Functional Characterization of Quiescent Keratinocyte Stem Cells and Their Progeny Reveals a Hierarchical Organization in Human Skin Epidermis

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ABSTRACT

Although homeostatic renewal of human skin epidermis is achieved by the combined activity of quiescent stem cells (SCs) and their actively cycling progeny, whether these two populations are equipotent in their capacity to regenerate tissue has not been determined in biological assays that mimic lifelong renewal. Using fluorescence activated cell separation strategy validated previously by us, human epidermis was fractionated into three distinct subsets: that is, x6CD71dim, x6CD71bri, and x6dim with characteristics of keratinocyte stem, transient amplifying, and early differentiating cells, respectively. The global gene expression profile of these fractions was determined by microarray, confirming that the x6CD71dim subset was quiescent, the x6CD71bri was actively cycling, and the x6dim subset expressed markers of differentiation. More importantly, functional evaluation of these populations in an in vivo model for tissue reconstitution at limiting cell dilutions revealed that the quiescent x6CD71dim fraction was the most potent proliferative and tissue regenerative population of the epidermis, capable of long-term (LT) epidermal renewal from as little as 100 cells for up to 10 weeks. In contrast, the cycling x6CD71bri fraction was the first to initiate tissue reconstitution, although this was not sustained in the LT, while differentiating x6dim cells possessed the lowest demonstrable tissue regenerative capacity. Our data suggest that in human skin, the epidermal proliferative compartment is not composed of equipotent cells, but rather is organized in a functionally hierarchical manner with the most potent quiescent SCs at its apex (i.e., x6CD71dim) followed by cycling progenitors (i.e., x6CD71bri) and finally early differentiating keratinocytes (i.e., x6dim).

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Adult stem cells (SCs) in many tissues are defined as rare and relatively quiescent or slow-cycling cells with the unique capacity to constantly self-renew and regenerate tissues during homeostasis. However, recent work on several epithelia including the interfollicular (IF) and hair follicle (HF) epidermis of the skin, and small intestinal mucosa, suggests that the more rapidly cycling progeny of SCs, termed transient amplifying (TA) cells or perhaps more accurately committed progenitors (CPs), also possess extensive capacity for tissue replacement and are multipotent [1–3]. Indeed, it has been postulated that cell replacement in the small intestine [4] and HF [5] is routinely performed by Lgr-5 positive “cycling stem cells,” although quiescent SCs exist in these tissues [6–12]. Similarly, it has been shown that the cycling TA cells or progenitor cells of the IF epidermis are long-lived and may be the workhorse executing significant long-term (LT) tissue renewal while SCs remain largely dormant [13].

It is likely that the proliferative compartment in rapidly renewing epithelia is hierarchically organized into a biological continuum at the apex of which lies the most potent quiescent tissue-regenerative SCs, and successive populations of cycling progenitor cells undergo a rather gradual loss of tissue-renewal capacity, concomitant with commitment to differentiation. This idea encapsulated in the “spiral model of diminishing stemness” [14] is best illustrated in the bone marrow, where a hierarchy was noted even within the SC compartment using differential ability to efflux Rhodamine 123 and Hoechst 33342 [15] identifying SC subsets. There is strong evidence to suggest that the murine HF epithelium is organized in a similar way. The cellular heterogeneity of the CD34/K15 positive HF bulge—a site enriched for quiescent,
multipotent SCs [16, 17]—has long been suspected, given the gradual loss of slow-cycling/DNA label-retaining cells in this niche [10]. This was confirmed recently by the detection of Lgr5+ cycling cells in the bulge, which are long-lived and contribute to all HF lineages [5]. However, these cells could equally represent either a narrow window during which quiescent SCs are in cycle, given their rare incidence, or represent the earliest progeny of quiescent SCs also described hypothetically as TA-1 cells [14]. However, TA cells of the IF epidermis are also not nearly as “transient” as previously thought [3, 13], causing a paradigm shift in our thinking of the actual properties of these cells, given their SC-like behavior and the recognition that sustained tissue renewal, is not restricted to a minor, quiescent, or slow-cycling SC population in many tissues. However, these data leave open the important question of whether all proliferative epithelial cells are “equally potent” as originally proposed by Leblond et al. [18], a particularly important issue for clinical applications, where the assumption is that quiescent SCs are necessary and critical to achieve permanent correction for genetic disorders [19]. Although, burns patients can be rescued with autologous grafts of “bulk” cultured keratinocytes, it remains unclear whether their LT-tissue regenerative ability (TRA) can be attributed to the activation of a minor population of quiescent SCs and/or their progeny during ex vivo expansion [20, 21]. Further work is required to elucidate the “comparative” LT-TRA properties of epithelial SCs and TA cells/CPs, because this has strong implications for the role of these subsets in homeostatic self-renewal as well as cancer development given that intrinsically high proliferative and self-renewal properties are characteristic of cancers.

