Skin tissue engineering*

David J. Wong[1] and Howard Y. Chang[1,§], [1]Program in Epithelial Biology, Stanford University, Stanford, CA 94305

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Abstract

The skin is the largest organ of the body and is critical to survival of the organism as a barrier to the environment and for thermal regulation and hydration retention. In order to serve these critical functions, the skin is constantly undergoing renewal and possesses the capacity for repair of wounds, which are dependent on the multiple types of stem cells in the skin. Engineered skin substitutes have a critical medical application to patients with extensive burn wounds. However, current skin substitutes do not restore the normal skin anatomy, lacking the normal appendages of skin including hair follicles, sebaceous glands, and sweat glands as well as the normal mechanical properties of the skin. Advances in stem cell biology and skin morphogenesis hold promise for the ability to markedly improve the engineering of skin substitutes that would ideally be indistinguishable from normal skin.

1. Introduction

Recent advances in our understanding of stem cells and tissue morphogenesis may enable the engineering of replacement tissues that restores the normal anatomy and physiology of the skin and other tissues. For tissues and organs to maintain a homeostasis between cell loss and cell replacement, they must possess stem cells that are capable of self-renewal as well as differentiation. Stem cells are also critical for response to trauma to replace damaged tissue. Sources of stem cells for tissue replacement include tissue-specific somatic stem cells. Alternatively, embryonic stem cells, which are pluripotent cells derived from the inner cell mass of the embryonic blastocyst, can be differentiated

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§To whom correspondence should be addressed. E-mail: howchang@stanford.edu

into the desired tissue-specific stem cell. The use of human embryonic stem cells has been controversial for ethical reasons because of the need to isolate them from human embryos. The recent finding that normal human fibroblasts can be reprogrammed into pluripotent cells (iPS) indistinguishable from embryonic stem cells (Jaenisch and Young, 2008), reopens the possibility of using such derived embryonic stem cells for tissue engineering. The challenge to engineer skin that is structurally and functionally equivalent to normal skin requires not only the ability to form the various skin-specific stem cells but to also induce proper signaling required for morphogenesis of this complex organ.

2. Current skin substitutes

The skin is the largest organ of the human body, representing approximately one-tenth of the body mass, and is necessary for animal survival. This organ serves several important functions, including physical barrier to the external environment, thermal regulation, and retention of normal hydration. When a large percentage of skin is lost, cultured epithelial sheets are routinely used to make autologous grafts, which can be lifesaving for patients with extensive burns (De Luca et al., 1989; Gallico et al., 1984; Romagnoli et al., 1990). Autologous keratinocytes can be isolated and cultured into cohesive sheets of epithelium that can be transplanted onto large skin defects on the patient. Clonogenic keratinocytes, termed holoclones, can be isolated from skin and serially propagated in culture for over 140 doublings, and have been shown to be bona fide multipotent stem cells based on their ability to renew multiple lineages in the skin (Claudinot et al., 2005; Mathor et al., 1996). These engrafted stem cells within these epithelial sheets ensure restoration and renewal of the epidermis for years.

Growing the epidermal stem cells on fibrin matrices or allogeneic dermis has proven to be advantageous. These support substrates have substantially improved the take rates of the grafts, improved the ease of handling and manipulation of the grafts, and decreased the wound edge retraction and scarring. Culturing autologous epidermal stem cells makes it possible to obtain large epithelial sheets for transplantation from a small skin biopsy from the patient, but this process requires several weeks. Growing the stem cells on a substrate decreases the time needed to make large epithelial sheets from a small skin biopsy because the epithelia on the substrate does not need to achieve full confluence prior to transplantation. Moreover, epidermal stem cells on fibrin matrices or allogeneic dermis confer the ability to regenerate the normal undulated dermal-epidermal junction and the superficial portion of dermis, termed the papillary dermis (Ronfard et al., 2000).

