# Human epithelial hair follicle stem cells and their progeny: Current state of knowledge, the widening gap in translational research and future challenges

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**Epithelial hair follicle stem cells (eHFSCs) are required to generate, maintain and renew the continuously cycling hair follicle (HF), supply cells that produce the keratinized hair shaft and aid in the reepithelialization of injured skin. Therefore, their study is biologically and clinically important, from alopecia to carcinogenesis and regenerative medicine. However, human eHFSCs remain ill defined compared to their murine counterparts, and it is unclear which murine eHFSC markers really apply to the human HF. We address this by reviewing current concepts on human eHFSC biology, their immediate progeny and their molecular markers, focusing on Keratin 15 and 19, CD200, CD34, PHLDA1, and EpCAM/Ber-EP4. After delineating how human eHFSCs may be selectively targeted experimentally, we close by defining as yet unmet key challenges in human eHFSC research. The ultimate goal is to transfer emerging concepts from murine epithelial stem cell biology to human HF physiology and pathology.**

**Keywords:**
bulge; CD34; CD200; human hair follicle stem cells; K15; K19; PHLDA1

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## Introduction

Ever since their identification in mice [1], the biology of epithelial hair follicle (HF) stem cells (eHFSCs) has become a very fertile and exciting frontier not only in the HF field, but also in general epithelial biology. The distinct localization of eHFSCs in morphologically well-defined and experimentally tractable niches has permitted exploitation of the HF as a highly instructive model for dissecting general principles of stem cell biology, such as the regulation of quiescence, self-maintenance, signalling, niche organization, activation and fate decision making [2–8].

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**Abbreviations:**
**APM,** arrector pili muscle; **Cp,** companion layer; **DP,** dermal papilla; **eHFSC,** epithelial hair follicle stem cell; **HF,** hair follicle; **HS,** hair shaft; **IFE,** interfollicular epidermis; **IRS,** inner root sheath; **ORS,** outer root sheath; **SG,** sebaceous gland; **SHG,** secondary hair germ; **TAC,** transit amplifying cell.

However, much of this seminal work has been performed in the murine system, and it is by no means clear which principles of eHFSC biology elaborated from the study of mice also apply to the human HF (Table 1) and are clinically relevant. Moreover, the murine HF carries limitations as a model for human HF research when one takes into account species' differences (discussed later). Therefore, the current review attempts to detail the emerging understanding of human eHFSCs, define the key areas to be explored in human eHFSC biology with respect to established or emerging murine concepts and discusses promising experimental approaches that can be used to study these concepts with respect to the clinical relevance of human eHFSCs.

## Mapping the 'stem cell maze' against the anatomy of the hair follicle

To begin, it is important to appreciate that "HF stem cells" are not a single multipotent entity, given that the pilosebaceous unit contains numerous stem cell populations and sub-populations, ranging from epithelial to mesenchymal and melanocyte stem cells. Thus, the HF has been appropriately described as a "stem cell maze" or "zoo" [9, 10], in which the functions of each distinct progenitor cell population needs to be characterized.

eHFSCs act as a regeneration pool for the cells needed to maintain and remodel the epithelium of the continuously cycling HF throughout life. This epithelium consists of several defined layers that undergo distinct epithelial differentiation pathways: the hair shaft (HS), inner root sheath (IRS), companion layer (Cp) (now regarded as a distinct cellular layer in mouse [11]) and outer root sheath (ORS) (Fig. 1A). These epithelial compartments longitudinally span the HF regions defined as the bulb, suprabulbar region, isthmus, infundibulum and the beginning of the interfollicular epidermis (IFE) (Fig. 1A) [12]. The isthmus is the region located between the entrance of the sebaceous duct and the insertion of the arrector pili muscle (APM). The isthmus is an important anatomical landmark because it includes the "bulge" region of the follicle where the main eHFSC niche is located.

The pigmented hair shaft (HS) begins its existence through synthesis in the precortical hair matrix (Fig. 1A). The matrix arises from the telogen secondary hair germ (SHG) during HF cycling (Fig. 1B) and is the site where transit amplifying cells (TACs) actively proliferate and differentiate during the active

**Table 1. Snapshot of epithelial progenitor markers in the human and mouse pilosebaceous unit**

| Marker | Human | Key compartments of localization | Mouse | Key compartments of localization |
|---|---|---|---|---|
| EpCAM/Ber-EP4 | ✓ | SHG. Epithelial strand. Early anagen matrix [67] | – | |
| Blimp1 | ✓ | SG, IFE, Intermittently in the ORS [101] | ✓ | SG [102] |
| Brg1 | ? | | ✓ | Lower bulge and hair germ of late telogen / early anagen HF<br>Negative in K15+ bulge but positive in matrix in mid to late anagen HFs [125] |
| CD34 | ✓ | Anagen sub-bulge and suprabulbar ORS [42-45, 68] | ✓ | Bulge eHFSCs [61, 80] |
| CD200 | ✓ | Bulge & SHG [30, 42, 43, 46] | ✓ | Bulge & SHG [46] |
| Fgf18 | ~ | Underrepresented in anagen bulge eHFSCs [30] | ✓ | Telogen bulge eHFSCs [103] |
| Gli1 | ~ | Bulge mRNA detected. ORS [104, 105] | ✓ | Upper telogen bulge & SHG [13] |
| Hopx | ? | | ✓ | Bulge & lower anagen matrix [81] |
| K15 | ✓ | Bulge. Suprabulbar ORS. Epithelial strand [30, 41, 43, 44, 67] | ✓ | Bulge eHFSCs [47, 48, 61] |
| K19 | ✓ | Bulge. Suprabulbar ORS. Epithelial strand [43, 50, 67] | ✓ | ORS [49] |
| Lgr5 | ~ | Bulge mRNA detected [46] | ✓ | Lower anagen ORS and matrix. Telogen bulge and SHG [106] |
| Lgr6 | ? | | ✓ | Isthmus above bulge [15] |
| Lhx2 | ~ | ORS [14, 43] | ✓ | Bulge eHFSCs [5, 14] |
| Lrig1 | ✓ | Basal IFE [107] | ✓ | Junctional zone between the bulge & infundibulum/periphery of SG [107] |
| NFATC1 | ~ | Upregulated in bulge [41] | ✓ | Bulge eHFSCs [108] |
| NFIB | ? | | ✓ | Bulge eHFSCs [40] |
| p75NTR/CD271 | ✓ | Sub-bulge/suprabulbar ORS [42] | ✓ | Anagen and catagen ORS [109] |
| PHLDA1/TDAG51 | ✓ | Bulge [36] | – | |
| MTS24/Plet1 | ? | | ✓ | Above bulge [107, 110] |
| S100a4 | ~ | Upregulated in bulge [104] | ✓ | Bulge eHFSCs [80] |
| S100a6 | ~ | ORS [111] | ✓ | Bulge eHFSCs [80] |
| Sox9 | ~ | ORS, SG & sweat glands [112] | ✓ | Bulge eHFSCs [14, 112] |
| TACE/ADAM17 | ? | | ✓ | Bulge eHFSCs [113] |
| Tcf3 | ~ | Upregulated in bulge [104] | ✓ | Bulge eHFSCs [114] |
| Tcf4 | ? | | ✓ | Bulge eHFSCs [14, 114] |

?, not characterized/no literature found; ~, further characterization may be warranted; ✓, characterized. For further comments and references regarding eHFSC markers please refer to Supporting Information Table 2.

