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Review

Advances in a Rapidly Emerging Field of Hair Follicle Stem Cell Research

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A B S T R A C T

Human skin maintains the ability to regenerate during adulthood, as it constantly renews itself throughout adult life, and the hair follicle (HF) undergoes a perpetual cycle of growth and degeneration. The study of stem cells (SCs) in the epidermis and skin tissue engineering is a rapidly emerging field, where advances have been made in both basic and clinical research. Advances in basic science include the ability to assay SCs of the epidermis in vivo, identification of an independent interfollicular epidermal SC, and improved ability to analyze individual SCs divisions, as well as the recent hair organ regeneration via the bioengineered hair follicular unit transplantation (FUT) in mice. Advances in the clinic include recognition of the importance of SCs for wound repair and for gene therapy in inherited skin diseases, for example epidermolysis bullosa. The study of the HF stem cells (HFSCs) started by identification of epidermal SC in the HF bulge as quiescent »label retaining cells«. The research of these cells emerged rapidly after the identification of bulge cell molecular markers, such as keratin 15 (K15) and CD34 in mice and CD200 in humans, which allowed the isolation and characterization of bulge cells from follicles. This paper provides an overview of the current knowledge on epidermal SCs in the HF, describing their essential characteristics and the control of follicle SCs fate, their role in alopecia, as well as their use in tissue engineering.

Key words: *hair follicle, bulge, stem cells, regenerative medicine*

Introduction

It is well accepted that human skin maintains the ability to regenerate during adulthood, which allows it to play the role as an interface with the external world and to maintain vital homeostatic functions throughout life[1]. The skin constantly renews itself throughout adult life, and the hair follicle undergoes a perpetual cycle of growth and degeneration. Adult skin is composed of a diverse organized array of cells deriving from different embryonic origins. During development, the neurectoderm cells form the stratified epithelium (sometimes called interfollicular epidermis), the hair follicles (HFs), sebaceous glands, and, in nonhaired skin, the apocrine (sweat) glands[1]. Each of these three epithelial lineages contains its own stem cell (SC) populations, residing in the epidermis and HF, and ensuring the maintenance of adult skin homeostasis and hair regeneration, but also participating in the repair of the epidermis after injuries[2,3].

Complications of normal regeneration of the skin result in chronic wounds, excessive scarring, alopecia or malignant transformation. As human populations prone to inadequate skin healing (in example the aged, obese, and diabetics) keep growing, treating the abovementioned conditions significantly contribute to the global health burden[4,5]. Therefore, novel therapies to treat dysfunctional skin repair and regeneration are critical, especially for the patients suffering from extensive burns or hereditary skin diseases[6].

The study of stem cells (SCs) in the epidermis and skin tissue engineering is a rapidly emerging field, where advances have been made in both basic and clinical research[7]. Advances in basic science include the ability to assay stem cells of the epidermis in vivo, identification of an independent interfollicular epidermal SCs, and improved ability to analyze individual SC divisions[7], as well as the recent hair organ regeneration via the bioengi-

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neered hair follicular unit transplantation (FUT) in mice[8,9]. Advances in the clinic include recognition of the importance of SCs for wound repair and for gene therapy in inherited skin diseases, for example epidermolysis bullosa[10].

This paper provides an overview of the current knowledge on epidermal SCs in the HF, describing their essential characteristics and the control of follicle SCs fate, their role in tumorigenesis and alopecia, as well as their use in tissue engineering.

Stem Cell Characteristics

SCs have the ability of self-renewal, which means that they can go through numerous cycles of cells division while maintaining the undifferentiated state and thereby preserving or expanding the SC pool. Additionally, SCs are characterized by their potency to differentiate into specialized cell types. SC differentiation potential can be described by five terms, depending on the number of cell types they can differentiate into: totipotent, pluripotent, multipotent, oligopotent and unipotent[11]. While embryonic stem cells have totipotent differentiation properties, most adult stem cells are lineage-restricted (multipotent) and are generally referred to by their tissue origin (mesenchymal stem cell, adipose-derived stem cell, endothelial stem cell etc.)[12]. Multipotent epidermal SCs, such as follicular cells, have been shown to be capable of forming HF, epidermis, and sebaceous gland[1,13,14].

