Androgenetic alopecia (AGA), also known as common baldness, is characterized by a marked decrease in hair follicle size, which could be related to the loss of hair follicle stem or progenitor cells. To test this hypothesis, we analyzed bald and non-bald scalp from AGA individuals for the presence of hair follicle stem and progenitor cells. Cells expressing cytokeratin15 (KRT15), CD200, CD34, and integrin, α6 (ITGA6) were quantitated via flow cytometry. High levels of KRT15 expression correlated with stem cell properties of small cell size and quiescence. These KRT15\(^{hi}\) stem cells were maintained in bald scalp samples. However, CD200\(^{hi}\)ITGA6\(^{hi}\) and CD34\(^{hi}\) cell populations — which both possessed a progenitor phenotype, in that they localized closely to the stem cell–rich bulge area but were larger and more proliferative than the KRT15\(^{hi}\) stem cells — were markedly diminished. In functional assays, analogous CD200\(^{hi}\)ITGA6\(^{hi}\) cells from murine hair follicles were multipotent and generated new hair follicles in skin reconstitution assays. These findings support the notion that a defect in conversion of hair follicle stem cells to progenitor cells plays a role in the pathogenesis of AGA.
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Here we analyzed KRT15, CD200, and CD34 expression with flow cytometry to assess the stem and progenitor cell compartments in bald and hair (i.e., non-bald) scalp from individuals with AGA. Surprisingly, we found that the stem cell population was maintained in bald scalp. However, CD200*ITGA6* and CD34* cells were greatly diminished. These lost cells likely represent early progeny of stem cells, based on their position in the follicle, stem cell marker expression levels, cell size, and cell cycle state. Functionally, an analogous murine CD200*ITGA6* population was capable of regenerating an entire hair follicle, consistent with a progenitor cell phenotype. These results suggest that loss of progenitor cells, but not stem cells, contributes to human male pattern baldness.

Results

KRT15 expression levels correlate with epithelial stem cell characteristics of small cell size and cell cycle quiescence. To evaluate changes in hair follicle stem cell numbers in human scalp, we used KRT15 expression as a stem cell marker. We focused on cells in the top 5% of KRT15 expression (KRT155%) by flow cytometry, since KRT15 is expressed at the highest levels in bulge cells (3, 14). To further verify that KRT155% cells possess epithelial stem cell properties, we analyzed the relationship of KRT15 expression to the known stem cell characteristics of quiescence and cell size (12, 17).

Small cell size has been associated with stem cells in multiple tissues (17–19). In epithelia, early studies indicated that small human epidermal keratinocytes were clonogenic and had the greatest proliferative potential (17). More recent analysis confirmed that cells isolated from the bulge are small in size and are highly proliferative in vitro, consistent with their role as stem cells (14). Small corneal epithelial cells also exhibit the highest proliferative potential (19).

Finally, cell size may regulate cell cycle progression, since large cell size triggers proliferation (20).

Quiescence remains a defining characteristic of epithelial stem cells in the skin and other tissues (11, 21, 22). Functional assays demonstrate that quiescent epidermal cells possess the greatest proliferative potential (9, 10). Label-retaining cell (4, 7), lineage (9), and cell ablation studies (2) confirm that quiescent keratinocytes in the bulge are responsible for constant regeneration of the hair follicle. Human basal bulge cells also retain label and have a quiescent proliferative profile (3, 23).

To assess the relationship between KRT15 expression and the stem cell properties of quiescence and small cell size, we measured the cell size and cell cycle characteristics of KRT15-expressing keratinocytes from human adult scalp. We analyzed viable keratinocytes for expression of ITGA6, KRT15, and Ki67 by flow cytometry. The gating strategy is listed in Supplemental Figure 1, A–G (supplemental material available online with this article; doi:10.1172/JCI44478DS1). Cells of increasing percentile of staining for KRT15 were measured to detect cell size by forward scatter (Figure 1A). Cells at the 50th percentile and higher of KRT15 staining were significantly smaller than cells at the 20th percentile (n = 5, P = 0.002 to P = 1.58 × 10–5). KRT15 expression positively correlated with quiescence. Cells at the 95th percentile for KRT15 expression were predominantly in G0 (B, n = 5, P = 3.85 × 10–5), whereas cells at the 10th percentile were predominantly in G1 (C, n = 5, P = 3.1 × 10–5) and S (D, n = 5, P = 0.0003). *P < 0.05.