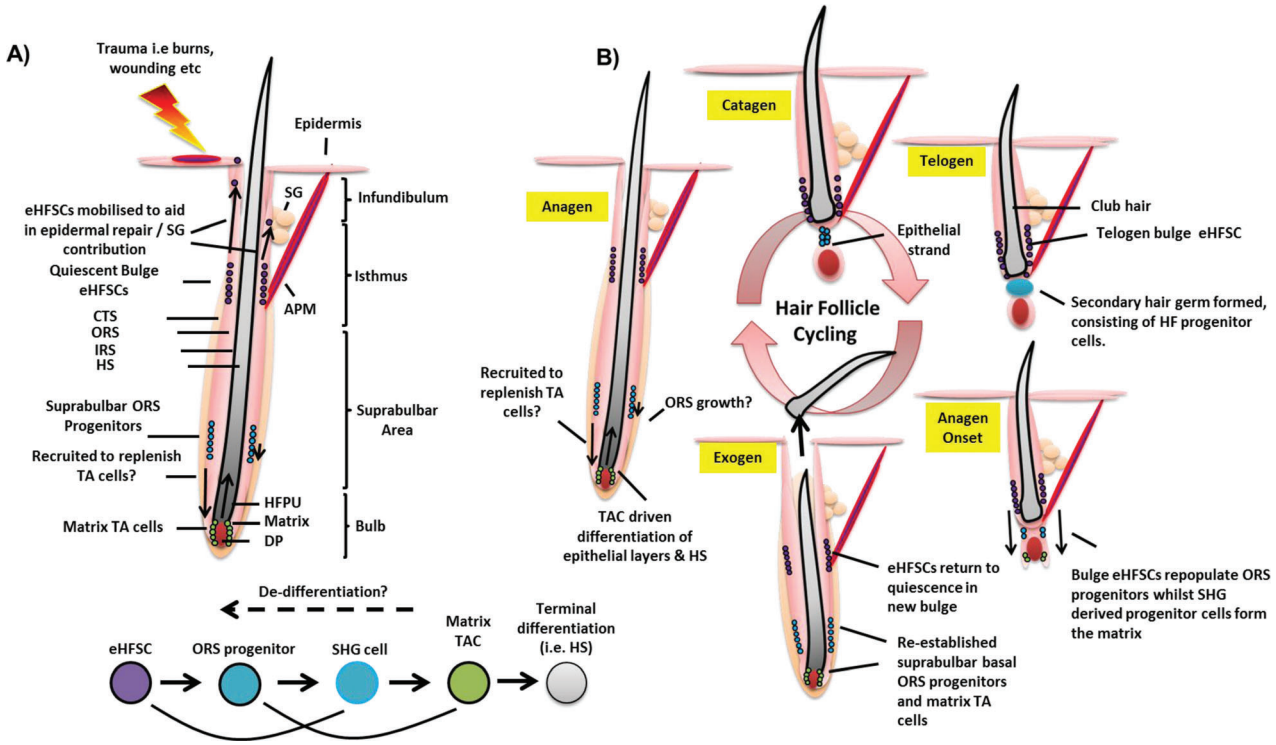


**Figure 1.** Proposed localization and behavior of epithelial stem and progenitor cells with respect to **A:** known hair follicle anatomy and **B:** during hair follicle cycling. The HF undergoes active growth (anagen) before cellular apoptosis and regression (catagen). Following this, the HF enters telogen (no longer regarded as simply a quiescent stage [120]). Subsequently, cells of the SHG are signaled by the underlying mesenchyme to proliferate and to bring about re-entry into anagen via reformation of the matrix. Formation of a new hair shaft in mid-anagen results in the shedding of the club hair in "exogen" [12]. Progenitor cell ability to re-populate the eHFSC niche has been documented in mouse [8]. APM, arrector pili muscle; CTS, connective tissue sheath; DP, dermal papilla; HFPU, hair follicle pigmentary unit; HS, hair shaft; IRS, inner root sheath; ORS, outer root sheath; TA, transit amplifying cells; SG, sebaceous gland; SHG, secondary hair germ.

growth phase (anagen) of the HF to produce the HS and its surrounding differentiated epithelial layers [12].

During HF cycling, descendants of bulge eHFSCs directly or indirectly contribute to these epithelial structures as well as to the sebaceous gland which develops from the ORS and remains associated with it via the SG duct. Furthermore, mouse models have repeatedly demonstrated that stem cells from within the HF possess an ability to reconstitute injured IFE, thereby contributing to repair [13–16] (Fig. 1A) and clinical evidence suggests that this also occurs in human skin [17, 18]. The human HF epithelium also contains a melanocyte stem cell population, that can be identified by Mitf and Pmel17 expression (recognized using NK1/beteb antibody) that may act as a source for follicular melanocytes (reviewed in [19]). Moreover, Merkel cells, with which the human ORS is richly endowed (in particular within the isthmus region [20]) in contrast to the majority of mouse HFs [21], may have their own intra-follicular epithelial stem cell population, just as has recently been shown for epidermal touch domes [22].

The surrounding connective tissue sheath (CTS) of the follicle and the dermal papilla (DP) at the base of the follicle are mesenchymal in origin, morphologically and structurally distinct from the HF epithelium (Fig. 1A). This human HF associated mesenchyme (and in the dermis of hairless skin) has been identified to harbor multipotent stem cell subpopulations, distinct from eHFSCs, such as skin-derived precursors, Nestin+ cells or Sox2+ cells [23–27]. These putative stem cells may be involved in overall follicular and dermal maintenance through the generation of progeny that populate adipose, vascular, and connective tissue as well as the glia of skin nerves. They may provide precursors to the inductive fibroblasts of the DP that signal to the hair matrix through epithelial-mesenchymal interactions [4, 12, 28, 29]. However, mesenchymal and other multipotent stem cells, as well as melanocyte stem cells and Merkel cell progenitors, although of great importance to the HF and the skin, are not within the scope of this review.