The IF epidermis of neonatal human foreskin has also been a valuable epithelial SC model. Cell surface markers that identify putative IF SCs in this tissue have been reported [22–25], although functional distinction between SCs and progenitors in terms of LT-TRA has either been untested or proven challenging [26]. Nevertheless, CD71/transferin receptor expression does successfully separate the integrin bright (z6bri) proliferative compartment of human IF epidermis into quiescent (z6bri,CD71dim) and cycling (z6CD71bri) tissue reconstituting cells; a third lineage marker-positive epidermal fraction (z6dim) can also be resolved representing the earliest differentiating keratinocytes of the skin [26, 27] (Fig. 1). Here, we report for the first time that the quiescent z6bri,CD71dim epidermal population can be distinguished from the cycling z6CD71bri and differentiating z6dim fractions in neonatal human foreskin by its molecular signature, greater potency for LT-TRA, and ability to maintain a self-renewing proliferative compartment, using a gold standard limit dilution transplant assay as described previously for hematopoietic and mammary epithelial SC populations [28, 29]. This work represents the first study to compare the LT biological function of human IF epidermal SCs and progenitor cells, with strong implications for their respective role in homeostasis, tissue repair, and cancer.

**Materials and Methods**

**Affymetrix Analysis**

The z6bri,CD71dim, z6bri,CD71bri, and z6dim populations as shown in Figure 1 were collected from pools of four to eight human foreskin keratinocyte isolates, from four independent fluorescence activated cell separation (FACS) experiments. The FACS fractions were washed with phosphate-buffered saline, counted, and 40,000 cells per cell fraction aliquoted for lysing in LysisBinding solution. Total RNA was isolated using mirVana RNA isolation Kit (Ambion, Austin, TX). mRNA quality was assessed with an Agilent 2100 bioanalyzer and RNA 6000 Pico Assays (Agilent Technologies, Santa Clara, CA), and concentrations were determined by means of the Quant-IT RNA assay kit and the Quibit fluorometer (Invitrogen, Carlsbad, CA). We then used a T7-primer-based two-round linear RNA amplification protocol (GeneChip Two-Cycle cDNA Synthesis Kit, Affymetrix, Santa Clara, CA, www.affymetrix.com/support/technical/manuals.affx [Expression Analysis Technical Manual, pdf], Expression Analysis Technical Manual.pdf, Affymetrix). Approximately 15 μg of biotin-labeled cRNA from each individual sample was fragmented and hybridized to Affymetrix high-density oligonucleotide arrays for human genes (HG-U133 Plus 2.0). All arrays were stained, washed, and scanned following the manufacturer’s protocol.

**Microarray Data Analysis**

Robust multiarray average normalized [30] and log2 transformed expression values for annotated probes were compared pairwise among the three fractions using moderated t-statistics to quantify the relative differences of probes between any two sample groups [31, 32]. After using the Benjamini and Hochberg multiple testing correction, those probes with fold change more than 2 and adjusted p values <.05 were considered differentially expressed and selected for further analysis. The 2,586 probes, which met the selection criterion for any of the three pairwise comparisons, were clustered using Eisen cluster software, and the hierarchical cluster with average linkage was plotted using Treeview. Pathway enrichment analysis was performed (Fig. 2) using the online program DAVID Bioinformatics Resources [33] and Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA).