Quiescence (%) Percentage of cells in G0

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Figure 1

KRT15 expression levels define a gradient of stem cell characteristics in isolated scalp keratinocytes. (A) Using flow cytometry analysis, basal layer (ITGA6*) cells at the indicated percentiles of KRT15 staining intensity were plotted against their cell size. Increasing levels of KRT15 correlated with smaller cell size (n = 5, P = 0.002 to P = 1.58 × 10–5). (B–D) Cell cycle analyses of cells expressing different levels of KRT15. Increasing levels of KRT15 correlated with quiescence. Cells at the 95th percentile for KRT15 expression were predominantly in G0 (B, n = 5, P = 3.85 × 10–5), whereas cells at the 10th percentile were predominantly in G1 (C, n = 5, P = 3.1 × 10–5) and S (D, n = 5, P = 0.0003). *P < 0.05.
B–D). Coincident with the increase in the percentage of cells in G0, there was a decrease in cells in G1, which indicated that most cells not in G0 were in G1. The percentage of cells in G1 was significantly decreased at the higher percentiles (Figure 1C; n = 5, P = 0.05 to P = 3.1 × 10⁻⁵). Similarly, cells expressing high levels of KRT15 were less likely to be in S phase (Figure 1D; n = 5, P = 0.04 to P = 0.0003). G2/M changes were more variable according to KRT15 levels, as has been published previously (9), but were significantly decreased at the 80th and 90th percentiles (Supplemental Figure 1K; n = 5, P = 0.04 and P = 0.05). Thus, high levels of KRT15 expression correlate well with a quiescent stem cell phenotype.

Bald scalp retains KRT15 hi stem cells. Having verified that cells with the highest level of KRT15 expression possess properties of epithelial stem cells, we next addressed whether hair follicle stem cell numbers decrease in bald versus haired scalp from men with AGA. We isolated single-cell suspensions of epithelial cells from bald and haired scalp from the same individuals. These cells were stained with antibodies against ITGA6 and KRT15 and then analyzed by flow cytometry (Figure 2). We defined KRT15 hi cells as those in the top 5% of staining in haired scalp. In each paired sample from the same individual, an identical gate defining the top 5% of cells in haired scalp was applied to the cells from bald scalp. The flow cytometry was performed on the same day with identical instrument settings (see Methods for details). On average, the percentage of KRT15 hi cells was the same in bald and haired scalp (Figure 2, A–C; 4.6% ± 0.9% vs. 5.0% ± 0.02%, P = 0.3, n = 8).

Immunohistochemical staining for KRT15 also showed many strongly positive cells in miniaturized follicles from scalp with androgenetic alopecia (Supplemental Figure 2, A and B), which supported the notion that hair follicle stem cells are maintained in bald scalp.

Progenitor cell populations distinct from KRT15 hi stem cells are depleted in bald scalp. Recently, CD200 expression was identified in human bulge cells in haired scalp from women (13, 14). In these studies, the CD200+ population overlapped substantially with the K15+ population. To define changes in CD200+ cells in men with AGA, we analyzed CD200 expression together with expression of the epithelial basal cell marker ITGA6 by flow cytometry in matched bald and haired scalp. We excluded CD45+ hematopoietic cells and CD117+ melanocytes from the starting population and confirmed that the CD200+ cells were negative for these nonepithelial markers (Supplemental Figure 1, L and M). Surprisingly, we found that a well-demarcated population of cells expressing high levels of both CD200 and ITGA6 was markedly decreased in hair versus bald scalp (Figure 2, D–F; 2.3% ± 0.7% vs. 0.28% ± 0.1%, P = 0.005, n = 9). This population represented 10.0% ± 0.1% (n = 9) of the entire CD200+ population; to our knowledge, it has not been studied previously.