The self-renewal and multi-lineage differentiation of SCs make these cells attractive for regenerative medicine, tissue repair and gene therapy. The key aim of SC research is defining the factors responsible for induction of SC commitment during skin and HF development, as well as those maintaining the »stem-ness« of follicular SC during development[15-17].

Localization and Identification of Hair Follicle Stem Cells

The epidermis is skin's outer covering, which protects us from dehydration and external environmental insults. The outer layer of the hair follicle is contiguous with the epidermis. Approximately 5 million follicles that are spread over the body generate hair in cyclical fashion. The duration of anagen (the period of hair growth), which varies from less than 60 to more than several thousand days on different body sites, determines the length of the hair shaft. After anagen, the follicle enters a stage of involution (catagen) and afterwards a stage of rest (telogen), when the hair that was produced dies but remains anchored in the follicle until it is shed during exogen.

Finally, stem cells at the base of the resting follicle, in an area known as the bulge, proliferate and regenerate a new lower follicle including the highly proliferative bulb cells that produce a new hair. After giving rise to the new hair-producing cells, the stem cells return to a quiescent state, which is a hallmark of these cells. Although surrounded by highly proliferating epidermal and hair follicle cells, the cells in the bulge rarely undergo mitosis. The extended state of dormancy is conform with other stem-like characteristics of these cells, including a prolonged lifespan[18].

Historically, hair follicle stem cells (HFSC) were believed to reside in the highly proliferative matrix cellular compartments[19], located in the »secondary germ« at the base of the telogen hair follicle. It was understood that the secondary germ moved downward to the hair bulb during anagen and provided new cells for production of the hair. At the end of anagen, the secondary germ was thought to move upward with the dermal papilla during catagen to come to rest at the base of telogen follicle. The notion of stem cell movement during follicle cycling was brought into question when Cotsarelis et al.[20] identified a population of long-lived presumptive stem cells, by administration of nucleotide analogs (pulse) followed by a chase period, which resulted in the presence of Label Retaining Cells (LRC) in an area of the follicle surrounding the telogen club hair, called the bulge, suggesting that bulge cells are more quiescent than the rest of the epidermal cells. Afterward, Lyle et al.[21] localized LRCs to the human HF bulge.

The bulge consists of a subpopulation of outer root sheath (ORS) cells located in the mid-portion of the follicle where the arrector pili muscle attaches to the HF, and marks the lower end of the »permanent« portion of the follicle[20]. The bulge area was already described in 1876 by Unna, who named the bulge area of the adult follicle the hair bed (Haarbett), believing that the club hair in catagen became implanted there and derived additional growth from it. Stöhr gave it a neutral name »Wulst« (bulge or swelling). Some papers described it as an area of marked proliferative activity, but it was fairly unclear since there were no mitotic figures observed even if other parts of the follicle contained them. Anyhow, these important morphological observations portended the characterization of the bulge cells in both mouse and human follicles as an area containing quiescent cells important for cell cycling[13,20–23].

Besides slow-cycling as one of the key features representing the SCs, there are also other properties of bulge SC to be investigated. Isolation of living bulge cells is required in clinical applications such as regenerative medicine and gene therapy. Although morphology based manual micro-dissection has been successfully used to isolate bulge cells from HF[24,25], this technique is time-consuming and requires profound experience and skill, as well as inadequate to ensure the purity of isolated cells. Therefore, the research of HFSCs had been delayed until the identification of bulge cell molecular markers such as keratin 15 (K15)[23] and CD34[26]. Adult transgenic mice that carry the enhanced green fluorescent protein (EFGP) under the control of K15 promoter form the basis for the successful isolation of living bulge cells using fluorescence-activated cell sorting (FACS)[23]. K15 expression in human bulge cells was first described by Lyle et al.[21]. K15 mRNA and protein are reliably expressed at high levels in the bulge, but lower levels of expression can be present in the basal layers of lower follicle ORS and the epidermis, so the use of K15 expression as a sole criterion for defining a bulge cell is not advisable[21,27]. K15 expression