## The bulge is a prototypic quiescent stem cell compartment

eHFSCs localize to the basal, outermost ORS layer of the distal HF epithelium at the proximal end of the isthmus; this region is otherwise known as the "bulge". The bulge can be further defined as the area of the ORS at and around the site where the APM attaches to the HF [30] and wraps around it [31]. In terminal human scalp HFs, this area is located well below the entrance to the SG duct (Fig. 1A).

The bulge has been identified to contain "label retaining" cells (LRCs) in both the mouse [1] and human HF [32]. LRCs are able to retain a label within their DNA, in this context

5-bromo-2'-deoxy-uridine (BrdU). These cells are regarded to be slow cycling, as in rapidly dividing cells the DNA incorporated label is lost through repeated rounds of cell division. This is generally considered a hallmark characteristic of stem cells that reside within a quiescent niche [33]. Thus, slow cycling bulge cells found within the HF are deemed representative of a quiescent eHFSC population. eHFSCs that reside in the human bulge epithelium can be effectively recognized by expression of the molecular markers such as: cluster of differentiation 200 (CD200) [30, 34], type I keratin 15 (K15) [32, 35] and pleckstrin homology-like domain, family A, member 1 (PHLDA1) [36, 37].

The bulge can be microscopically identified with relative ease in mouse HFs and human fetal HFs as a distinctive protuberance of the ORS at the inferior portion of the isthmus. It is often assumed that this protuberance is not detectable in human adult HFs. However, morphologically distinct protrusions of the ORS (provocatively termed "follicular trochanter") can often be identified in the bulge region of human terminal and vellus hairs [38] (Fig. 1A). In the context of adult human HF research, the term "bulge" typically refers to the quiescent eHFSC niche as a biological concept rather than an anatomical epithelial protuberance.

This makes it difficult to delineate the exact borders of the bulge in the human HF. To complicate matters further there is a lack of consensus and a tendency to group parts of the isthmus and the infra-bulge area of the ORS into what is summarily termed "bulge" or "bulge region". Therefore, it becomes problematic when attempting to compare the expression patterns for putative eHFSCs and putative progenitor cells reported by different authors. We propose that the bulge be defined strictly as an ORS region that lies within the lower isthmus around the APM attachment site but well below the entrance to the SG duct (Fig. 1A).

## The difficulty of distinguishing human eHFSCs from their progeny

As in the murine system, one of the central challenges in human eHFSC biology is to reliably distinguish bona fide slow cycling stem cells from their immediate lineage-restricted but not yet highly proliferative progeny, and these again from rapidly proliferating yet still immature HF keratinocytes that have descended from that progeny (i.e. TACs; Fig. 1B). In mice, in vivo tools such as lineage tracing experiments [8, 39] and the selective deletion of defined cell populations [5, 40] have facilitated the distinctions between parent stem cells and their diverse progeny.

For ethical reasons, in vivo tracing experiments are not possible in human individuals, and instructive techniques for cell tracking in human HF organ culture remain to be developed. This has greatly limited stem cell research in the human HF. Currently, determining parent-progeny distinctions in human eHFSCs relies on classical protein and RNA expression techniques (i.e. immunohistology, in situ hybridization, laser capture microdissection followed by qRT-PCR or microarray analysis, flow-cytometry cell sorting (FACS) and on functional studies with isolated HF cell populations, including clonogenic assays [30, 41–46]). Using these techniques, a limited number of instructive markers have been studied in greater detail and have allowed the field to at least approximate distinctions between true eHFSCs and their progeny in the human HF throughout HF cycling.

## Which markers are most useful to identify human eHFSCs and their immediate progeny?

### K15

Although the biological function of this epithelial progenitor cell-associated keratin is still obscure, K15 has become one of the most widely used markers to identify human eHFSCs (Fig. 2), and its identification in the human bulge ranks among the landmarks of modern hair research [32]. In the murine bulge, K15 expressing cells are label retaining [47], are capable of regenerating all epithelial HF lineages throughout cycling, typically reside in $G_0/G_1$ phase of the cell cycle and exhibit high clonogenic capacity [48]. Furthermore, they are capable of contributing to the epidermis and sebaceous gland [48]. Due to these properties in mouse, K15 bulge cells are regarded as HF stem cells.

Interestingly, in situ K15 promoter driven GFP expression can be seen within, but not limited to, the defined human bulge ORS [41] as much weaker, focal K15 protein expression is also found in the infra-bulge ORS and then increases in intensity again in the suprabulbar ORS (Figs. 3 and 4), depending on the specific primary anti-K15 antibody employed (see Supporting Information). However, non-bulge K15+ cells show a decreased clonogenic capacity when compared to bulge-derived K15+ cells [41]. This suggests that outside of the niche K15+ cells are not necessarily bona-fide stem cells; this is supported by the finding that K15 can be expressed in differentiated epidermal keratinocytes in vitro [35]. Nevertheless, K15 may label suprabulbar progenitor cells important to HF maintenance and cycling (Fig. 1B).

Moreover, human bulge ORS cells exhibiting the highest levels of K15 expression ($K15^{hi}$ cells), display lower expression of the proliferation marker Ki67 and a greater tendency to reside within the $G_0$ phase of the cell cycle [46]. Also, $K15^{hi}$ cells are smaller and less proliferative compared to $CD200^{hi}$ $ITGA6^{hi}$ and $CD34^{hi}$ cells [46], suggesting that CD200 and CD34 mark different HF progenitor cell sub-populations (discussed below).

### K19

Type I Keratin 19 (K19) is another important human eHFSC marker (it also marks HF LRCs in mouse [49]). Like K15, its function in epithelial stem cells remains unclear. K19 is expressed within the bulge and suprabulbar ORS in the human anagen follicle but also intermittently between these two regions [50] (Figs. 3 and 4). Focal K19 immunoreactivity (IR) can also be found within the basal layer of human epidermis [43, 49, 51]. Interestingly, bulge K19+ cells have been hypothesized to arise from K15 cells, as cells positive only