To better characterize CD200+ITGA6+ cells with respect to their stem cell properties, we determined their level of KRT15 expres-
sion, cell size, and degree of quiescence. We compared cells gated as CD200^hiITGA6^hi (Figure 2D), as well as cells gated as KRT15^hi ITGA6^hi (Figure 2A), with an otherwise un gated population of all ITGA6^hi cells (Supplemental Figure 1E). CD200^hiITGA6^hi cells expressed lower levels of KRT15 compared with KRT15^hi cells (n = 3, P = 0.036), and higher levels of KRT15 (n = 3, P = 0.046) compared with ITGA6^hi cells (Figure 3A). In line with this, we found almost no CD200 expression among the KRT15^hi cells (Figure 3D; n = 3, P = 0.008), which indicates that these populations are distinct. Given the intermediate expression of KRT15 in the CD200^hiITGA6^hi cells, the gradient of stem cell characteristics (Figure 1) predicts that these cells would be intermediate in cell size and cell cycle; indeed, this was the case (Figure 3, B and C). CD200^hiITGA6^hi cells were 75% ± 2% as large as all ITGA6^hi cells (Figure 3B; n = 6, P = 3 × 10^-5), but were significantly larger than the KRT15^hi cells (n = 6, P = 0.007). Thus, CD200^hiITGA6^hi cells were of intermediate size compared with the KRT15^hi cells.

To determine the level of quiescence of the CD200^hiITGA6^hi cells, we performed cell cycle analysis. The CD200^hiITGA6^hi population showed 69% ± 5% of cells in G0 (Figure 3C; n = 2), significantly higher than all basal cells (21% ± 1.9%, P = 0.02), but lower than the percentage of KRT15^hi cells in G0 (98% ± 0.6%, P = 0.05). Thus, CD200^hiITGA6^hi cells were of intermediate size and quiescence compared with KRT15^hi cells.

To assess whether other progenitor cell populations were depleted in bald scalp, we quantitated the number of CD34^+ cells, which juxtapose the bulge and localize below it in the outer root sheath. We found that CD34^+ cells were diminished roughly 10-fold in bald versus haired scalp (Figure 2, G–I; 1.9% ± 1% vs. 10.5% ± 0.3%, P = 0.01, n = 3). CD34^+ cells expressed low levels of KRT15 and were larger than the KRT15^hi stem cells (Figure 3, E and F). These findings are consistent with a role for these cells as progenitors descend from the bulge cells (14).

Human CD200^hiITGA6^hi cells localize to the hair follicle bulge and to the secondary germ. To further define the location of the CD200^hiITGA6^hi cells that did not localize to the bulge, we used the Ber-EP4 antibody, which detects epithelial cell adhesion molecule (EPCAM), to stain for secondary germ cells (Figure 4, D–F, Supplemental Figure 3, A and B, and ref. 24). Of the CD200^hiITGA6^hi cells, 16% were positive for Ber-EP4 (Figure 4F), indicative of their localization to the secondary germ. By immunohistochemistry, we detected CD200 expression in the bulge region and in secondary germ cells in telogen human hair follicles from hair scalp (Figure 4E and Supplemental Figure 4, D and E). In agreement with our fluorescence-activated cell sorting (FACS) analysis, we found a qualitative decrease in staining for CD200^+ cells in bald scalp (Supplemental Figure 2, C and D).
To further investigate the relationship of CD200hiITGA6hi cells to the secondary germ cells, we evaluated expression of LGR5 by quantitative PCR (qPCR). LGR5 recently has been touted as a marker of hair follicle progenitor cells in the lower bulge and secondary germ (11, 25). We found LGR5 mRNA elevated 1,443-fold in CD200hiITGA6hi versus CD200−ITGA6− cells (Figure 4G; n = 3, P < 0.01). As another test of the hypothesis that loss of the CD200hiITGA6hi population in AGA represents a loss of activated bulge cells, we evaluated expression of secondary germ cells, we compared changes in cell cycle features in mouse bulge (CD200hi) versus CD200− cells that localized to the secondary germ (Figure 5, A and B, CD34hi−ITGA6−). We found that approximately 82% of the CD200hiItga6hi cells were CD34+ and therefore localized to the bulge (Figure 5F). Together with the immunostaining data, these results indicate that roughly 18% of CD200hiItga6hi cells localized to the secondary germ. This corresponds closely to the 15% of human CD200hiITGA6hi cells that localized to the secondary germ based on their Ber-EP4 status (Figure 4F). Thus, the mouse and human CD200hiITGA6hi populations localize to both bulge and secondary germ in equivalent ratios.