in the epidermis is prominent in neonatal mouse and human skin but decreases with age[27,28]. The expression of CD34, as first described by Trempus et al.[26], represents the most specific marker for mouse HF bulge cells and is a valuable tool for bulge cell isolation[14]. CD34 is, however, not expressed in the human bulge region. Instead, CD200 has been identified as a common marker of human HFSCs and is used for the enrichment of living human HFSCs. HFSCs are also marked by Sox9[29], transcription factor 3 (Tcf3)[30], LIM homeobox 2 (Lhx2)[16], and leucine-rich G protein-coupled receptor 5 (Lgr5)[31]. However, these markers are not uniquely expressed by bulge cells, as they are also expressed on the cells of the hair germ and lower ORS of cycling follicles[3].

Tumbar et al.[32] established the gold standard for detecting SCs in the epidermis by isolating HFSCs using the LRC technique. They engineered a mouse expressing histone H2B-green fluorescent protein (H2B-GFP), which can be turned off when tetracycline is administered into the animal's diet (Tet-off system). Keratin 5 (K5) promoter controls the expression of a tet repressor-VP16 transgene, which leads to the expression of H2B-GFP in the epidermis until 4 weeks of age. At this point, tetracycline is administered for 4 weeks so that the dividing cells dilute out the label and differentiating cells are shed from the skin. This leaves only the slow-cycling bulge cells detectable as H2B-GFB LRCs, and being easily isolated by FACS[32].

In addition to epidermal SCs, there are also other types of SCs that reside in HF: melanocyte SCs in the bulge area wall, and mesenchymal SCs present in the dermal papilla and dermal sheath surrounding the HF[33,34].

The localization of HFSCs in the bulge area also gives an illustrative explanation on the differences between permanent and reversible hair loss alopecias. In cicatricial alopecias (lichen planopilaris and discoid lupus erythematosus), inflammation involves the superficial portion of the follicle, including the bulge area[35,36], suggesting the damage of SCs necessary for the HF regeneration. The inflammatory injury of alopecia areata, however, involves bulbar region of the HF that is composed of bulge cell progeny[36,37]. Although the bulbar area is immediately responsible for hair shaft production and its destruction leads to hair loss, the bulge area in this case remains intact, and a new lower anagen follicle and subsequent hair shaft can be produced.

In Vitro Assessment of the Bulge Cells' Proliferative Potential

Since stem cells are responsible for continual renewal of the tissue, they are supposed to possess a high proliferative capacity. Approximately 5% of adult epidermal basal cells are shown to have a »holoclone« phenotype characterized by high proliferative capacity and low level of terminal differentiation, and these cells are thought to represent stem cells[38]. Another indicator of proliferative capability is colony-forming efficiency (CFE; colonies per number of cells plated), which correlates to the number of the stem cells in a tissue[24,39].

In order to determine the clonogenic potential of bulge cells, Barrandon et al.[38] dissected the skin epidermis into fragments and cultured the cells originating from these different epidermal regions in vitro. Bulge cells gave rise to more holoclones than the epidermal cells coming from other regions, suggesting that bulge cells, although quiescent in vivo, present a much greater proliferative potential during in vitro culture[25,38]. Also, more recent studies[23,26] showed that isolated bulge cells formed larger and more numerous colonies than non-bulge basal keratinocytes. However, Blanpain et al.[40] reported that, although majority of holoclones originate from the bulge in the adult mouse while basal epidermis did not form any holoclones, both populations had similar CFE. The inconsistence of this method may be solely due to the fact that the bulge is enriched by stem cells, while the basal epidermis contains stem, transient amplifying and even differentiated cells, so a higher percentage of holoclones from the bulge is anyhow expected[14].