**Real-Time PCR Validation of Microarray Data**

The RNA used was from the same samples used for the Affymetrix arrays. The reverse transcription reaction was performed on 100 ng of total RNA template, and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and random primers as instructed by the manufacturer (Promega, Madison, WI). Real-time polymerase chain reactions (PCR) for Wnt-1 and PDGFA was carried out using SYBRgreen (Applied Biosystems, Carlsbad, CA) on a StepOne Plus real-time PCR system (Applied Biosystems). The reaction profile consisted of an initial denaturation at 95°C for 15 minutes followed by 40 cycles of PCR at 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing and extension). The fluorescence emitted was captured at the end of the extension step of each cycle at 530 nm. Primers for the genes platelet-derived growth factor a polypeptide (Pdgfa) and Wnt-1 were obtained from GeneWorks (Hindmarsh, Australia). Primers: PDGFA: 5¢ caagacagacagctgctGGG 3¢; 5¢ ctcgaatttctcatactcgbgg 3¢; Wnt-1: 5¢ ccaaaaagggcccttta 3¢; 5¢ gigtgctcagcaaacatgt 3¢. Real-time PCR for Tcf4 and Bmi-1 was carried out using a custom made TaqMan array micro fluidics card (Applied Biosystems), TaqMan universal PCR mastermix following manufacturer’s (Applied Biosystems) instruction, and carried out on a ABI7900 real-time PCR machine (Applied Biosystems). Triplicate results were normalized to the expression of the housekeeping gene Gapdh, and the ΔΔCt was calculated using Excel and plotted in relation to the gene expression of the cycling z6bri,CD71bri cell population.

**Transplantation Assay**

Devitalized rat tracheas (RTs) were inoculated with specified numbers of freshly fractionated quiescent z6bri,CD71dim, cycling z6CD71bri, or differentiating z6dim populations of primary human foreskin keratinocytes together with 5 × 10^5 cultured and irradiated support keratinocytes per RT in 30 μl medium as described...
Two RTs were implanted per fraction per mouse and four replicate experiments were performed. Severe combined immunodeficiency (SCID) mice were used as recipients for transplants. Control transplants consisting of \(5 \times 10^5\) irradiated support keratinocytes only per RT were performed for each time point per experiment to test whether the support keratinocytes alone could contribute to epithelial reconstitution. Both human tissue use and animal studies were approved by Institutional Ethics Committees.

**Histology and Immunostaining of Transplanted Tissue**

Mice were sacrificed 6 or 10 weeks post-transplant, and the RTs were excised and processed for cryosections and paraffin sections [26]. Paraffin sections were stained for histological analysis of the reconstituted epithelial tissue to determine the extent and rate of epithelialization. Cryosections were stained for a number of markers indicative of a proliferative basal layer and differentiating suprabasal layers. Primary antibodies used were mouse anti-human Ki67 (DAKO, Carpinteria, CA); guinea pig anti-human K14 (Progen, Heidelberg, Germany); guinea pig anti-human K15 (Progen); and rabbit anti-human involucrin (a gift from Dr. Bob Rice, University of California, San Francisco, CA); mouse anti-human Keratin 10 (neat, LHP2, hybridoma supernatant was kindly provided by Dr. Irene Leigh (Royal London Hospital, London, U.K.) and rabbit anti-human Loricrin (1:500, Covance, Princeton, NJ). Secondary antibodies are as follows: goat anti-mouse Alexa 555, goat anti-guinea pig Alexa 488, goat anti-rabbit Alexa 488, and goat anti-rabbit Alexa 555 (Invitrogen). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (1:10,000; Sigma, St. Louis, MO), and sections were analyzed by Olympus BX51 and processed with Adobe Photoshop.

**RESULTS**

Molecular Signature of \(\alpha_6^{\text{br}}\)CD71\(^{\text{dim}}\), \(\alpha_6^{\text{br}}\)CD71\(^{\text{bri}}\), and \(\alpha_6^{\text{dim}}\) Keratinocytes of the Human IF Epidermis