To further compare the human CD200hiITGA6hi population with mouse CD200hiItga6hi cells, we performed cell cycle analysis of the mouse as we did on human CD200hiITGA6hi cells (Figure 3). Specifically, we compared cell cycle features in mouse bulge (CD200hiCD34+hi) and secondary hair germ (CD200−CD34−) cells (Figure 5G) with the human CD200hiITGA6hi population (Figure 3).

To enable functional studies of the CD200hiITGA6hi cells, we sought to define an analogous cell population in the mouse hair follicle. In mice, CD200hiItga6hi cells accounted for approximately 8% of the total viable epithelial cell population from back skin (Figure 5C and Supplemental Figure 6). To localize these cells, we took advantage of the known specific expression of CD34 by hair follicle bulge cells in the mouse epithelium (15) and compared CD34 and CD200 staining patterns. Immunostaining demonstrated overlap of their expression in the bulge, but extension of CD200 staining into the CD34hi− population (Figure 3). By FACS analysis, approximately 82% of the CD200hiItga6hi cells were CD34+ and therefore localized to the bulge (Figure 5F). Together with the immunostaining data, these results indicate that roughly 18% of CD200hiItga6hi cells localized to the secondary germ. This corresponds closely to the 15% of human CD200hiITGA6hi cells that localized to the secondary germ based on their Ber-EP4 status (Figure 4F). Thus, mouse CD200hiITGA6hi and human CD200hiITGA6hi populations localize to both bulge and secondary germ in equivalent ratios.
Consistent with previous studies demonstrating quiescence of the bulge cells (4, 9, 15), the proportion of bulge cells in S phase (0.84% ± 0.1%) was significantly lower than in all cells (Figure 5G; 1.44% ± 0.004%, n = 3, P = 0.02). The mouse bulge did show increased numbers of cells in G2/M (all cells 1.46% ± 0.5%, bulge cells 2.84% ± 0.5%, P = 0.02), as described previously (9). The 4.44% ± 1.5% of secondary germ cells (CD34–) in G2/M was higher than that of the bulge (26). The cells demonstrating the highest percentage of G2/M were CD200hiItga6hi in the secondary hair germ (Figure 5G; 6.07% ± 0.3%, n = 3, P = 0.01). In an inverse pattern, the percentage of cells in G1/G0 was decreased in cells with elevated levels of G2/M. Therefore, CD200hiItga6hi cells of the secondary hair germ showed significantly lower levels of cells in G1/G0 (89.5% ± 0.4%, n = 3) than did bulge cells (95.7% ± 0.2%, n = 3, P = 0.02). In summary, the decreased proportion of cells in G1/G0 in the mouse CD200hiItga6hiCD34− population compared with CD34+ bulge cells was similar to our cell cycle analysis demonstrating decreased G0 levels in human CD200hiITGA6hi cells compared with KRT15hi bulge cells (Figure 3). We conclude that both mouse and human CD200hiITGA6hi cells show evidence of cell cycle activation compared with cells of the bulge.