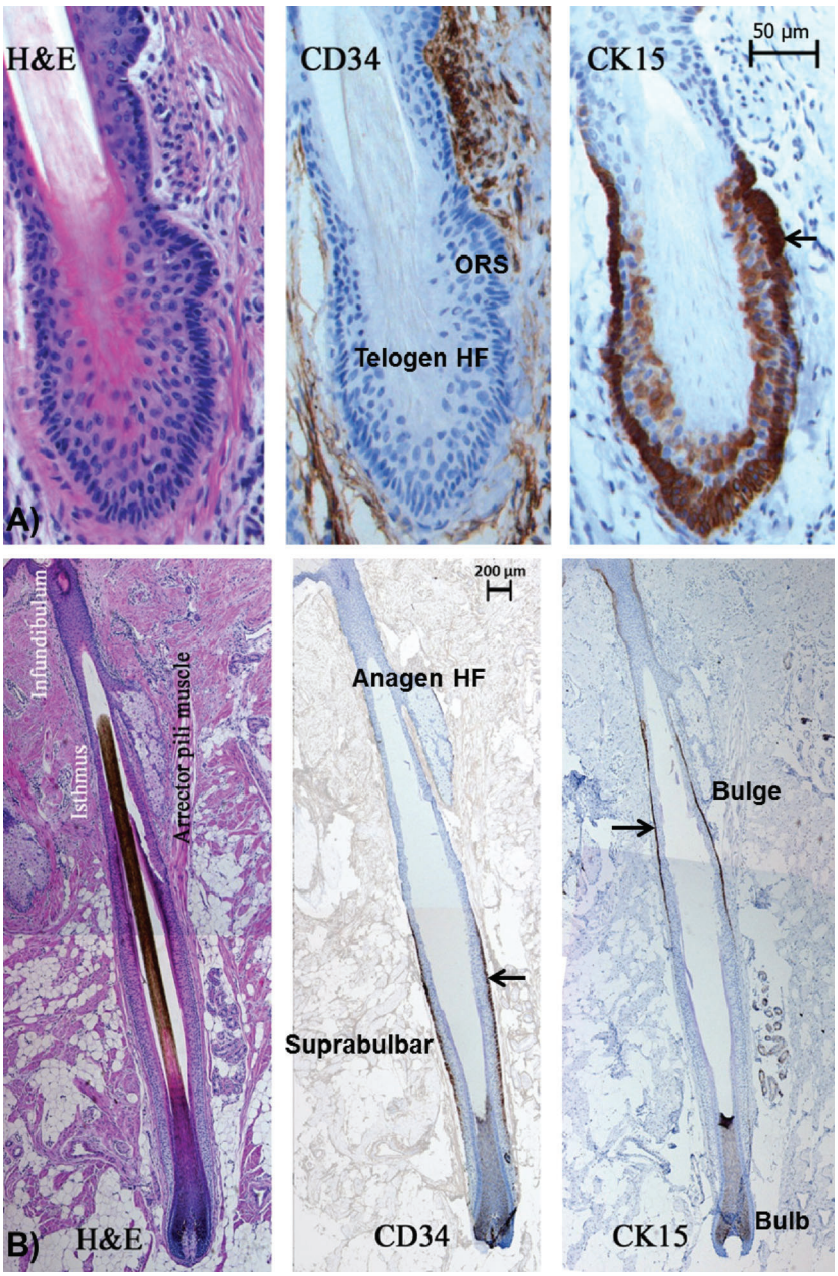


**Figure 2.** H&E, CD34 (QBEND10), and K15 (LHK15) in serial sections of a human telogen and anagen hair follicle. **A:** Hair follicle progenitor marker CD34 is not present in the human telogen HF bulge as epithelial outer root sheath CD34 immunoreactivity is negative (whereas connective tissue sheath CD34 immunoreactivity remains, center panel). On the other hand, immunoreactivity for bulge stem cell marker K15 is sustained in telogen (arrow, far right panel). Modified figure originally published in [44], RightsLink permissions obtained. **B:** CD34 immunoreactivity can be seen within the suprabulbar and lower ORS (arrow, center panel) of the human anagen follicle. K15 immunoreactivity prominently marks ORS cells of the defined bulge (and infra-bulge) region, found below the sebaceous gland duct and near the arrector pili muscle attachment site of the human anagen follicle (arrow, far right panel). K15 immunoreactivity can also be found in the suprabulbar ORS (see core text and Figs. 3&4). Mesenchymal CD34 immunoreactivity has been attenuated for representative purposes.

for K15 have been reported to be localized above K15/K19-double-positive cells in the human bulge, which again are localized above cells only positive for K19 [51]. A similar pattern of expression can be seen in the suprabulbar area (Fig. 3C). Moreover, K19+ cells in the suprabulbar ORS co-localize with Ki67, (Fig. 3D), whereas upper ORS/bulge cells do not [50], suggesting quiescence is predominant in the latter compartment.

## CD200

Cell surface glycoprotein CD200 is an effective means to identify putative eHFSCs within the human bulge in the outermost layers of the ORS [30, 43] (Fig. 3E). In the basal layer of the human bulge, epithelial cells express CD200 alongside K15, whereas in the suprabasal layers, cells typically lack expression of K15 but maintain expression of CD200 [42].

CD200 functions as an immune inhibitor and mediates immune privilege (IP) in a number of tissues [52–55]. Interestingly, it has been implicated in mediating the IP of murine [56, 57] and human eHFSCs [58, 59], since it is strongly expressed in the bulge area where major histocompatibility complex (MHC) class Ia, MHC class II and β2-microglobulin are prominently downregulated [59]. Therefore, insufficient CD200 expression may be involved in the collapse of IP in the eHFSC niche and may contribute to the pathogenesis of cicatricial alopecias (permanent inflammatory hair loss disorders) such as lichen planopilaris [58, 60].