We undertook global gene expression analysis of fractionated epidermal cells to determine their proliferation and differentiation status at a genome-wide level and firmly establish their distinct identity following separation by sorting. Thus, microarray analysis was performed on mRNA isolated from freshly sorted \(\alpha_6^{\text{br}}\)CD71\(^{\text{dim}}\), \(\alpha_6^{\text{br}}\)CD71\(^{\text{bri}}\), and \(\alpha_6^{\text{dim}}\) keratinocytes using the Affymetrix HG-U133 Plus 2.0 arrays. Unsupervised
Figure 2. Molecular profile of $a^{ab\,6\,CD71^{dim}}$, $a^{ab\,6\,CD71^{bri}}$, and $a^{dim\,6\,epidermal}$ fractions of neonatal human foreskin. Microarray analysis of the mRNAs expressed differentially by the quiescent $a^{ab\,6\,CD71^{dim}}$ (Quies), cycling $a^{ab\,6\,CD71^{bri}}$ (Cycl), and differentiating (Diff) $a^{dim\,6\,epidermal}$ fractions from four independent fluorescence activated cell separation experiments. Treeview software was used to display a hierarchal cluster with average linkage analysis of normalized gene expression (more than twofold change, adjusted $p$ values of <.05). Differentially overexpressed (red) or underexpressed (green) genes in the quiescent $a^{ab\,6\,CD71^{dim}}$ (lanes 1–3), Cycl $a^{ab\,6\,CD71^{bri}}$ (lanes 4–6), and Diff $a^{dim\,6\,epidermal}$ (lanes 7–10) related to cell cycle progression were under-represented in the quiescent cells but transcribed in the cycling and differentiating cells (A), as were the steroid, fructose, and mannose metabolic pathways (B). Genes in the WNT, Hedgehog (Hh), and insulin pathways (C) reveal overexpression of inhibitors of these pathways particularly in the quiescent cells, while epidermal differentiation markers were expressed in the differentiating population. Note the gradual transition in expression patterns of gene subsets from the quiescent $a^{ab\,6\,CD71^{dim}}$ to cycling $a^{ab\,6\,CD71^{bri}}$ to the differentiating $a^{dim\,6\,epidermal}$ fractions indicating a possible parent-progeny relationship between these subsets. Abbreviation: Hh, Hedgehog.
hierarchical clustering revealed 2,585 differentially expressed genes (i.e., more than twofold change; \( p < .05 \)) among these fractions (Supporting Information Table S1, data from four separate experiments). Analysis for genes indicating proliferation, metabolic activity, epidermal differentiation, and activity of signaling pathways described in several SC types [12, 34] revealed that the \( x^{+}\text{CD71}^{\text{dim}} \) fraction known to be enriched for G0/G1 cells (Fig. 1 [27]) was characterized by “reduced” mRNA levels for markers of proliferation, for example, Ki67, cell cycle progression genes: cyclins D1, E1, and F (Fig. 2A), and epidermal differentiation markers K1, K10, and K2 (Fig. 2D). A major distinguishing feature of the \( x^{+}\text{CD71}^{\text{dim}} \) population (referred to from hereon as Quiescent) was its overexpression of negative regulators of key signaling pathways (Fig. 2C), known to be active in the epidermis most notably Wnt, transforming growth factor \( \beta \) (Tgf-\( \beta \)), sonic hedgehog (Shh), and insulin pathways as shown for HF bulge SCs [35]. The Gli3 and Ptc1 genes were upregulated in the quiescent (Quies) keratinocytes, which are indicative of Shh pathway blockade. Inhibitors of the Wnt signaling pathway (Dkk3, Fzd1, Tcf4, Tcf712, Wf1, and Wnt3) and insulin pathway (Igfbp3, Igfbp5, Igf1r, Irs1, and Irs2) were also upregulated (Fig. 2C). Similarly, inhibitors of the Notch pathway (Dil1 and Mamli2), regulators of the Fgf (Fgfr2, Spry1, and Sryc2), Jak/Stat (Socs-2 and Socs-5), Bmp (Fst and Znfs22), and Tgf-\( \beta \) pathways (Lhbp1, Lhbp2, and Tgfl1) were also overexpressed in Quies keratinocytes (Supporting Information Table S1). For instance, the overexpression of Igfbp3 and Igfbp5, known to bind to and sequester insulin growth factor, and down-regulation of downstream target genes implicated in the insulin signaling pathway (i.e., Id1, Id3, and Igf2; Supporting Information Table S1) may ensure that the \( x^{+}\text{CD71}^{\text{dim}} \) cells do not respond to this potent stimulant of epithelial proliferation [36, 37] in situ when compared with the \( x^{+}\text{CD71}^{\text{dim}} \) and \( x^{+}\text{CD71}^{\text{dim}} \) populations. This data suggest that the \( x^{+}\text{CD71}^{\text{dim}} \) fraction is held in a quiescent state in vivo by keeping in check a number of signaling pathways that would otherwise result in proliferation given the presence of growth factors in their microenvironment. Interestingly, Bmi-1 and Nfatc1, transcription factors critical for self-renewal of several different SCs [38–40], were both overexpressed in the Quies \( x^{+}\text{CD71}^{\text{dim}} \) population (Supporting Information Table S1). Notably, Nfatc1 has a demonstrated role in balancing quiescence versus proliferation in HF bulge SCs [40].

