Review Article

A Review: The Location, Molecular Characterisation and Multipotency of Hair Follicle Epidermal Stem Cells

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Abstract

Introduction: Recent work has focused on the hair follicle as the main repository of multipotent stem cells in skin, which is a neat model to study the mechanisms regulating the proliferation, migration and final fate of adult stem cells. This review examines the available literature for its location, molecular markers and multipotency. Methods: Peer-reviewed journals and monographs on the subject were covered. Results: With the application of stem cell-labelling techniques and clonogenicity assay, it is clear that most of the hair follicle stem cells are located at the bulge region, but the base of the hair follicle does contain some clonogenic cells; whether they are stem cells is still unknown. Extensive works have been done in identifying hair follicle stem cells. The potential markers for hair follicle stem cells include: b1-integrin, keratin 19, a6-integrin, CD71, p63, and CD34. Most of these markers are expressed in high levels in hair follicle stem cells, but there is still difficulty in distinguishing hair follicle stem cells from their transit-amplifying progeny, and the sorted hair follicle stem cells with these markers are far from pure. As hair follicle stem cells might have been activated after leaving the stem cell niche, the markers for cells in vitro might not be identical to those in vivo. Using double-labelling techniques with BrdU and ^{3}H-Thymidine, and the creation of novel chimera transgenic mice, it was proved that hair follicle stem cells can repopulate wound epidermis, forming epidermis, hair follicles and sebaceous glands, but it contributes little to the epidermis in physiological condition, except the hair follicle. Conclusions: Slow cycling, label-retaining cells exist at the bulge of the hair follicle, with high proliferative potential and clonogenicity. The putative bulge stem cells can contribute to the epidermis, outer root sheath, inner root sheath, hair shaft and sebaceous gland. However, they still lack certain markers to distinguish bulge stem cells from their progeny, and much work needs to focus on the interrelations between bulge cells and interfollicular keratinocyte stem cells, the relations between bulge cells and dermal papilla mesenchyme cells, and the mechanism of hair growth.

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Introduction

There have been significant advances in the understanding of keratinocyte stem cells since the 1970s, when the concept of interfollicular epidermis was initially proposed; later much work was focused on the specific region of the hair follicle outer root sheath, especially the bulge region (Fig. 1). Hair follicle stem cells are multipotent, capable of giving rise to all cell types of the hair, the epidermis and the sebaceous gland.[1]

The characterisation and manipulation of stem cells have led to a wide range of clinical applications. For example, the identification of haematopoietic stem cells led to autologous peripheral blood stem cell transplantation and gene therapy; and the characterisation of limbus epithelial stem cells led to the development of new techniques for cornea transplantation.[2] The study of skin stem cells will lead to the treatment of skin loss, skin disease and hair loss.

Hair Follicle Stem Cell Location

Hair follicle stem cells, like other adult stem cells, are thought to be slow-cycling cells or rarely cycling cells, with a superior clonogenicity and proliferative capacity,[3] and the capacity to proliferate and generate large amounts of tissue for a long time.[4] With slow cycling, the stem cells conserve their proliferative potential and minimise DNA replication-related errors. The rare division of stem cells gives rise to 1 stem cell and 1 transient amplifying (TA)

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cell, which has a limited proliferative potential. After exhausting their proliferative potential, the rapidly proliferating TA cells undergo terminal differentiation.

The main method of identifying adult stem cells makes use of their slow-cycling nature. Normally, all the cells within the investigated tissue are labelled with a DNA precursor, such as tritiated thymidine (^{3}H-T) or bromodeoxyuridine (BrdU), during the 4- to 8-week chasing period. The rapidly dividing TA cells lose most of their labels due to dilution. Only stem cells that divide rarely and still reside within the tissue over time will retain their labels. In this way, the stem cells can be detected as the label-retaining cells (LRCs).

There were 2 studies which investigated the relevance of label retention of bulge cells, hair follicles from rat whisker and human scalp. In 1993, Kobayashi et al[5] made fine dissections of rat vibrissae and checked the colony-forming efficiency of keratinocytes in different parts of the hair follicle in vitro. They found that 95% of the total colonies formed in culture from fractionated vibrissae were in the bulge region, and fewer than 4% were located in the matrix area of the follicle. This study suggests that the bulge region might be the reservoir of stem cells of the hair follicle. In 1994, Rochat et al[6] examined the growth capacity of keratinocytes isolated from human scalp hair follicles. The base of hair follicles in 3 separate experiments contained 0.5%, 0.8% and 1.7% of colony-forming cells, in contrast to 45.3%, 17.7% and 26.7% for the upper segment of the hair follicle. The result suggests that most of the clonogenic cells in hair follicles reside in the bulge region.