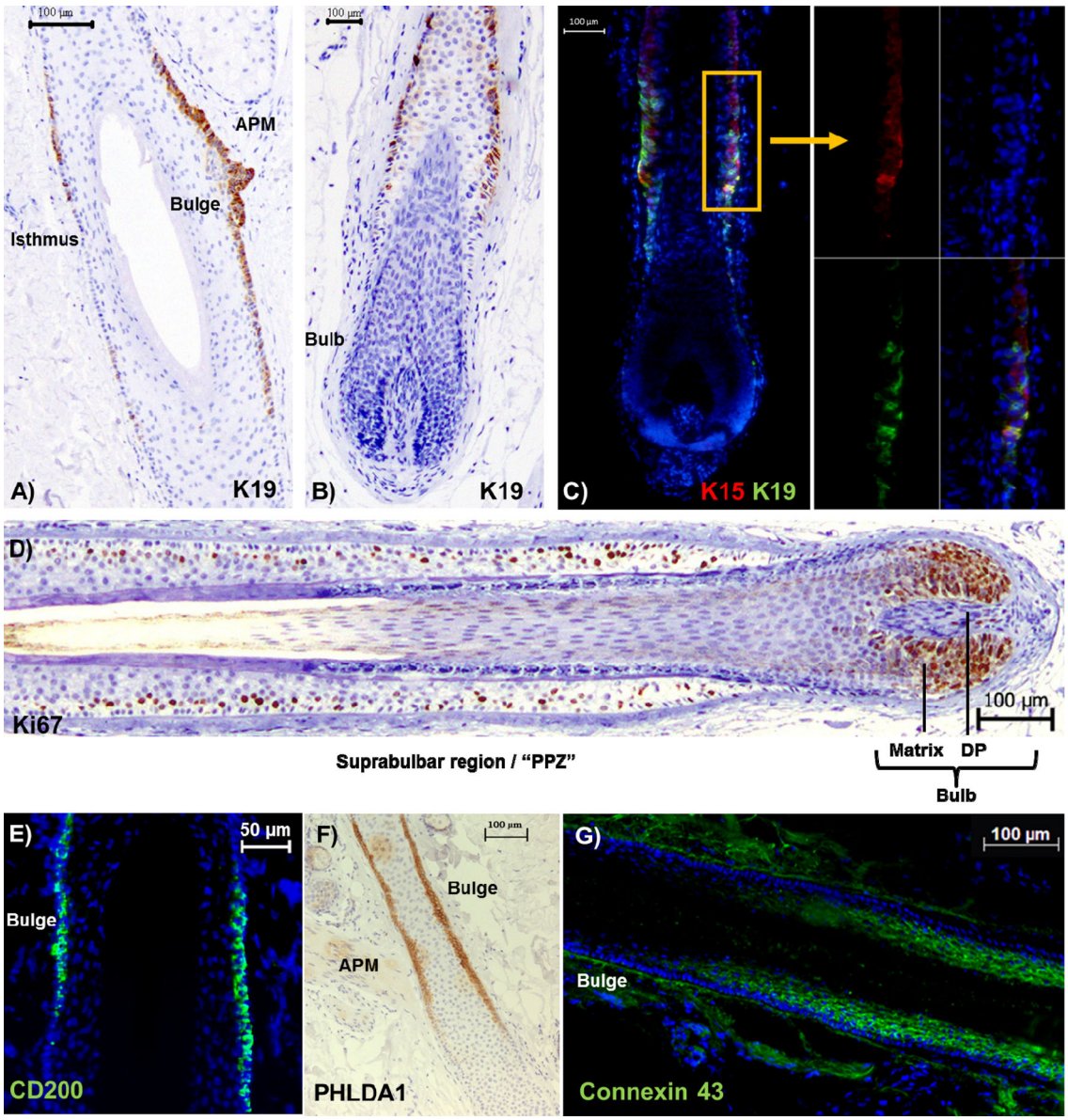


**Figure 3.** Localization of stem cell markers K19, CD200 and PHLDA1, proliferation marker Ki67 and negative bulge marker Connexin 43 in the human HF. **A:** Intermittent asymmetrical K19+ cells in the bulge. **B:** K19+ cells in the suprabulbar area. **C:** Immunofluorescence depicting co-localization of anti-K19 and anti-K15 (EPR1614Y, see Supporting Information) staining in the suprabulbar ORS. Anti-K15 EPR1614Y clone shows weak immunoreactivity (relative to bulge immunofluorescence, not shown) in the suprabulbar ORS that is otherwise minimal or undetectable using C8/144B antibody, see Supporting Information Discussion. **D:** Ki67 immunohistochemistry showing proliferating cells localizing to the suprabulbar ORS region where it has been proposed that progenitors elongate the ORS [11] and within the matrix where differentiation into hair shaft, companion layer and inner root sheath occurs. **E:** Anti-CD200 immunofluorescence in the bulge. **F:** Anti-PHLDA1 immunohistochemistry in the bulge. **G:** Connexin 43 is positive throughout the ORS but is negative in the bulge and the basal ORS layer of the infra-bulge region.

## CD34

CD34 is used as an epithelial stem cell marker in the murine HF, where it co-localizes with K15 in the bulge [61–62]. In contrast, CD34 is not found within the human HF CD200+ K15+ bulge region. CD34 marks epithelial cells in the most external layer of the ORS below the level of the isthmus (sub-bulge to suprabulbar regions) in the human anagen HF [30, 43–45, 63] (Fig. 2). The immunohistochemical expression of CD34 changes according to the phase of the HF cycle, being present in anagen but not in telogen follicles (Fig. 2). This underscores that CD34 can be expressed by different cell populations of the human HF, possibly depending on the degree of maturation or on the functional activities that these cells acquire. In addition, human CD34+ cells have a lower clonogenic potential relative to putative eHFSCs [42, 64], and thus may represent a step in the maturation of the ORS towards lineage restriction. This highlights potential