To further compare the mouse and human CD200hiITGA6hi cells, we analyzed their global gene expression patterns using microarrays. Given that mouse CD200hiItga6hi cells were composed of both bulge and secondary hair germ cells (Figure 5, E and F), we compared the expression of human CD200hiITGA6hi cells with those of mouse bulge and mouse secondary hair germ in a cross-species comparison. Gene lists of enriched genes for each population were created (see Methods) and compared for overlap (Figure 5H). Although mouse bulge and mouse secondary hair germ gene
expression patterns showed the most overlap (499 genes), it is likely that this is explained by species homogeneity. All 3 populations shared 178 genes. Interestingly, of the populations uniquely shared between the human CD200\(^{hi}\)ITGA6\(^{hi}\) cells and the mouse cell populations, more genes were shared with mouse bulge (151 genes) than with mouse secondary hair germ (39 genes). The greater overlap with the bulge compared with the secondary hair germ matches the cellular composition of the CD200\(^{hi}\)ITGA6\(^{hi}\) populations in both mice and humans. Further analysis of these gene lists (Supplemental Figure 9) showed that shared human and mouse genes present in the bulge were enriched in biologic adhesion proteins, whereas transcripts of the secondary hair germ were enriched in genes regulating death and apoptosis. These results are consistent with the concept that substrate attachment maintains the quiescent phenotype of the bulge cells and that the loss of these adhesions is associated with differentiation to secondary hair germ cells. Mouse CD200\(^{hi}\)ITga6\(^{hi}\) cells are multipotent and capable of regenerating hair follicles in a skin reconstitution assay. We performed functional analysis on the CD200\(^{hi}\)ITga6\(^{hi}\) population using a reconstitution assay, which tests the ability of isolated cell populations to regenerate hair follicles. Isolated single cells from epithelium are combined with neonatal dermal cells and injected intradermally into an immunodeficient mouse host. After 4 weeks, the injected tissue is examined for the presence of newly formed hair follicles, epidermis, and sebaceous glands (9, 27).

We sorted CD200\(^{hi}\)ITga6\(^{hi}\) or CD200\(^{lo}\)ITga6\(^{lo}\) cells from ROSA26 reporter mice. Grafting of CD200\(^{hi}\)ITga6\(^{hi}\) keratinocytes together with neonatal dermis successfully reconstituted hair follicles (Figure 6, A, B, and E). CD200\(^{lo}\)ITga6\(^{lo}\) keratinocytes produced few hair follicles, despite injection of equal numbers of cells (Figure 6, C–E). Contaminating neonatal epidermis from neonatal dermal preparations contributed to some hair follicle formation in both samples (Figure 6, A–D), but could be distinguished by its lack of β-galactosidase activity. Histologic sectioning of reconstituted hair-bearing cysts demonstrated contribution of CD200\(^{lo}\)ITga6\(^{lo}\) cells to all hair follicle lineages, including outer root sheath, inner root sheath, and sebaceous gland (Supplemental Figure 7, A–C), indicative of the multipotency of these cells.

**Discussion**

To our knowledge, the status of the hair follicle stem cell compartment in AGA has not been previously addressed. We hypothesized that the miniaturization of the hair follicle seen in AGA may result from loss of hair follicle stem cells. We showed that high levels of KRT15 expression correlated with a stem cell phenotype and found that KRT15\(^{hi}\)ITGA6\(^{hi}\) stem cells were preserved in bald scalp, whereas distinct populations of CD200\(^{lo}\)ITGA6\(^{lo}\) and CD34\(^{lo}\) cells were markedly diminished. The diminished cell populations likely arise from the KRT15\(^{hi}\)ITGA6\(^{hi}\) stem cells, since they were located within or adjacent to the stem cell population and possessed a more activated phenotype. Thus, our findings suggest that AGA results from diminished conversion of hair follicle stem cells to progenitor cells.

**Preservation of hair follicle stem cells in AGA.** The preservation of KRT15\(^{hi}\)ITGA6\(^{hi}\) cells in AGA is consistent with the current clinical concept that AGA is a nonscarring type of alopecia. Dermatologists classify alopecias into scarring and nonscarring categories. Some types of alopecia (e.g., lichen planopilaris, discoid lupus erythematosus, and graft-versus-host disease) are associated with destruction of hair follicle stem cells in the bulge and permanent hair loss. Ablation of the stem cell compartment leading to scarring alopecia has been replicated experimentally in mice through transgene expression of a cytotoxic gene in the bulge (2). In reversible types of alopecia (e.g., alopecia areata), inflammation targets hair follicle progenitor cells but spares hair follicle stem cells. In these disorders, regrowth occurs with suppression of inflammation and subsequent regeneration of the hair follicle from uninjured stem cells (5). Our finding that AGA, in the clinical category...
demonstrate here that a subset of these cells, the CD200+ population, was lost in AGA and was intermediate in the stem cell features of cell size and quiescence compared with the KRT15+ population and with all cells. Therefore, we speculate that the loss of CD200+ cells reflects the pathologic loss of a daughter population, owing to lack of activation of parent KRT15+ cells in AGA. Given the capacity of CD200+ cells to generate multiple lineages of the hair follicle (Figure 6), the lack of these cells might contribute to the miniaturization of the hair follicle during AGA.