We had previously inferred that the \( x^{+}\text{CD71}^{\text{cri}} \) subset was actively cycling (named Cycl), given its enrichment for cells in S/G2M (Fig. 1); and that the \( x^{+}\text{CD71}^{\text{dim}} \) population most likely represented the earliest differentiating keratinocytes (termed Diff) given the expression of differentiation markers [27, 41]. Consistent with this, \( x^{+}\text{CD71}^{\text{cri}} \) cells expressed positive regulators of cell cycle progression and proliferation, that is, cyclin D1, D2, and E1, cyclin dependent kinase, cdc45, E2f, mcm5, and Ki67, and genes for several metabolic pathways, for example, fructose, mannose, glucose, and galactose metabolism and steroid biosynthesis, are also expressed in \( x^{+}\text{CD71}^{\text{cri}} \) cells despite the onset of differentiation (Fig. 2A, 2B) indicated by the abundance of early differentiation markers (Fig. 2D). The differential gene expression observed by microarray analysis was verified independently by performing real-time PCR for a number of selected genes including PDGFA, BMI-1, TCF-4, and WIF-1 (Supporting Information Fig. S1). For example, WIF-1 was the single most upregulated mRNA in the Quies \( x^{+}\text{CD71}^{\text{dim}} \) population by microarray analysis (Table S1) and this preferential expression was confirmed by real-time PCR (Supporting Information Fig. S1).

### Tissue Reconstitution Ability of Quies \( x^{+}\text{CD71}^{\text{cri}}, \) Cycl \( x^{+}\text{CD71}^{\text{cri}}, \) and Diff \( x^{+}\text{dim} \) Keratinocytes

Given that LT-TRA from small numbers of cells is considered to be a hallmark of SCs, we titrated the skin reconstitution activity of the Quies \( x^{+}\text{CD71}^{\text{cri}}, \) Cycl \( x^{+}\text{CD71}^{\text{cri}}, \) and Diff \( x^{+}\text{dim} \) epidermal subsets by inoculating 1, 10, 100, 1,000, or 10,000 cells each into devitalized RTs, before subcutaneous transplantation onto SCID mice (Fig. 1) [26]. In this model, the traasses were vascularized by the host and the inoculated keratinocytes reconstitute a stratified epidermis on the tracheal lumenal surface over several weeks. Reproducible epithelialization was obtained by transplanting 100 cells or more per fraction in pilot experiments (\( n = 4 \)). While transplanting more than 1,000 cells resulted in indistinguishable TRA at 6 and 10 weeks as shown by us previously [26], differential tissue reconstitution was obtained from each fraction in the 100–1,000 cell range and is presented below.

### Transplantation of 1,000 Quies \( x^{+}\text{CD71}^{\text{cri}}, \) Cycl \( x^{+}\text{CD71}^{\text{cri}}, \) and Diff \( x^{+}\text{dim} \) Keratinocytes Demonstrates Significant but Differential LT-TRA of These Epidermal Subsets