However, these epidermal stem cell grafts are not capable of restoring a fully functional skin. Epidermal appendages, including hair follicles, sebaceous glands, or sweat glands are not regenerated after transplanting these grafts of epidermal stem cells, suggesting that complex epithelial and mesenchymal interactions are necessary to form appendages. In addition, the grafts do not restore the mechanical properties or aesthetic appearance of the original skin. Advances in stem cell biology and skin morphogenesis have the potential to improve the engineering of skin that can replace the normal functionality and aesthetics of normal skin.

3. Skin anatomy

Engineering skin equivalent to normal skin has been challenging because of the structural and functional complexity of the skin organ. The skin organ is composed of diverse cells derived from three distinct embryonic origins: neurectoderm, mesoderm, and neural crest. The skin is composed of three layers: the epidermis, dermis, and hypodermis (see Figure 1). The epidermis, the outermost layer, is primarily composed of stratified squamous epithelium of keratinocytes, which is derived from neurectoderm and comprises over ninety percent of epidermal cells. Keratinocytes are responsible for the cohesion of the epidermal structure and the barrier function. Pigment-containing melanocytes of neural crest origin, antigen-processing Langerhans cells of mesoderm origin, and pressure-sensing Merkel cells of neural crest origin also reside within the epidermis.

The dermis is a connective tissue that is responsible for the mechanical properties of the skin. It is composed of fibroblasts of mesoderm origin, which lie within an extracellular specialized matrix. Collagens are interwoven with elastin, proteoglycans, fibronectin, and other components. The epidermis and dermis are connected by a basement membrane that is composed of various integrins, laminins, collagens, and other proteins that play important roles in regulating epithelial-mesenchymal cross-talk. The superficial papillary dermis is arranged in ridge-like structures called the dermal papillae, which contains microvascular and neural networks and extends the surface area for these epithelial-mesenchymal interactions. Sebaceous glands, eccrine glands, apocrine glands and hair follicles are of neurectoderm origin and develop as downgrowths of the epidermis into the dermis. In addition, the dermis also contains blood vessels and lymphatic vessels of mesoderm origin, and sensory nerve endings of neural crest origin. The hypodermis, which