Our studies demonstrated heterogeneity of the human hair follicle bulge stem cell area as well as the secondary germ area, findings that we believe to be novel. Although bulge cells as a whole were shown to generate all epithelial cell types in the lower anagen hair follicle of the mouse, prior evidence suggests that subpopulations of cells within the bulge are responsible for generating different segments of the follicle epithelium (11, 26, 30). Cells from the lower bulge formed the secondary germ, for example (1, 26). Our more detailed analysis of the secondary germ delineated further subdivisions within this structure based on proliferative properties. We showed that the human bulge also had subpopulations of cells with differing cell cycle properties that could be recognized by different markers. These insights provide tools for analyzing hair disorders and other skin conditions with respect to their stem and progenitor complement. Understanding the signals responsible for transition of stem cells to progenitor and more differentiated cells will be the next step in developing new treatments for skin disorders.

Methods

Tissue samples

This study used only normally discarded human scalp obtained anonymously and was approved by University of Pennsylvania’s IRB office as an exempt protocol. Normally discarded human scalp was obtained during hair transplantation. During the hair transplantation procedure, 2-mm-wide by several-centimeter-long specimens are taken from the donor occipital scalp and then dissected into various graft sizes (single hair grafts to multiple hair grafts). Some tissue is deemed unsatisfactory for transplantation, for example, because of inadvertent sectioning. These discarded donor samples from the occipital scalp were used as hair specimens. Likewise, 1.2-mm- and 1.7-mm-diameter cylindrical punches are performed to remove bald frontal scalp and create vacant recipient sites for donor scalp. This normally discarded tissue from the frontal scalp was used as the bald samples. Hair and bald samples were taken randomly and included both interfollicular epidermis as well as hair follicles. Tissue was all from white males ranging in age from 40 to 65 years. None of the subjects was using finasteride; 1 subject used minoxidil and was found to have a FACS profile similar to that of the others. Discarded tissue was obtained from a total of 54 males.

All animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. ROSA26 and SCID mice were acquired and used as published previously (9).

Immunohistochemistry

Previously published protocols were followed (20). Briefly, paraformaldehyde-fixed, paraffin-embedded slides containing human scalp hair from excisions were deparaffinized and rehydrated. Microwave antigen retrieval was used with 10 mM citrate buffer (pH 6.8). Slides were treated with hydrogen peroxide to block endogenous HRP activity. Slides were washed and treated with blocking reagent and then primary antibody (mouse anti-KRT15, Lab Vision clone LHKRT1; rat anti-mouse CD34, MEC 14.7; goat anti-human CD200, R&D Systems; goat anti-mouse CD200, R&D Systems; and mouse anti-human Ber-EPA, Dako). Secondary biotinylated anti-mouse antibody (Kirkegaard and Perry Laboratories Inc.) and biotinylated anti-goat/rat (Vector) were added following a wash. Slides were again washed, treated with streptavidin-HRP (Vectastain), developed using ABC kit, and counterstained with Methyl Green (Vectastain). For immunofluorescence, Streptavidin–Texas red or anti-rat FITC (Abcam) were used. Frozen sections were air-dried, washed with PBS, fixed with acetone, washed with PBS, and incubated with the above antibodies, followed by staining as described above. Staining and preparation of β-galactosidase expressing tissues was performed as previously described (9).

