells in submerged and organotypic culture.
(A) Large clone grown from a single human epidermal stem cell. Stem cells were selected by 10-min adherence to collagen type IV-coated culture dishes and grown for 10 days in submerged culture. Note that small proliferative cells appear to grow as a cluster (arrow) in the middle of the clone, while larger differentiated cells are found at the outer edge of the clone. (B) Re-formation of an epidermis by preselected human epidermal stem cells grown on AlloDerm in organotypic culture for 6 weeks. Note the proliferating cell (arrow) in the basal layer, indicating that this bioengineered skin is still living and producing well-structured suprabasal layers, despite the extensive piling up of the stratum corneum cells (SC).

Figure 2. Expression of an integrated gene transduced into preselected human epidermal stem cells via a retroviral construct (MFG.LacZ).
Preselected human stem cells were transduced with MFG.LacZ in a submerged culture, then grown on AlloDerm in an organotypic culture, and stained with x-gal, which stains LacZ-expressing cells blue. (A) One preselected transduced stem cell produced a βgal + (blue) clone of basal cells shown here at 2 weeks in vitro. The blue stain denotes βgal + colonies in the AlloDerm organotypic culture at 2 weeks (B), 6 weeks (D), 8 weeks (E), and 12 weeks (F). (C) Ten-fold higher power of the clones in B. Note the increase in number of βgal + colonies with the time of growth on AlloDerm.
ever, autologous grafts of cultured cells, which have been expanded at least ten-fold, have been shown to last for years (27–29). Although these grafts may not form all of the epidermal appendages, such as hair follicles and sweat glands, they do re-form a structured epidermis with spinous, granular, and cornified cells overlying the basal cell compartment, suggesting that epidermal stem cells have survived and possibly replicated during the culturing procedure.

In this study, we tested the ability of the preselected stem cells to re-form a stratified squamous epidermis. Since keratinocytes must be associated with a subepithelial connective tissue component or with fibroblasts in order to form a structurally complete epithelium (27,30,31), we recombined the preselected human epidermal stem cells with the dermal substrate, AlloDerm (LifeCell Corporation, The Woodlands, TX), and grew these bioengineered tissues in organotypic cultures. AlloDerm is a de-epithelialized, freeze-dried human dermis from which all cells have been removed, leaving the basement membrane complex and the dermal connective tissue complex intact. It has been shown to replace the lost dermis in severe burn wounds by allowing the immediate infiltration of the human host’s fibroblasts and endothelial cells, and the migration of epidermal cells (32). In this study, the preselected epidermal stem cells re-formed a stratified squamous epidermis 1 week after the AlloDerm was raised to the air-liquid interface. These organotypic cultures grew continuously and, even at 6 weeks in culture, still maintained proliferation in the basal cell layer (Fig. 1b). The re-formed epidermis appeared morphologically normal, except for the piling up of squames that is to be expected in a 6-week culture.

**EPIDERMAL STEM CELLS EXPRESS AN INTEGRATED RECOMBINANT GENE**

Several techniques for expressing recombinant genes using retroviruses in keratinocytes have been previously developed (33–35), although most report that DNA expression drops rapidly after 4 days (34,36). Fenjves et al. (37) suggested that the failure to maintain long-term expression of a retroviral-transduced gene in keratinocytes may be due to selective inactivation of viral promoters in vivo after transplantation. However, since three recent studies have found long-term expression in a subpopulation of the transduced cells (38–40), it seems more likely that the earlier procedures failed to transduce stem cells. This may have happened because integration of retrovirally transported DNA occurs only if there is active cell division at the time of infection (41). When the total basal cell population is transduced, the slowly cycling stem cells would be less likely to be transduced than the more rapidly cycling transient amplifying cells. Furthermore, since the stem cells make up a very small percentage of the total basal cell population, transduction of this small population may be hard to detect in the much larger population of transient amplifying cells.

To overcome these problems in this study, we preselected the stem cell population, thereby reducing the number of transient amplifying cells in the population that would be transduced. We preselected human stem cells from dissociated basal cells by 10-min adherence to collagen type IV, then concentrated the preselected stem cell population for growth in the culture. To increase the chances of transducing these slowly cycling stem cells, we allowed the cells to begin replication by growing them in culture for 17 hr before transducing with the retroviral vector MFG carrying a LacZ reporter gene (42). This method resulted in transduction of ≈50% of the preselected epidermal stem cells. A future approach could include the neomycin resistance gene in the MFG vector, which would allow for G418 selection of the transduced stem cells, yielding a 100% transduced population. For this study, we used the 50% transduced cultures. After transduction, the stem cells were expanded by further culture, then transferred to AlloDerm for growth in organotypic culture. Clones of β-galactosidase (Bgal+) basal cells were clearly visible in the submerged culture (Fig. 2a) and on top of the AlloDerm as early as 2 weeks later (Fig. 2, b and c). The number of colonies increased throughout the time course of the experiment and expression of the LacZ reporter gene was still evident at 6, 8, and 12 weeks in organotypic culture (Fig. 2, d–f). These results demonstrate the feasibility of retroviral transduction for preselected human epidermal stem cells and that, in organotypic culture, the transduced stem cells show persistent expression of the recombinant gene.

**FUTURE PROSPECTS**

**Selection of a Target Disease for Gene Therapy**

Having demonstrated the feasibility of genetically modifying an enriched population of epidermal stem cells and observing long-term expression in organotypic culture, it is possible to contemplate applying this strategy in the development of a gene therapy approach for skin diseases. As discussed in the introduction, considerable progress has been made in defining the molecular basis of most of the major types of inherited blistering skin diseases. Of particular interest is EBS, since blister formation occurs within the basal
layer of the epidermis, a putative location for epidermal stem cells. On the basis of anecdotal data, it has been suggested that wild type stem cells may have a growth advantage over EBS stem cells. Some skin disorders are characterized by a mosaic pattern with alternating stripes of affected and unaffected skin that follow the lines of Blaschko. These nonrandom patterns are thought to be caused by a postzygotic mutation that occurs during embryogenesis. Paller et al. (43) have recently discovered that one of these disorders, epidermal nevus of the epidermolytic hyperkeratotic type, is caused by a mutation in the K10 gene. With the exception of the mosaic pattern, the clinical features of this disease are similar to those of the autosomal dominant disease, epidermolytic hyperkeratosis, which can be caused by mutations in either the K1 or K10 genes (8). Interestingly, a mosaic form of EBS has never been reported. It has been suggested that basal stem cells carrying a postzygotic mutation in K5 or K14 would have a selective disadvantage and would be rapidly displaced by wild type basal stem cells, which can move laterally (44). In contrast, keratinocytes exhibiting fragility only in the suprabasal layers, as in the mosaic form of epidermolytic hyperkeratosis, would not be selected against because stem cells in the basal compartment do not manifest the defect.

We are currently developing two models to test a gene therapy approach to ameliorate EBS, which is based on the premise that genetically corrected EBS stem cells will have a growth advantage over defective EBS stem cells. One model consists of growing human EBS epidermal cells on the backs of severe combined immunodeficiency (SCID) mice. The other model is a transgenic mouse that will mimic the human disease at the genetic level by the introduction of a single point mutation into one K14 allele, using embryonic stem cells and a knockin strategy. We will select defective human or mouse EBS stem cells and attempt to correct the defect either by replacement of the mutant allele with a wild type allele, or by suppressing expression of the mutant allele. Corrected EBS stem cells will be expanded and transplanted back to the appropriate model to determine whether a growth advantage will allow selective repopulation of the graft site by corrected stem cells.

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