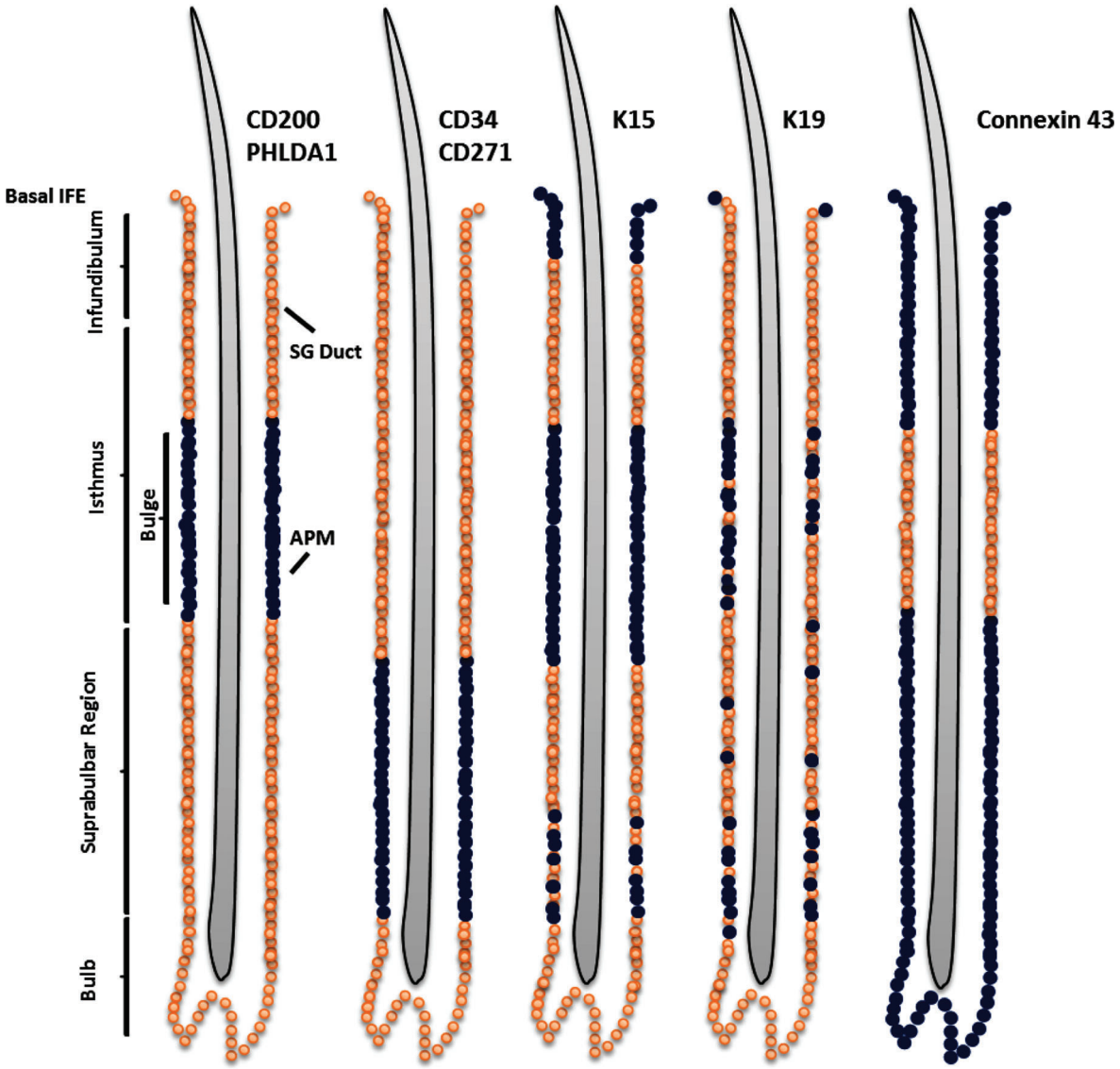


**Figure 4.** Localization of markers commonly used to identify bulge eHFSCs and other progenitor populations of the human outer root sheath. From left to right: markers CD200 and PHLDA1 identify stem cells of the quiescent bulge region. CD34 and CD271 can be used to identify outer root sheath cells outside of the bulge region within the sub-bulge/suprabulbar region. K15 and K19 mark stem cells within the bulge and infra-bulge region where staining patterns for the former can be irregular. K15 and K19 can also identify cells within the suprabulbar region where K15 staining is non-contiguous with bulge immunoreactivity. Gap junction protein Connexin 43 is expressed throughout the outer root sheath but is negative within the defined bulge region.

differences in the organization of the stem cell niche between human and murine HFs.

## PHLDA1

PHLDA1, also known as TDAG51 (T cell death-associated gene 51) is a proline and glutamine rich protein, and was first identified in the human HF bulge via DNA microarray analysis [30]. PHLDA1 may mediate resistance to apoptosis within cells that express it, but this concept remains controversial [37]. PHLDA1 IR is prominent in the bulge region [36] (Fig. 3F), and shows an IR pattern within the bulge, which resembles that of K15 and CD200 (Fig. 4). However, PHLDA1 is not specific to eHFSCs as it reportedly also demarcates the lower IRS and some human sweat gland cells [65].

## EpCAM/Ber-EP4

Epithelial cell adhesion molecule (EpCAM), when detected via Ber-EP4 antibody, is considered to be a useful marker of the human telogen SHG [66, 67]. The SHG is reportedly negative for K15 (LHK15 but not C8/144b, see Supporting Information) and K19 [67] as well as for CD34 (CD34 IR is not sustained in the catagen or telogen human HF [44, 68] (Fig. 2)) but *is* partially positive for CD200 [46]. The SHG is responsible for de novo hair matrix formation during anagen development and thus it could be posited that it must contain critical epithelial

progenitor cells [67, 69] (Fig. 1B). EpCAM/Ber-EP4 is also expressed in the lower part of the epithelial strand of late catagen follicle, but is typically absent in terminal anagen HFs [67, 70]. Therefore, EpCAM/Ber-EP4 could allow one to identify functionally unique subpopulation of epithelial HF progenitor cells during a distinct phase of HF cycling.

### Negative bulge markers: Connexin 43 and MHC class Ia

As no single positive marker allows one to definitively distinguish between the bulge compartment and the adjacent regions of the ORS, it is very useful to also employ negative bulge markers. Notably, connexin 43 IR is absent from the bulge (Fig. 3G) (Supporting Information) [43] suggesting that eHFSCs shut down gap junction communication. Moreover, as already described, the ORS in the bulge region has markedly lower expression of MHC class Ia and β2-microglobulin IR, supporting the immuno-privileged nature of the human bulge [58, 59]. Additionally, CD24, CD71, and CD146 have also been suggested to be useful negative bulge markers as confirmed by both immunohistochemistry and microarray analysis [30].

### Additional putative markers of epithelial progenitor cells in the human HF

In addition to this selection of markers employed for the identification of human eHFSCs and/or their immediate progeny, further details for other potentially important eHFSC markers in the human HF can be found within Table 1 and the Supporting Information. However, with rapidly expanding research on eHFSC in mice, this list is unlikely to be final as an ever-increasing number of putative murine eHFSC markers awaits systematic characterization in the human system (Table 1). Since space limitations preclude their discussion here, Supporting Information Table 1 highlights the functions of some stem cell-related proteins in murine HFs, but is by no means exhaustive.

Some of these markers have already been studied in more detail in the human HF, for instance Lhx2 was found to be overexpressed in and above the bulge region of the human ORS. In response to wounding, the number of intra-follicular Lhx2 expressing cells increases, localizing to the human upper infundibulum and wound edge [14]. However, it has been shown that Lhx2 mRNA steady-state levels are underrepresented in the human bulge ORS [41], and that Lhx2 can localize throughout the ORS but is predominantly outside of the bulge region [43]. The example of Lhx2 illustrates the difficulties one faces when attempting to define useful and definitive markers of human eHFSCs.

## The signature of human eHFSCs and their progeny: How many sub-populations?

The above literature synthesis suggests that bulge cells in the human ORS epithelium can best be identified by a combination of positive markers, namely K15, CD200, and PHLDA1 (Table 1; Fig. 4), and negative markers such as connexin 43 (Fig. 4). To identify putative sub-bulge to lower ORS epithelial progenitors we propose CD34 and, possibly, p75NTR (CD271) [42, 71] (Table 1; Fig. 4). K19 is also a useful, yet not selective eHFSC marker, since it clusters prominently to both the bulge and the suprabulbar ORS (importantly, the same argument can be made for K15, especially if the C8/144b antibody is not used [see Supporting Information]).

The differential expression of markers observed throughout the ORS may represent a divergence between an active ORS progenitor pool in the lower follicle and the permanent, largely quiescent and repopulating niche in the bulge/upper ORS [42, 50, 63, 64](Supporting Information Fig. 1). This is supported by the observation that the proliferation marker Ki67 associates with K19 cells in the suprabulbar ORS rather than the upper ORS [50] (Fig. 3D). Conversely, CD34+ and K19+ cells in the suprabulbar ORS appear to co-localize with the expression of hypoxia-associated genes, carbonic anhydrase and glucose transporter 1 (SLC2A1) [63]. Hypoxia is considered a global characteristic of adult stem cell niches [72] and as such, this lends support to the argument that these cells might be located within their own distinct stem cell niche.