Flow cytometry

Samples were treated with 5 U/ml Dispase (Sigma-Aldrich) overnight. The epidermis and dermis were separated, including removal of individual hair shafts, surrounding follicle, and surrounding interfollicular epidermis simultaneously. Isolated epidermis was then washed in PBS and treated with tryptic for 15 minutes at 37°C in the presence of DNase. The sample was vortexed and filtered through a 70-μm filter. Cells were spun at 200 g for 5 minutes and counted using a hemocytometer. For every stage of the above procedure, tissue was saved for monitoring of successful purification by hematoxylin and eosin staining. We grossly and histologically verified tissue identity and success of sample preparation (Supplemental Figure 8). Between 0.5 × 106 and 1 × 106 cells were used for counting each sample, with attempts made to quan-
tify the maximum possible during flow analysis. Unless otherwise indicated, all antibodies used were identical to those used for immunohistochemistry. Samples were stained with various combinations of antibodies to CD200 (MRC OX-104) and ITGA6 (BD Biosciences — Pharmingen clone GoH3), fixed and permeabilized (Caltech labs), and stained with the above anti-human antibodies against actin (Sigma-Aldrich clone AC-15), KRT15, FST, Ki67 (BD Biosciences — Pharmingen clone B56), CD117 (BD Biosciences clone 104D2), CD45 (BD Biosciences clone 2D1), Ber-EP4, DAPI, and/or an isotype control (IgG2a, Sigma-Aldrich). Staining of mouse skin was done as previously described (9). For murine staining, we used anti-mouse CD200 (Serotec OX-90, CD117 (BD Biosciences clone 2B8), and CD45 (BD Biosciences clone 30-F11). Mouse and human staining of CD200 probes were filtered to retain only those that were flagged as present in the arrays; default values provided by Affymetrix were applied to all analysis parameters. Border pixels were removed, and the average intensity of pixels within the 75th percentile was computed for each probe. These values were exported as .cel files. The average of the lowest 2% of probe intensities occurring in each of 16 microarray sectors was set as background and subtracted from all features in that sector. Probe pairs were scored positive or negative for detection of the targeted sequence by comparing signals from the perfect match and mismatch probe features. The number of probe pairs meeting the default discrimination threshold (τ = 0.015) was used to assign a call of absent, present, or marginal for each assayed gene, and a P value was calculated to reflect confidence in the detection call. The flag values were additionally exported as .csp files.

Affymetrix probe intensities were imported into GeneSpring (version 7.2, Agilent Technologies), where probe set signal values were calculated using the GC-RMA algorithm. Upon import of the Affymetrix flag values, the probesets were filtered to retain only those that were flagged as present in at least 2 of the repeat samples. This list was used for condition-based principle components analysis to assess global trends in sample similarity. This analysis demonstrated groupings based on sample identity and prompted the use of mixed-model 2-way ANOVA as a means of finding differentially regulated genes between the sites of interest.

Affymetrix signal data were imported into Partek Genomics Solution (version 6.2, Partek Inc.), where the data were log2 transformed. Probesets not shared between mouse and human chips were removed. Genes less than P < 0.05 for the identity of sample term of the ANOVA, and with less than 2-fold change between positive and negative populations, were also excluded. The resulting enriched gene lists for bulge, secondary hair germ, and CD200–ITGA6hi cells were then examined for intersection using Partek. Primary data are available at the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/, GEO accession no. GSE21569).

Hair reconstitution
Hair reconstitution was performed as previously described (9). Briefly, epidermis from 7-week-old ROSA26 mice (Jackson Labs) was sorted to yield the identified population. Dermal preparations from 2-day-old neonate wild-type mice as previously described (27) were frozen in advance. Frozen dermal cells (6 × 10⁶) were thawed, combined with the listed number of epidermal cells in 60 μl PBS, and injected intradermally onto the backs of SCID mice. After 4 weeks, grafts were harvested and processed for β-galactosidase activity, paraffin embedded, and sectioned.

Statistics
For all P value calculations, paired Student’s t test with a 1-tailed distribution was used, and P values less than 0.05 were considered significant. All averages listed are mean ± SEM.

Acknowledgments
We gratefully acknowledge the assistance of Charles H. Pletcher and William Murphy of the Penn Flow Cytometry and Cell Sorting Department.
Tumbar T, et al. Defining the epithelial stem cell identity for any analyses, interpretations, or conclusions; the Edwin & Fannie Gray Hall Center for Human Appearance at University of Pennsylvania Medical Center; the American Skin Association; the Dermatology Foundation; and L’Oreal.