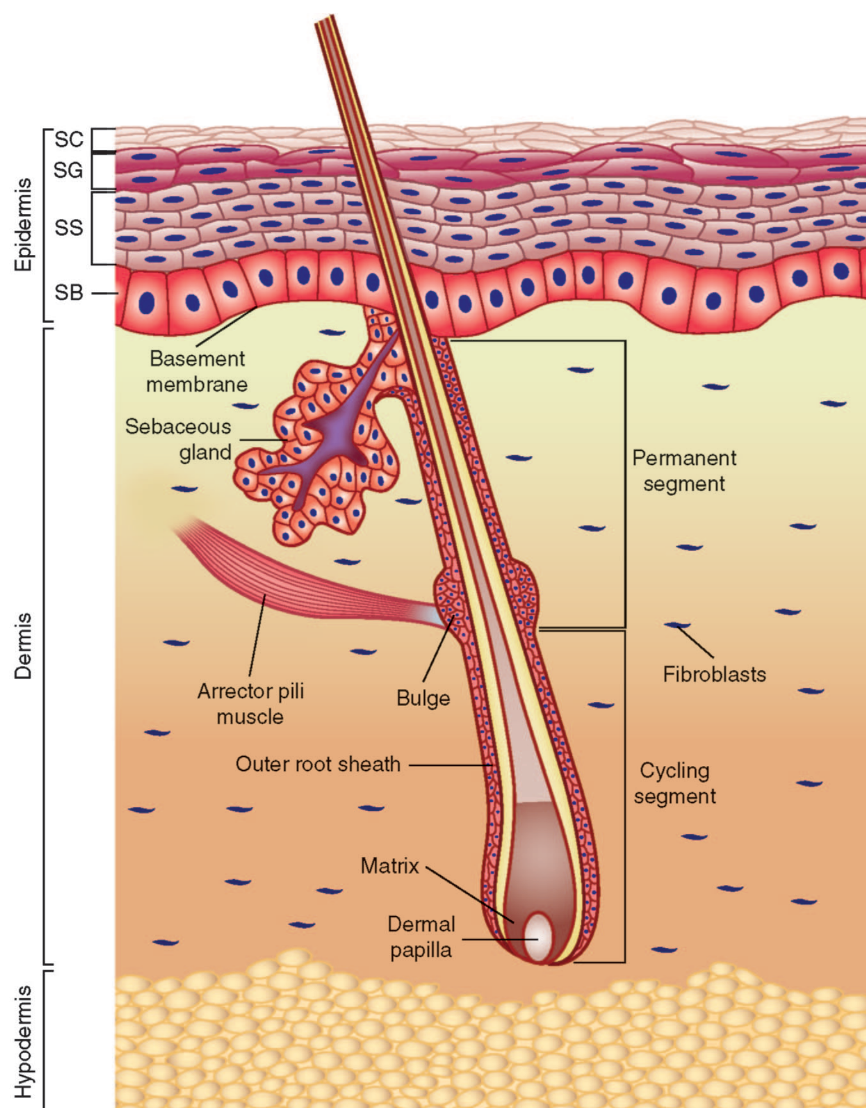

Figure 1. Anatomy of the skin. Skin is composed of three layers, starting with the outermost layer: the epidermis, dermis, and hypodermis. Epidermis is a stratified squamous epithelium that is divided into four layers, starting with the outermost layer: stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB). Outer root sheath of the hair follicle is contiguous with the basal epidermal layer. Stem cell niches include the basal epidermal layer, base of sebaceous gland, hair follicle bulge, dermal papillae, and dermis.

is deep to the dermis, is composed primarily of adipose tissue of mesoderm origin, and separates the dermis from the underlying muscular fascia.

4. Skin stem cells

In order to perform and maintain its barrier function while continually exposed to mechanical and chemical trauma from the environment, skin must undergo constant turnover throughout adult life and have the ability for wound repair to replace damaged cells. Similar to the role of hematopoietic stem cells in the bone marrow to continuously generate mature blood cells, the ability for skin tissue to self-renew is conferred by skin-specific stem cells. Multipotent or unipotent skin stem cells are slowly-cycling cells that reside in at least five distinct niches in the skin: basal (innermost) layer of epidermis, hair follicle bulge, base of sebaceous gland, dermal papillae, and dermis (see Figure 1). Not only are these stem cells critical for the long-term maintenance of the skin tissue but also are activated by wounding to proliferate and regenerate the tissue.

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Skin epidermis is undergoing constant rejuvenation by shedding off its surface cells while replacing them with new cells in the proliferating basal layer that differentiate and move outward into the multiple layers of terminally differentiating cells. This self-renewing structure maintains an effective barrier for protection from the environment and maintaining normal hydration. Through asymmetrical cell division, epidermal stem cells continually give rise to progeny that execute the terminal keratinocyte differentiation program: withdraw from the cell cycle, suppress their integrin and laminin expression, move outward toward the skin surface until they slough off after approximately 4 weeks, and are continually replaced by new progeny. Lineage tracing analyses have demonstrated that the basal epidermal layer is organized into epidermal proliferative units with at least one epidermal stem cell per unit. Chimeric skin generated by infection with retrovirus expressing LacZ revealed discrete columns of blue cells extending from the basal layer to the outermost layer of differentiated cells, the stratum cornuem (Ghazizadeh and Taichman, 2001; Kolodka et al., 1998; Mackenzie, 1997). Further support for epidermal proliferative units of self-renewal was demonstrated by patchy GFP-positive columns of cells observed in stop-EGFP transgenic mice from sporadic mutations (Ro and Rannala, 2004). However, the number of epidermal stem cells within an individual epidermal proliferative unit and their ability to give rise to other lineages within the skin, such as sebaceous glands and hair follicles, remains unclear. Future studies are needed to identify markers to be able to efficiently purify and analyze interfollicular epidermal stem cells.