Interestingly, in bald scalps of patients with androgenetic alopecia (AGA) $CD200^{hi}$ $ITGA6^{hi}$ and $CD34^{hi}$ cells are markedly diminished, whereas K15 positive cells are maintained [46]. These findings suggest that a defect in the conversion of eHFSCs to progenitors may play a role in the pathogenesis of AGA [46]. These different epithelial progenitor cell populations in the human ORS might have different, though not mutually exclusive, functions with respect to normal HF cycling and overall HF maintenance. This is further elaborated in hypothetical form in the Supporting Information (see Supporting Information Fig. 1). As a conclusion, emerging studies allow one to paint a picture of eHFSCs and their progeny, and this is important to understand the HF not only during homeostasis but also during a pathological state.

## What can we learn from emerging eHFSC concepts that have arisen from mouse models?

Beyond translating the marker expression profile of murine eHFSCs into the human system, it is imperative that we probe the extent to which key regulatory concepts that have been identified in mice (i.e. eHFSC activation, quiescence and overall cellular dynamics during HF cycling and morphogenesis), also apply to human HFs. We adapt some of these concepts to the human HF in the Supporting Information and illustrate them in Supporting Information Fig. 1.

The regulation of the mouse HF is mediated by a complex interplay between transcription factors implicated in, as well as proteins belonging to, several signalling pathways, including the Hedgehog, Wnt/β-Catenin, Tgf-β, BMP and Notch pathways, which are essential for normal HF function and cycling (reviewed in [3]). The maintenance of murine eHFSCs in a quiescent state is mediated by dermal, adipose and epithelial derived bone morphogenic protein (BMP) signalling [73–75]. The BMP signalling threshold needs to be

overcome and/or inhibited via DP signals before eHFSCs can be activated via environmental cues such as fibroblast growth factors (FGFs) that arise from the DP, Wnt signalling from the hair germ and platelet-derived growth factor (PDGF) from adipocyte progenitors [73, 76, 77]. Interestingly, enforced inhibition of BMP signalling in mice causes K15+ eHFSCs to become hair germ-like and primes them for activation via Wnt signalling as shown by upregulation of Wnt receptor Fzd10 and ligands Wnt7a, Wnt7b and Wnt16 [78].

At anagen onset in the mouse HF, proliferation occurs within the bulge and other parts of the ORS. This later becomes restricted to the ORS and the hair matrix as anagen progresses, whereas the bulge cells return to their characteristic quiescent state [79]. As the HF continues to cycle, upper ORS stem cells that had exited the mouse bulge and avoided apoptosis in catagen form the "new" basal bulge consisting of CD34+ cells as well as the SHG [79]. This lends support to the concept that the SHG descends from bulge cells during late catagen (Fig. 1B) [76, 80], although it remains possible that SHG progenitor cells form a separate lineage that has split from the bulge after the first hair cycle following HF morphogenesis [69].

During HF regression towards telogen, it has been proposed that cycling/proliferative lower ORS cells of the anagen follicle also evade apoptosis and eventually form the innermost keratin 6 (K6) expressing layer of the telogen bulge [79] (whether this is related to the K6 expressing Cp layer is unclear, see discussion within reference [81]). Despite being marked by Tcf3, Sox9, and Lhx2 (but CD34−), these innermost K6+ lower ORS derived cells lack stem cell properties; instead, they help to anchor the telogen club hair in murine HFs and maintain quiescence of the CD34+ve basal layer through BMP6 and FGF18 signals [79].

Another recently identified mouse eHFSC marker, Hopx, is expressed within basal bulge cells [81]. An alternative argument states that it is not lower ORS cells (Lgr5+ Hopx-) that form the innermost K6 layer of the ORS in telogen, but an Lgr5+ Hopx+ Shh- pool of cells that exist in the lower anagen hair bulb. These are believed to subsequently downregulate *Lgr5* and *Hopx* and upregulate *Tcf3*, *Lhx2*, and *Sox9* upon formation of the innermost K6 layer of the telogen bulge [81]. This concept coincides with the documentation of a "lower proximal cup" (LPC) in the lower anagen bulb of mice that is contiguous with the ORS but is clonally distinct, and may be distinct from the "matrix germinative layer" (GML) which gives rise to the layers of the Cp, IRS as well as the HS [11]. The same authors propose a model in which the suprabulbar ORS (for which they coin the term "privileged proliferation zone" (PPZ)) shows distal to proximal descending growth that elongates the whole ORS during growth in anagen but the PPZ itself remains at a fixed point from the bulb [11]. However, whether these elaborated concepts are applicable to the human HF remains largely unaddressed (see Supporting Information for further discussion).

## Future challenges: How can we selectively target human eHFSCs for research and therapy?

Enhancing our knowledge of human eHFSCs is likely to have a substantial impact on numerous areas such as in the treatment of alopecias, in wound healing, in regenerative medicine or in understanding the origins of epithelial derived cancers (for details and references, see Table 2).

Bulge eHFSCs could eventually be therapeutically targeted for such purposes, especially as research into the use of HF bound drug and/or gene delivery systems, such as nanoparticles, progresses [82]. For instance, such research could pave the way for direct or indirect therapeutic eHFSC gene regulation mediated by small-interfering RNAs (siRNAs) delivered using a suitable nanoparticle system, although challenges remain for perfecting this particular approach for siRNA delivery [83].

But even with this exciting potential on the horizon, human studies are fundamentally hindered by the lack of flexibility that laboratory experimentation on ex vivo human tissue confers (as well as relatively limited availability of accessible tissue). For example, we cannot readily make use of transgenic reporter methods (i.e. labeling of K15+ cells via Cre/lox system [39]) to trace distinct cell lineages and behaviors from embryonic development of the HF through each HF cycle stage.

Human HF biologists can already employ a HF organ culture system to experimentally manipulate isolated anagen human HFs in situ [84–85]. In this system, one can visualize the specific behaviors of human eHFSC subpopulations under various experimental conditions. For example, it becomes possible to visualize K15+ cells intravitally via transfection of human HFs using a K15 promoter-tagged GFP non-viral vector, including the use of 2-photon microscopy [41]. Also, lentiviral vector gene delivery systems could be employed [86] to image distinct eHFSC sub-populations by utilizing specific promoters placed upstream of a fluorescent reporter.