Multipotency of the Bulge Cells

If the HFSCs are located in the bulge, then it is expected that these cells should give rise to all of the lower hair follicle epidermal cell types[14]. When bulge cells were transplanted onto immunodefficient mice, bulge cells can differentiate into all cell lineages of the skin epidermis including HF, interfollicular epidermis and sebaceous glands, demonstrating that bulge region contains SCs with different potential lineages[13]. Later on, Blanpain et al.[40], as well as Claudinot et al.[41] confirmed the multipotency of bulge cells by using clonal analysis and showing that the progenies of one single bulge SC can reconstitute all the epidermal lineages of the skin epidermis. Fate mapping studies also indicated that bulge cells are multipotent SC, which means that they are able to give rise to all cells of the hair follicle, sebaceous gland and intefollicular epidermis[23,32,42].

A common finding of these studies was that there is a little if any contribution of bulge SC to the maintenance of intefollilular epidermis during the adult skin homeostasis, suggesting that unipotent progenitors ensure renewal of interfollicular epidermis[23,43]. However, upon wounding, bulge SC are activated, and migrate rapidly toward the skin lesions and participate actively in the repair of epidermis[22,42,44,45]. Once the epidermal repair is completed, the flux of bulge cells stops, and the bulge cells that had migrated in the interfollicular epidermis gradually disappear overtime[42].

In addition to the bulge and interfollicular SCs, there are also other types of epidermal progenitors recently described that participate in the homeostasis of other epidermal compartments such as sebaceous gland and the infundibulum, the portion of epidermis connecting the HF to the interfollicular epidermis. Experiments performed by Ghazizadeh et al.[46] suggested the existence of unipotent sebaceous lineage progenitors, while Horsley et al.[47] identified rare cells located at the juncture between HF and sebaceous gland able to give rise to the entire sebaceous gland. These findings show that sebaceous glands homeostasis, just like homeostasis of interfolli-

cular epidermis, can be maintained by the presence of unipotent progenitors. In contrast to these, Nijhof et al.[48] identified the multipotent MTS24 expressing cells in a region located above the bulge but below sebaceous gland, within the upper isthmus, which are able to give rise to all three epidermal lineages[48,49]. Furthermore, considering the presence of other, nonepithelial SCs in the HF, HF bulge SCs can also differentiate into neurons, glia, smooth muscle cells, and melanocytes in vitro, which provides an effective and accessible autologous source of SCs[50].

Regulation of the Bulge Stem Cells' Activations and Maintenance – The Bulge as a Stem Cell Niche

All SC populations exist in specific anatomic locations, so-called niches, where they are protected from depletion and kept in the microenvironment of specific growth factors, which regulate their activity. So far, HF bulge area is now the best characterized SC niche in the skin.

The evidence acquired from the gene profiling[23,32,51] and immunohistological analyses[52] prove that the bulge region HFSCs enjoy a relative immune privilege, primarily due to down-regulation of MHC class I molecules and the local up-regulation of potent immuno-suppressants such as α-MSH and CD200[53]. This immune privilege presumably protects the HF epithelial stem cell reservoir from auto-aggressive immune attack, whereas a loss of bulge immune privilege may play a central role in the pathogenesis of cicatricial alopecias[52].

Also, the activation and self-renewal of HFSCs should be tightly regulated to maintain HF and epidermal homeostasis. HFSCs isolation and microarray analysis of multiple gene expression were used to investigate the molecular signatures of the HFSCs that are considered to participate in controlling the fate of SCs[3].

Gene expression analysis in the telogen HFSCs and interfollicular epidermal proliferative basal layer cells revealed that genes that participate in maintaining the resting and undifferentiated state of HFSCs were up-regulated – components of the bone morphogenetic protein/transforming growth factor-β (BMP/TGF-β) signaling pathway, including latent TGF-β binding protein 1 (Ltbp1), Ltbp2, Ltbp3, TGF-β2, Gremlin, and the phosphorylation of Mothers against dpp homolog 1 (phospho-Smad1) and phospho-Smad2[23,32]. Additional upregulated genes included inhibitors of the Wnt/β-catenin signaling pathway, such as Tcf3, Tcf4, Dickkopf-related protein 3 (Dkk3), secreted frizzled-related protein 1 (SFRP1), disabled-2 (Dab2), and C-terminal banding protein 2 (Ctbp2). In contrast, those genes involved in the regulation of HF proliferation were down-regulated in HFSCs[23,32]. These findings have elucidated, at least in part, how the HFSCs are maintained in a quiescent and undifferentiated state in adult mouse HFs.