At 6 weeks, transplants of 1,000 Quies, Cycl, and Diff fractions gave rise to a well-formed multilayered epidermis with normal morphology, that is, a well organized, polarized basal layer, differentiating suprabasal layers and squames (Fig. 3A, 3A’, 3C, 3C’, 3E, 3E’). However, the extent of epithelial coverage regenerated by the Quies cell transplants was consistently lower than that regenerated by the Cycl and Diff fractions in four individual experiments (two transplants/fraction/time point/experiment; \( n = 8 \)). Despite this initial delay in initiating TRA, the Quies \( x^{+}\text{CD71}^{\text{cri}} \) population achieved a more complete and normal multilayered lining of the RT lumen (Fig. 3B, 3B’) by 10 weeks. Over the same time course, the LT-TRA of the Cycl \( x^{+}\text{CD71}^{\text{cri}} \) population showed an overall decrease with an epithelium only partially filling the lumen (Fig. 3D, 3D’ vs. Fig. 3C, 3C’) indicating the absence of sustained TRA (\( n = 8 \)). Similarly, the keratinocyte-lineage marker positive \( x^{+}\text{dim} \) Diff cells displayed a burst of TRA at 6 weeks (Fig. 3E, 3E’) regenerating significant epithelial tissue initially; however, by 10 weeks, the epithelial sheets appeared terminally differentiated, with flattened nucleï typical of suprabasal cells and the absence of a morphologically normal “basal” layer indicating loss of tissue and self-renewing activity (Fig. 3F, 3F’), in four separate experiments (\( n = 8 \)) consistent with their differentiation status. Importantly, control transplants did not yield any epithelialization (Supporting Information Fig. S2, \( n = 16 \)).

These histological data were further substantiated by immunohistochemical analysis for proliferation and by epidermal markers, which were indicative of the maintenance of a proliferative compartment with a keratin 15 positive (K15+) basal layer containing K67+ cells to ascertain the quality of the regenerated tissue. K15 was selected because of its exclusive expression in the basal layer in vivo, whereas K14 is also present in suprabasal cells [42]. At 6 weeks, all epidermal fractions had a K15+ basal layer (Fig. 4C, 4G, 4K) containing rounded K67+ cells (Fig. 4A, 4E, 4I) indicating the maintenance of a healthy proliferative compartment. Notably, quantification of the percentage of K67+ cells across all four replicate experiments confirmed that the proliferative index was comparable among the three fractions (Fig. 5A), providing strong indication of a steady-state replicating epidermis. Further immunostaining for differentiation markers at 6 weeks from all three fractions, specifically involucrin (Fig. 4C, 4G, 4K), loricrin, and K10 (Supporting Information Fig. S3A, S3C, S3E), indicated appropriate spatial-temporal expression of these proteins, that is, K10 and involucrin in suprabasal layers and loricrin in the granular layers, consistent with a homeostatic epidermal differentiation program [43–45]. By 10
weeks, the epidermal sheets derived from the originally Quiescent populations still contained Ki67+ cells and a K14+/K15+ basal layer (Fig. 4B, 4D) demonstrating maintenance of a self-renewing proliferative compartment, despite an overall decrease in proliferative index (Fig. 5A); this fraction also continued to display normal involucrin (Fig. 4D), K10, and loricrin expression (Supporting Information Fig. S3B) demonstrating its ability to maintain a normal differentiation program. In contrast, the Cycla bri-derived epithelial sheets showed loss of K14 and K15 (Fig. 4F, 4H),
Although Ki67\(^+\) cells were still detectable (Figs. 4F, 5A), while involucrin (Fig. 4H) and loricrin (Supporting Information Fig. S3D) expression was maintained, decreased K10 expression was evident suggesting an altered differentiation pattern. The Diff\(_{\text{a dim}}\) fraction-derived epithelia were the least normal with a significant drop in proliferative index at 10 weeks (Fig. 5A). Notably, Ki67\(^+\) cells, where present, were found within a flattened, K14\(^-\) and rarely K15\(^+\) “basal” layer (Fig. 4J, 4L). Moreover, the differentiation markers involucrin (Fig. 4L) and loricrin (Supporting Information Fig. S3F) were lost, while K10 was found only in the most superficial layers indicating degeneration of the epithelial sheet. The combined data suggest that the Quies\(_{\text{a bri}}\)bri\(_{\text{6}}\) CD71\(_{\text{dim}}\) population was the only one capable of LT-maintenance of a steady-state...