Hair follicles cycle through growth and degeneration throughout adult life, a process which requires stem cells for continual hair regeneration. The hair cycle is divided into three stages: growth phase called anagen, degenerative stage called catagen, and resting stage called telogen. Hair follicle stem cells are typically quiescent but are stimulated to give rise to progeny that proliferate and differentiate to regenerate the hair follicle during the anagen phase of the hair cycle and in response to wound. Based on their quiescent state and their ability to form large colonies in vitro, these stem cells have been found to be located in a discrete location in the hair follicle called the bulge, which does not degenerate during the hair cycle. The bulge is a part of the outer root sheath that is contiguous with the epidermis located just below the sebaceous gland near the attachment site of the arrector pili muscle to the hair follicle (see Figure 1). These stem cells were initially identified by label retaining experiments in the hair bulge, which mark the slowest proliferating cells by administering BrdU for a week in newborn mice and then assessing label retention in the skin after four weeks (Cotsarelis et al., 1990). In addition, cells dissected from segments in the bulge region from rat whisker follicles and segments extending from the bulge to the lower outer root sheath from adult human skin more efficiently formed highly proliferative colonies and can be passaged long-term in vitro (Kobayashi et al., 1993; Rochat et al., 1994). Unlike epidermal stem cells, hair bulge stem cells have been successfully enriched and isolated by three approaches. Fuchs and colleagues used a tetracycline-regulated histone H2B-GFP transgenic mouse in which four weeks after tetracycline administration, only the bulge cells label brightly (Tumbar et al., 2004); they also employed a K14-GFP transgenic mouse in combination with antibodies against α6-integrin and CD34 (Blanpain et al., 2004). Alternatively, Cotsarelis and colleagues used K15-GFP transgenic mouse with antibody against α6-integrin (Morris et al., 2004). Bulge stem cells normally only generate the hair follicle lineages to maintain homeostasis, but they do contribute to the epidermis as an initial repair response when the epidermis is wounded (Ito et al., 2005; Oshima et al., 2001; Taylor et al., 2000). However, the molecular signals that mobilize the bulge stem cells for epidermal repair remains unknown.

Melanocyte stem cells also reside in the hair follicle bulge and give rise to the melanocytes in both the hair matrix and epidermis (Nishimura et al., 2002). Use of melanocyte stem cells will be important for matching the color of the patient complexion as well as the relative color of the body site. In addition, the ability to engineer skin with melanocyte stem cells will have applications to pigmentary disorders such as vitiligo.

Sebaceous glands, which are attached to the hair follicles, also undergo continual turnover. Stem cells at the base of the sebaceous gland are needed to continually generate terminally differentiated sebocytes, which degenerate to release lipids and sebum through the hair canal and lubricate the skin surface (Ghazizadeh and Taichman, 2001).

5. Fibroblasts and dermal stem cells

Although the dermis may seem to be just a support substrate for the epidermis, it has become clear that it is a complex structure that has important signaling communication with the epidermis, which is vital to the homeostasis of the skin. The primary cellular component of the dermis is the fibroblast, which is of mesenchymal origin. The dermis is continually being remodeled which is maintained by mesenchymal stem cells. These mesenchymal stem cells give rise to not only fibroblasts, but also nerves and adipocytes. Fibroblasts produce and organize extracellular matrix and communicate with each other and with the epidermis through various signaling pathways. Previous work using genomic expression profiling demonstrated that fibroblasts from different anatomic regions of the body are

heterogeneous (Rinn et al., 2006). They have distinct genome-wide gene expression patterns that are site-specific and can be divided anatomically into anterior-posterior, proximal-distal, and dermal-nondermal. There is also heterogeneity in fibroblasts from the same anatomic site; fibroblasts in the papillary dermis (superficial dermis) are distinct from those in the reticular dermis (deep dermis). The composition and organization of the extracellular matrix is also different between the papillary dermis and reticular dermis. In addition, there is a unique group of fibroblasts that lie around the hair follicles.

A stem cell distinct from mesenchymal stem cells, which has been termed SKP for skin-derived precursor, has been isolated from the dermis as floating spheres using a technique previously devised for isolating neural stem cells from the brain (Toma et al., 2001). SKPs are neural-crest derived, at least in facial skin, have a niche in the dermal papillae of hair and whisker follicles, and appear to be multipotent based on their ability to differentiate into cells of both neural and mesodermal origin in vitro (Fernandes et al., 2004). However, whether SKPs give rise to endogenous cells such as fibroblasts, Merkel cells, Schwann cells, or melanocytes remains unclear.