So far, the Wnt/β-catenin and BMP/TGF-β signaling pathways are considered to be the most important regulators of HFSC. Wnt signaling is important for activating HFSCs and has a sequential role in regulating HFSC lineages[42,54], whereas β-catenin is required for SC maintenance and niche biology, and its activation is essential for promoting the transition of quiescent HFSCs into proliferating transit amplifying cells that terminally differentiate along the hair cell lineage[55–60]. On the other hand, inhibitory effect of BMP/TGF-β signaling pathway mediates the maintenance of the special quiescent nature of HFSCs[61–65].

BMP signaling inhibits SC activation, at least partly, by inhibiting the nuclear localization of β-catenin[65,66]. Activation of the Wnt pathway and inhibition of the BMP pathway are likely to converge to regulate bulge SC activation, which is dependent on β-catenin stabilization, the junction point of both signaling pathways. Additionally, BMP signaling may also coordinate with transcription factor of activated T cells c1 (NFATc1), a transcription factor specifically expressed in HF bulge cells, that favors SC quiescence partly through the transcriptional repression of the cell cycle regulatory gene cyclin dependent kinase 4 (CDK4)[32,67]. Nonetheless, the exact mechanism by which BMP signals are coordinated with TGF-β and their antagonists in order to regulate the activation, proliferation and maintenance of HFSCs needs further research and clarification.

Hair Follicle Stem Cells as a Hot Target for Regenerative Medicine

Due to their location at the body surface, HFs constitute a promising source of readiliy accessible adult SCs[68]. The development of molecular techiniques that enabled isolation and culture of autologous adult epidermal, mesenchymal and neuronal HFSCs from a patient's own HFs opened a spectrum of therapeutic perspectives for: 1) treatment of third-degree burn victims, 2) promotion of the healing of large, chronic leg ulcers, 3) de novo generation of new human hair follicles and 4) gene therapy strategies[53]. Gene therapy would have a role in treatment of inherited structural and enzymatic skin defects (in example epidermolysis bullosa), where genetically engineered and modified, fully functional autologous HFSCs would be transplanted in order to gradually replace defective tissue[69,70]. Also, melanocytic SCs could be used to prevent or revert heir graying, or in treatment of epidermal depigmenting disorders like vitiligo. On the other hand, even permanent removal of cosmetically unwanted hair could be approached by targeted damage of SC regenerative capacity of the HF[53].

Although the knowledge about HFSCs has substantially increased in recent years, many unanswered questions remain and the whole field is still kept in the domain of preclinical research. By now, only interfollicular basal layer epidermal SCs are in clinical use for treatment of patients suffering from severe burnt injuries. The method was described in 1979 by Green et al.[71], when they found that human epidermal SCs have an enormous proliferating potential, discovering that only few in vitro cultured cells can regenerate, differentiate and reform a functional skin barrier that can be transplanted onto the severely burnt patients. This method is

very powerful since it allows transplantation of much larger pieces of autologous skin that it is permissible by the classic autologous skin grafting. The limitations of this method are the time needed to grow the confluent epithelial sheets in vitro and the huge cost of the treatment. Also, the grafted skin grown by this method does not contain HFs and sweat glands. With the intention of achieving HF regeneration in grafted epidermal sheets coming from cultured epidermal SCs, dermal papilla cells need to be co-transplanted in order to stimulate epidermal SCs to adopt HF fate[72]. The underlying reason is that HF morphogenesis and regeneration is much more complex since its development, like in all ectodermal organs (teeth, salivary glands), depends on the reciprocal epithelial-mesenchymal interactions between epidermal SCs and mesenchymal SCs in dermal papilla[73].