With ^{3}H-T labelling of mice skin, Morris and Potten[7] identified highly persistent LRCs at the bulge region 14 months after completion of continuous labelling; no LRCs were found in the interfollicular epidermis or sebaceous glands 14 months after labelling. Later, Taylor et al[8] labelled newborn mouse skin with BrdU, which affords a high sensitivity compared with ^{3}H-T labelling. After 8 weeks, the only labelled cells that remained were located in the bulge area of hair follicles.

These results imply that the LRCs of follicles are mostly restricted to the bulge region (Fig. 1), a portion in the outer root sheath of the hair follicle, which is at the insertion site of the arrector pili muscle. The cells from the bulge region display the greatest in vitro growth capacity and clonogenicity, compared with cells from other regions of the hair follicle and epidermis. The normally quiescent bulge cells undergo a transient period of cell proliferation and give rise to TA cells during early anagen or after stimulation.[9] The bulge cells are in a relatively undifferentiated ultrastructural state; they live in a well-protected region and they will likely persist throughout the lifetime of the organism.[7] Also, with topical application of tumour initiator to mouse back skin at the onset of anagen, the bulge showed greater tumour susceptibility.[10] Taken together, all these data suggest that the bulge region is the site of hair follicle stem cells.

On the other hand, although most of the stem cells of the follicle are located at the bulge region, the base of the hair follicle does contain some clonogenic cells. Whether these cells are stem cells is yet unknown.

Hair Follicle Stem Cell Markers

Identifying the hair follicle stem cells is a major advance in the field of skin biology, which allows scientists to investigate their biochemical properties, lineage and their relation to other cells. Hair follicle stem cells can be identified in vivo by label retention or in vitro by clonogenicity, but neither of these methods allows easy isolation of stem cells for analysis. Therefore, there is a strong need for specific hair follicle stem cell markers to be identified.

One of the major controversies in the study of skin stem cells is related to the existence of specific markers. Many attempts have been made in recent years to identify keratinocyte stem cells. The potential candidate hair follicle stem cells markers include b1-integrin, keratin 15, keratin 19, CD71, transcription factor P63 and CD34.

Keratinocytes express several integrins, including a2b1 (collagen receptor), a3b1 (laminin receptor), a5b1 (fibronectin receptor), a6b4 (laminin receptor) and avb5 (vitronectin receptor). Integrins not only mediate adhesion to the underlying extracellular matrix, but also regulate the initiation of terminal differentiation.[11,12] Integrins play a role in controlling epidermal differentiation and morphogenesis. This fact suggests that differences in integrin function or expression might provide markers for different subpopulations of proliferating keratinocytes.

Jones and Watt[13] used fluorescent-activated cells sorter (FACS) to fractionate basal layer keratinocytes into groups on the basis of surface integrin levels. Cells with the highest levels of the a2b1 showed a higher colony-forming efficiency (CFE) than cells with the lowest levels, and similar results were obtained with a3b1 and a5b1 integrins. When plated on extracellular matrix (ECM) proteins, such as type IV collagen, fibronectin, the basal keratinocytes adhered progressively over a short time, and the cells' CFE correlated with the speed of cell adherence to ECM. Also, the b1 integrin dull keratinocytes tended to form small, abortive colonies, and underwent terminal differentiation.

In investigating the transcriptional profiles of haematopoietic, neural and embryonic stem cells, Ivanova et al[14] and Ramalho-Santos et al[15] found integrins to be up-regulated in these stem cells compared with their transiently amplifying progeny. It is possible that keratinocyte stem cells require strong adherence by integrins to the basement membrane to maintain their stem cell characteristics or

their position in the stem cell niche. But the usefulness of integrins as stem cell markers is limited by the uncertain expression levels relative to transit-amplifying stem cell progeny. In addition, Jones and Watt[13] showed that the cells with the highest integrin had the highest CFE, and was not related to cell size, but Barrandon and Green's work[16] suggested that the keratinocyte stem cells have the smallest size among the keratinocyte population.

Keratinocyte exhibit characteristic keratin intermediate filaments. In the epidermis, keratins 5 and 14 are expressed in the basal layer, while keratins 1 and 10 are found in the suprabasal layer. The hair follicle stem cells expressed the above keratins and keratins 6, 16 and 17 .

Michel et al[17] labelled LRCs in mouse hair follicle with ^{3}H-T , checked keratin 19 expressions by immunoblotting, and found that keratin 19 positive cells are also ^{3}H-T-label-retaining cells. In checking the expression in mouse and human skin, keratin 19 was expressed in the hair follicle, and was absent at interfollicular epidermis at hairy sites. In contrast, at glabrous sites, K19-positive-cells were found in deep epidermal rete ridges. K19-expressing cells also contained high levels of a3b1 integrin. There was more keratin 19 expression in newborn than older foreskin, which correlated with keratinocytes culture lifespan. This result strongly suggests that keratin 19 is a valuable marker, or at least a co-marker, for keratinocyte stem cells.