**Table 2. Potential therapeutic benefits arising from translational human eHFSC research**

| Type of benefit | Further reading |
|---|---|
| eHFSCs could be exploited for their capacity to mobilize out of their niche, thereby contributing to reepithelialization during skin wound repair. | [16, 115, 116] |
| Determining novel mechanisms by which eHFSCs can be protected from cytotoxic damage; i.e. in cases of permanent chemotherapy-induced alopecia, would provide significant benefits to patient health. | [117, 118] |
| If we could determine how to re-activate eHFSCs in vivo, hair loss associated with androgenetic alopecia and perhaps even primary and secondary cicatricial alopecias may be halted or even reversed. | [46, 58, 60] |
| Understanding the nature of eHFSCs could shed light on cancers suspected to arise from this stem cell pool, most notably basal cell carcinoma. | [36, 97, 119] |

Moreover, this HF culture model system can be employed to manipulate isolated human scalp HFs to selectively intervene with key pathways of eHFSC biology, such as Wnt, BMP, and FGF signalling, and test how hormones, nutrients, and drugs long appreciated to impact on human hair growth affect human eHFSCs in situ. For example, thyroid hormones $T_3$ and $T_4$, prolactin and spermidine (a polyamine) have already been shown to impact the behavior of human eHFSCs using this culture system [87–89]. It is also possible to perform gene silencing experiments in human HF organ culture [90] so that the effect of knocking-down selected genes thought to be functionally relevant in murine eHFSCs can also be elucidated in human HFs (Supporting Information Table 1).

However, human HF culture as of yet only permits limited HF survival and studying of the anagen-catagen transformation in vitro. This constitutes a major limitation of human HF organ culture and research given that mouse bulge eHFSCs are dynamic during HF cycling (i.e. formation of the SHG following catagen or activation of the telogen bulge [79]) (Supporting Information Table 2). This limitation can be overcome by transplanting human scalp skin onto immunocompromised mice [91].

This draws our attention to another limitation of human eHFSC research, which has largely been centered on anagen scalp HFs (due to the relative ease of isolating them), but remains fairly ignorant of hair cycle-associated changes in the human bulge during catagen and telogen [85, 92]. As illustrated by the example of CD34 expression (see above), hair cycle-dependent alterations in human eHFSC activities remain to be dissected and understood, and one should exercise caution when extrapolating from (strikingly hair cycle-dependent) murine hair biology concepts to the human HF and its mosaic cycling behaviour [92].

## From mouse to man: Why is it so important?

As discussed above, a key challenge is to determine if, when and where the established murine eHFSC markers are expressed in the human HF, how their expression changes with HF cycling and what their true functional relevance is in human eHFSC biology.

Elucidating these open questions is clearly important, given both the anatomical and functional differences (e.g. HF cycle length, synchronized versus mosaic HF cycling, wave pattern formation [92]) between murine and human HFs. The murine HF also responds differently to hormonal and growth factor stimulation (i.e. estrogens [93]). Furthermore, as well as the known difference in CD34 expression in HF cells between mouse and human, it has recently been shown that the murine eHFSC marker CD133 is *not* expressed in the human bulge [94]. Therefore, whilst the abnormal HF phenotypes of mutant mice (reviewed in [95]) and the concepts of eHFSC biology that have arisen from mouse models provide invaluable pointers, it remains a major unmet challenge to translate our existing understanding of murine eHFSC biology to the human HF.

Bridging this gap will be an important milestone, given that existing dermatopathological studies are typically reliant on well-established human stem/progenitor cell markers such as K15, K19, PHLDA1, CD200, Ber-EP4, and CD34. Some of these markers are currently used in research and in diagnostic pathology to assess the histogenesis and/or follicular differentiation of a variety of cutaneous tumors [36, 96, 97] or to study epithelial progenitor cells in distinct forms of scarring and non-scarring alopecias [46, 58, 98, 99]. Systematically exploring eHFSC markers characterized in mice (Supporting Information Table 1) in human HFs in health and disease should further enrich our repertoire of diagnostic markers and may provide valuable new insights into the pathobiology of human hair diseases, especially within HF disorders that cannot effectively be recapitulated within the murine system, such as AGA.

## Do cells of human HF epithelium undergo endoreplication?

Finally, understanding eHFSC control requires an understanding of where and when the epithelial progenitors within the human HF multiply and cease to divide to initiate post-mitotic terminal differentiation. Namely, cell cycle dynamics in the human HF epithelium are still unclear. It remains to be systematically characterized whether these involve continuous cell growth and post-mitotic DNA replication ("endoreplication") as it has been proposed for the IFE [100].

## Conclusions and outlook

Understanding epithelial stem cells and their immediate progeny in the human HF has become clinically essential (Table 2). Here, we have described the emerging understanding of human eHFSCs and have discussed key molecular markers. Moreover, we have delineated a number of key open questions within human HF research as well as promising experimental approaches that can be used to answer them. Although mouse research provides data that today cannot be obtained on humans, we must make a concerted effort to perform research on the human HF. This not only will enhance our understanding of the physiology of the human hair, but also the molecular and cellular alterations leading to hair loss disorders and skin cancer. Only by systematic characterization of the physiology and pathology of human eHFSCs in situ shall we manage to gradually close the widening gap between major progress in the defining of the biology and molecular controls of murine eHFSCs [3–6, 8, 73, 116, 121]. Recent progress, for example by linking human eHFSC function to defined types of alopecia (e.g. [46, 58]), in defining the topobiology of human HF epithelial progenitor cells [122], and in the differentiation of human induced pluripotent stem cells into eHFSCs that are capable of generating all epithelial lineages of the human HF [123], promises that this gap can be closed, if a concerted effort is made to do so. Moreover, as eHFSCs are in close contact with the immediately adjacent, specialized HF mesenchyme (Fig. 1A [4, 8, 124]), it is also imperative to better define the interactions of human eHFSCs with the CTS and with potential neural, Merkel cell, and immunocyte inputs into human eHFSC function at the level of the bulge.

In the absence of long-term lineage tracing experiments, the upcoming major leaps in understanding human eHFSC biology may arise from the fast paced methodological advances in cellular analysis technologies. For instance, RNAseq and mass cytometry at the single cell level may provide a better grasp of cellular heterogeneity within eHFSC and progenitor cell pools, as well as suggest candidate gene targets for pharmacological manipulation of eHFSC self-renewal and differentiation. In principle, we envisage that such interventional studies might include reverse engineering approaches, for instance in alopecia-induced HF miniaturization. Importantly, these single cell analyses should help circumvent the current problems associated with stem cell availability in low cell numbers and thus they might improve our understanding of the cross talk between the different stem cells residing in the HF maze and their respective niches. Similarly, the development of models that better reflect human HF cycle phases and corresponding stem cell behavior will no doubt impact our understanding of the dynamic nature of cell survival, migration and differentiation within the HF in both homeostasis and in response to exogenous signalling cues such as wounding, inflammation, cancer and aging.

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