6. Skin morphogenetic signaling

Skin engineering has also been challenging because of the complex signaling pathways that control self-renewal, proliferation, and differentiation, which are critical for maintaining homeostasis of the constantly regenerating skin organ. One of the key regulators is p63, which plays a critical role in regulating self-renewal and long-term proliferative capacity of epidermal stem cells. Its critical role was first suggested by the observation that p63 knockout mice have impaired ability to form epidermis (Mills et al., 1999; Yang et al., 1999). The delta N isoform of p63 is normally expressed in basal cells in the epidermis, and p63 is down-regulated once cells migrate away from the basal layer. Reducing p63 by small interfering RNAs (siRNAs) in an organotypic skin culture system reduced cell proliferation and terminal differentiation (Truong et al., 2006). When both p63 and p53 are knocked-down by siRNAs, cell proliferation was normal, suggesting that p63 regulates cell proliferation by inhibiting p53. However, p53 siRNA did not rescue the block of terminal differentiation, suggesting that p63 deficiency inhibits differentiation independent of p53. p63 was recently shown to be regulated by a skin microRNA, miR-203, which is expressed in suprabasal epidermis and inhibits p63 expression, thereby repressing proliferation and promoting differentiation (Yi et al., 2008).

Complex epidermal and dermal signals act together to regulate hair morphogenesis in embryonic skin. During embryonic development, a cluster of cytokeratin 17-expressing epidermal cells termed an epithelial placode forms over a dermal condensate that has alkaline phosphatase activity. Epithelial-mesenchymal communication through Wnt activation and subsequent downstream sonic hedgehog signaling, drives the downgrowth of the epidermis to form a follicle that will cyclically produce hair. Signals that stabilize beta-catenin are central to the decision of stem cells to differentiate into either hair follicle rather than epidermis. When excess beta-catenin is stabilized in the epidermis of transgenic mice, ectopic hair follicles form in the interfollicular epidermis (Gat et al., 1998). In contrast, hair follicle morphogenesis is inhibited by conditional ablation of beta-catenin or ectopic expression of Wnt inhibitor Dickoff 1 (Dkk1) (Andl et al., 2002; Huelsken et al., 2001). When levels of stabilized beta-catenin are transiently elevated, hair follicles precociously enter anagen (Lo Celso et al., 2004; Van Mater et al., 2003). It was previously thought that hair follicles could only form during embryonic development and that loss of the adult hair follicle is permanent. However, the remarkable finding that a wound stimulus and Wnt pathway activation can trigger de novo hair follicle formation from epidermal stem cells that parallels embryonic follicle development (Ito et al., 2007), suggests that engineering skin with normal hair follicles is a real possibility. Intriguingly, hair follicle neogenesis occurred only in the center of large wounds away from the wound edge, which suggests that unwounded skin may possess a diffusible, lateral inhibitor of hair and skin regeneration.

Dermal signals have been shown to have the ability to program epidermal cell identity. Pioneering work studying embryonic development in birds demonstrated that the mesenchymal component of the skin controls the formation and patterning of feathers (Lin et al., 2006; Sengel, 1990). Similarly, human palmoplantar epidermis has unique properties compared to skin on other parts of the body, notably expressing keratin 9, lacking hair, and having a lower density of melanocytes, that are regulated by the underlying dermis. Palmoplantar fibroblasts have been found to express DKK1, which inhibits not only hair follicle development, but also melanocyte proliferation and production of melanosomal proteins and melanin (Yamaguchi et al., 2004). In addition, coculturing nonpalmoplantar keratinocytes with palmoplantar fibroblasts caused the keratinocytes to induce keratin 9 expression (Yamaguchi et al., 1999). This ability of palmoplantar fibroblasts to induce epidermal keratin 9 expression was markedly reduced with depletion of the distal-specific gene HOXA13 by siRNA (Rinn et al., 2008). HOXA13 is required to maintain the distal-specific transcriptional program, including WNT5A expression, in adult human fibroblasts, and remarkably, addition of recombinant purified WNT5A protein to the coculture with HOXA13-depleted distal fibroblasts or to the

keratinocytes in the absence of the cocultured fibroblasts, rescued epidermal keratin 9 expression (Rinn et al., 2008). These results suggest that similar to feather patterning in birds, the embryonic HOX pattern in dermal fibroblasts serves as a source of positional memory for human skin in both homeostasis and regeneration.