Recently, it was also reported that expression of keratin 15 is restricted to the cells at the bulge region of the human hair follicle, and keratin 15 was suggested to be a stem cell marker.[18] However, later work proved that keratin 15 is present in the entire outer root sheath of the human hair follicle,[19] and further work is needed to investigate the relation between stem cells and keratin 15 expressions.

Later, multiple criteria associated with stem cells have been applied in searching for stem cell markers. Li et al[20] isolated a subpopulation of keratinocytes from the basal layer of neonatal human foreskin; these cells express high levels of a6 integrin (a6^{bright}), and weakly express transferrin receptor (CD71). These cells (described as a6^{bright} and CD71^{dim}) are quiescent at the time of isolation from the epidermis, have a long-term proliferative potential and a high short-term colony-forming efficiency. ^{3}H-T-labelled LRCs are predominantly a6^{bright} and CD71^{dim}. When using cell size as a criterion for identifying stem cells (small size or high nuclear to cytoplasmic ratio), it was found that small size correlated with a6^{bright} and CD71^{dim}. These a6^{bright} and CD71^{dim} cells were suggested as epidermal stem cells, but when using fluorescent-activated cell sorting, it was found that CD71^{dim} cells were also present in situ in the hair bulb region, which is supposed to be lacking in epidermal stem cells.

In 2001, p63 was identified as a marker for keratinocyte stem cells. p63 transcription factor is a homologue of the p53 tumour suppressor gene. It belongs to a family that includes structurally related proteins such as p53 and p73. Pellegrini et al[21] isolated cornea and skin epidermal keratinocytes, checked the p63 expression and colony forming efficiency in vitro, and found that in cultured keratinocytes, p63 is highly expressed in the holoclones and greatly reduced in the meroclones and paraclones, and transient amplifying keratinocytes have greatly reduced p63 levels. However, p63 expression alone is not sufficient to identify epidermal stem cells in vivo, as it is expressed in all basal cells as well as in a significant number of suprabasal cells of the epidermis and of the outer root sheath of the hair follicle. Moreover, p63 is also highly expressed in the hair matrix, which is thought to contain only cells with limited growth capacity.

It was recently proposed that CD34 is specifically expressed on stem cells isolated from the bulge region hair follicles. Trempus et al[22] identified CD34 staining on keratinocytes in the bulge region of mouse hair follicles, CD34 expression co-localised with label-retaining cells, cells were quiescent when isolated from the bulge region, and cells showed a high proliferative potential in vitro. This implies that CD34 is a specific marker for bulge keratinocytes. CD34 is a specific marker for haematopoietic stem and progenitor cells. The expression of CD34 in the bulge region suggests potential relations between haematopoietic stem cells and epidermal stem cells. However, epidermal cells that are CD34-negative also show stem cell properties.[23,24] Epidermal stem cells sorted by Hoechst 33342 dye exclusion method from newborn mice did not express CD34[25] either. Much more work is needed to clarify these conflicting results.

The importance of the lymphoid enhancer-binding factor (LEF) family of transcription factor has been highlighted recently, as LEF-1 knockout mice have apparent stem cell defects in hairs.[26] As b-catenin and wingless-type (WNT) protein regulate this pathway, these molecules might also be potential markers for keratinocyte stem cells.

One of the variables is the biological state of keratinocyte stem cells in culture. In vitro clonogenicity and proliferative potential provide useful assays for stem cells,[27,28] but cultured cells are released from the in vivo stem cell niche, which keep them in a slow-cycling state. Thus, keratinocyte stem cells in culture become "activated" in culture, which is evidenced by increased cell size, proliferation and migration.[29,30] The in vitro expansion of keratinocyte stem cells in tissue culture conditions is also accompanied by exhaustion of the cell proliferative potential[31] and the loss of slow-cycling features. So those markers present in the in vivo keratinocyte stem cells might not be identical to those for cells in vitro culture conditions. This might explain some of the inconsistencies in experimental results. Clearly, further work is required to search for definite stem cell markers.

Hair Follicle Stem Cell Multipotency

To investigate the function and fate of hair follicle stem cells, Taylor et al[8] devised a double-labelling technique, in which newborn mouse skin was first labelled with BrdU and after a short interval, labelled with ^{3}H-T. The fact that keratinocytes have a heterogeneous cycle time; this technique establish the follicle cells with a cycle time of 16 to 28 hours and tag the follicle TA cells. The fate of these TA cells was followed, and there was a decrease in the number of double-labelled cells, accompanied by a dramatic increase of such cells in the epidermis. These results showed that bulge cells give rise to lower follicles, including the outer root sheath, matrix and medulla; and bulge cells contribute to the neonatal epidermis and the repair of the epidermis after a full-thickness wound.