So in order to achieve the HF regeneration in the hair cycle, it is essential to regenerate various stem cells and their niches[74,75]. Many studies attempted to develop technologies to renew the variable lower region of the hair follicle[72,76], to achieve de novo folliculogenesis via replacement with HF-inductive dermal cells[77], and to direct the self-assembly of skin-derived epithelial and mesenchymal cells[78–83]. Nakao et al.[84] reported that a bioengineered HF germ, reconstituted from embryonic follicle germ-derived epithelial and mesenchymal cells can generate a bioengineered HF and shaft. The latest study of Toyoshima et al.[9] demonstrated fully functional orthotopic hair regeneration via the intracutaneous transplantation of bioengineered hair follicle germ. Also, Asakawa et al.[8] proved fully functional regeneration using ectopically bioengineered HFs transplanted via the FUT method that are practical for clinical therapies. The bioengineered hair had the correct structures of the naturally occurring hair follicle and shaft, and it formed proper connections with surrounding host tissues (epidermis, arrector pili muscle and nerve fibres). The bioengineered HFs showed full functionality, including the ability to undergo repeated hair cycles through the rearrangement of various stem cell niches, as well as responsiveness to the neurotransmitter acetylcholine (ACh). This study hence demonstrated the potential for not only hair regeneration therapy but also the realization of bioengineered organ replacement using adult somatic stem cells. The studies of Toyoshima et al. and Asakawa et al.[8,9] provided novel evidence of fully functional hair follicle regeneration through the rearrangement of various stem cells and their niches in bioengineered hair follicles, and gave a substantial contribution to the development of bioengineering technologies that will enable future regenerative therapy for hair loss caused by injury or by diseases such as alopecia and androgenic alopecia.

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NAPREDAK U POLJU ISTRAŽIVANJA MATIČNIH STANICA DLAČNOGA FOLIKULA

S A Ž E T A K

Koža odrasle osobe zadržava sposobnost regeneracije, s obzirom da se trajno obnavlja tijekom odrasle dobi, a dlačni folikuli neprekidno prolaze kroz ciklus rasta i degeneracije. Istraživanje epidermalnih matičnih stanica i inženjering kožnoga tkiva je polje u kojemu je u zadnje vrijeme došlo do značajnih napredaka kako u bazičnim, tako i u kliničkim istraživanjima. Napretci bazičnih istraživanja uključuju mogućnost istraživanja epidermalnih matičnih stanica in vivo, prepoznavanje nezavisnih interfolikularnih epidermalnih matičnih stanica, bolju sposobnost analize pojedinih dioba matičnih stanica, kao i nedavno uspjelu regeneraciju dlačnoga organa putem transplantacije bioinženjeringom stvorene dlačne jedinice u miševa (FUT, prema engl. follicular unit transplantation). Napredak u kliničkim istraživanjima uključuje prepoznavanje važnosti epidermalnih matičnih stanica u cijeljenju rane, kao i u genskoj terapiji nasljednih bolesti kože, kao što je npr. bulozna epidermoliza. Istraživanje folikularnih matičnih stanica započeto je identifikacijom epidermalnih matičnih stanica kao nijemih stanica koje zadržavaju biljeg (LRC, prema engl. label retaining cells) u izbočini dlačnoga folikula. Istraživanje ovih stanica ubrzano se razvilo nakon utvrđivanja specifičnih molekularnih biljega stanica izbočine dlačnoga folikula, kao što su keratin 15 (K15) i CD34 u miševa, te CD200 u ljudi, jer su oni omogućili izolaciju i utvrđivanje značajki stanica izbočine iz dlačnih folikula. U ovome članku donosimo pregled dosadašnjih spoznaja o folikularnim epidermalnim matičnim stanicama, opisujući njihove temeljne značajke, način regulacije njihova usmjeravanja, ulogu u alopeciji, te mogućnosti njihova korištenja u tkivnom inženjeringu.