7. Skin gene therapy

Skin substitutes derived from skin stem cells also hold promise for feasible gene therapy for disabling genetic diseases of the skin, such as epidermolysis bullosa. The finding that the epidermis is organized into epidermal proliferating units that are each self-renewed by at least one epidermal stem cell, suggests that transduction of epidermal stem cells from the basal layer of the epidermis for gene therapy should result in permanent expression of the transgene. Ex vivo transduced keratinocytes of holoclones has been shown to have transgene expression that lasts for over 150 cell generations in culture and more importantly, has been shown to express the transgene protein when grafted in epidermal sheets in vivo (Mathor et al., 1996). Autologous epidermal stem cells isolated in culture by formation of holoclones were retrovirally transduced with laminin 5 and were successfully transplanted on patients with junctional epideromolysis bullosa (Mavilio et al., 2006). The grafts regenerated a normal epidermis at day eight and the normal epidermis was maintained throughout the one year of follow-up.

8. Future

The continued advancement of iPS cell reprogramming technology opens the door to patient-specific stem cell sources for tissue replacement. The efficiency of iPS generation was recently found to be markedly improved with the use of keratinocytes compared to fibroblasts (Aasen et al., 2008). In addition, iPS can be generated from keratinocytes isolated from a single adult person's hair. Thus, plucking a single hair from a person provides the only cellular starting material needed to generate iPS cells, which can then be differentiated to form tissue-specific stem cells, including intraepidermal, hair follicle bulge, melanocyte, sebaceous gland, mesenchymal, and SKP stem cells. The recent demonstration that introduction of three defined transcription factors can reprogram pancreatic exocrine cells in adult mice to islet β-cells suggests that directed reprogramming may even be possible without reversion to a pluripotent stem cell state (Zhou et al., 2008). In other words, it may be possible for keratinocytes from a plucked hair to be directly reprogrammed to the various skin stem cells without an intermediary iPS state. The ability to form all of the different skin stem cells that can be combined into an engineered skin tissue will enable regeneration of all of the complex cell types within the skin.

In addition, the ability to manipulate the morphogenetic signaling pathways in engineered skin tissue will be necessary to form a properly differentiated tissue as well as to form skin appropriate for different body sites, for example, facial skin versus hair-bearing scalp skin. Future work is needed to determine whether introducing a wound stimulus and activating the Wnt pathway will trigger hair follicle formation not only in mouse skin, but also human engineered skin. Liposomal packaged Wnt3a protein was recently developed and shown to have enhanced Wnt3a activity and have the ability induce hair follicle neogenesis *in vivo* by subcutaneous injection into mice (Morrell et al., 2008). Similar development of efficient tissue delivery forms of agonists or antagonists of other signaling pathways will open additional doors for controlling morphogenesis in engineered skin.

9. Summary

Advances in the biology of stem cells in the skin and signaling pathways that regulate morphogenesis of the skin have provided profound insights into the complexity of the skin organ. However, our knowledge remains limited regarding the precise mechanisms that maintain the unique identities and sustain the niches for the various stem cells, as well as the precise signaling pathways that coordinate proper growth and differentiation from these stem cells to form and sustain the constantly regenerating skin organ. Answers to these questions will be critical to the use of regenerative medicine to improve engineering of skin tissue for such important therapeutic applications as extensive burns or severe genetic blistering disorders. Ongoing genetic and cell biological studies on skin stem cells and skin morphogenesis will undoubtedly bring us closer to enabling the engineering of skin substitutes that are structurally and functionally indistinguishable from skin generated by normal embryonic development.

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