In another experiment, Oshima et al[32] used transgenic mice Rosa 26, which has a Lac Z reporter gene. They dissected fragments of the bulge region from Rosa 26 mice, and transplanted them onto the back of wild-type mouse embryos. When stained for b-galactosidase, the typical blue staining for Rosa 26 transgenic mice was observed throughout the grafted follicle and epidermis, which implies that cells from the donor bulge can repopulate the entire follicle, including the outer root sheath, inner root sheath and hair shaft, and that the bulge cells can contribute to all elements of the epidermis and sebaceous glands.

Recently, with the identification of a Krt1-15 promoter with specific activity in adult bulge cells, Liu et al[33] created Krt1-15-EGFP transgenic mice, and traced the fate of labelled bulge cells.[1] Bulge cells were found throughout all epithelial cell types of the new lower hair follicle and hair shaft, and were also present in the epidermis and sebaceous glands. Tumbar et al[34] engineered transgenic mice to express histone H2B-green fluorescent protein (GFP) controlled by a tetracycline (tet)-responsive regulatory element and crossed them to mice harbouring a keratin5 (K5) promoter-tet repressor-VP16 transgene. With this model, they purified LRCs that mark the skin stem cell niche, checked their multipotentiality and transplantability, and defined the stem cell niche. They got almost the same result as Liu et al.[33] These results indicate that bulge cells normally contribute to 3 major epithelial cell types of the cutaneous epithelium: the entire hair follicle, epidermis and sebaceous glands (Fig. 1), but the major flux of bulge cells is towards the generation of the new hair follicle and hair at anagen onset during hair follicle cycling.

It is clear that the basal layer of glabrous epidermis and the interfollicular epidermis contain unipotent epidermal stem cells. The hair follicle bulge contains multipotent stem cells that can repopulate a wounded epidermis, forming epidermis, hair follicles and sebaceous glands. But it is proved that the epidermal interfollicular keratinocytes can

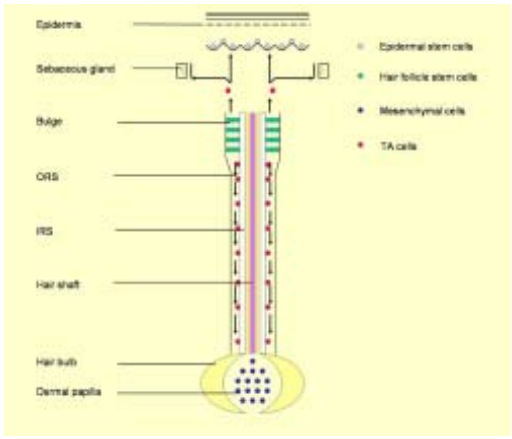

Fig. 1. Illustration of the structure of the hair follicle. Hair follicle stem cells are mainly located at the bulge region (in green), and generate TA (transient amplifying) cells, which migrate to the sebaceous glands, epidermis and hair bulb, and contribute to specific progenitor cells. ORS: outer root sheath, IRS: inner root sheath.

self-renew for months without any contribution from the hair follicle, which implies that hair follicle stem cells contribute little to epidermal renewal under physiological conditions in adult mice.[35] There are still difficulties in establishing a definite lineage hierarchy for skin stem cells.

Clinical Application of Hair Follicle Stem Cells

Human epidermal stem cells can be cultured in vitro under appropriate conditions, leading to the preparation of cultured epithelium sheets. Treating with dispase, the autologous epithelium sheet can be removed from culture dishes, and transplanted to re-epithelialise burn wounds, chronic wounds and ulcers.[36] This technology is effective in providing a temporary wound cover and reducing infection rates; but the epithelial sheet is fragile and does not adhere well to some skin wounds. It is also time-consuming to culture epithelial sheets in vitro.

Hair follicle stem cells have been used in preparing skin equivalents, and can form epithelium in deep burn wounds after implantation.[37,38] This makes it possible to culture autologous epithelium grafts in vitro in a short time. As it shows multipotency to differentiate into almost all epithelial cell types, hair follicle stem cells can be used in preparing composite skin substitutes. In the near future, it might be possible to prepare a real composite skin substitute, which involves both the epidermis and dermis.

Conclusion

There exist slow-cycling, label-retaining cells in the bulge region of the hair follicle, with high proliferative potential and clonogenicity. The putative bulge stem cells can differentiate into the epidermis, outer root sheaths, inner root sheaths, hair shafts and sebaceous glands.

However, there are no definitive markers to distinguish bulge stem cells from their transient amplifying progeny, and much work needs to be done on the interrelations between bulge cells and interfollicular keratinocyte stem cells, the relation between bulge cells and dermal papilla mesenchyme cells, and the mechanism of hair growth.

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