**Figure 4.** Expression of proliferative markers of the basal layer confirm that quiescent $a_{\text{bri}}^{6}$ CD71\(_{\text{dim}}\) cells regenerate the most normal epidermal tissue long-term upon transplantation of 1,000 cells per rat trachea. Illustration of immunostaining of epithelial sheets derived from 1,000 quiescent $a_{\text{bri}}^{6}$ CD71\(_{\text{dim}}\), Cycling $a_{\text{bri}}^{6}$CD71\(_{\text{dim}}\), and differentiating $a_{\text{dim}}^{6}$ cells for Ki67 (red) and K14 (green) (A, B, E, F, I, J) or K15 (red) and involucrin (green) (C, D, G, H, K, L). Nuclei are stained blue with 4',6-diamidino-2-phenylindole. Staining for Ki67 revealed that the quiescent, cycling, and even the differentiating fractions contained Ki67\(^+\) cells within a polarized basal layer expressing K15 (C, G, K) and appropriate K14 and involucrin expression at 6 weeks (A, E, I) indicating a homeostatic epidermis. However, at 10 weeks, only the quiescent cells (B, D) maintained a uniformly K14\(^+\)/K15\(^+\) basal layer, whereas the cycling (F, H) and differentiating cells (J, L) did not. Note that in panel (j), a region containing Ki67\(^+\) cells was selected for display, although the “basal layer” regenerated by the differentiating fraction is not homogeneously proliferative as shown in Figure 3 panel (F), illustrating its limited tissue regenerative ability. Scale bar = 50 \(\mu\)m.
proliferative basal layer and both differentiating and mature progeny. Although significant TRA and differentiation capacity were present in the Cycl and Diff fractions, it was short-lived.

**Transplantation of 100 Keratinocytes Reveals that Quiescent Keratinocytes Are the Most Potent LT-Tissue Reconstituting Fraction of the Epidermis**

Further discrimination between the Quiescent, Cycling, and Differentiated fractions was possible by transplanting 100 cells per trachea. Once again, a delay in the initiation of TRA was observed in the Quiescent fraction, with only a cluster of keratin-positive cells detectable 6 weeks post-transplant (Figs. 6A, 6A'). However, by 10 weeks, this fraction was the only one capable of regenerating a normal multilayered epithelium lining the tracheal lumen (Fig. 6B, 6B'), complete with a self-renewing proliferative basal layer with a significant number of Ki67-positive cells (Figs. 5B, 7B), that were uniformly K14+ and K15+ (Fig. 7B, 7D), while simultaneously maintaining differentiated layers expressing involucrin (Fig. 7D), K10, and loricin (Supporting Information Fig. S4B). In the correct spatial and temporal sequence in four separate experiments (n = 8). In contrast, transplantation of 100 Cycling keratinocytes gave the best short-term epithelial coverage of the RT lumen at 6 weeks (Figs. 5B, 6C, 6C'), confirming their ability to rapidly reconstitute an epidermis complete with a self-renewing proliferative basal layer with significant numbers of Ki67+ cells (Figs. 5B, 7B), that were uniformly K14+ and K15+ (Fig. 7B, 7D), while simultaneously maintaining differentiated layers expressing involucrin (Fig. 7D), K10, and loricin (Supporting Information Fig. S4B). Although some epithelial tissue was still maintained at 10 weeks (Fig. 6D), it was markedly thin (Fig. 6D') showing morphological signs of terminal differentiation (n = 8). The number of Ki67+ cells regenerating by this fraction also declined substantially by 10 weeks when compared with the Quiescent keratinocytes (Fig. 5B). Notably, the basal layer regenerating from 100 Cycling keratinocytes contained very few K15+ cells at 10 weeks (Fig. 7H), demonstrating limited self-renewal capacity. Similarly, the differentiation program was deregulated—thus, although involucrin expression remained (Fig. 7H), K10 was expressed only in the most superficial layers and loricin was lost completely (Supporting Information Fig. S4D). In comparison, transplantation of 100 quiescent keratinocytes resulted in very little or no detectable epithelialization at either 6 (Fig. 6E, 6E') or 10 weeks (Fig. 6F, 6F') despite extensive histological analysis of serial sections at 10 µm intervals along the entire length of the tracheal lumens, (n = 8; four independent experiments).

**DISCUSSION**

Lifelong self- and tissue-renewal is considered to be a unique property of relatively quiescent SCs, but is difficult to fully recapitulate experimentally. Nevertheless, surrogate assays that approximate LT-TRA of unpurified [8, 46, 47] and/or SC-enriched keratinocytes [12, 16, 26, 47] have been successful especially for murine HF's given the tractability of this animal model. It has been assumed that only SCs are capable of LT tissue reconstitution and indeed this has formed the basis of quantitative calculations of SC frequency in the murine IF epidermis, where it has been suggested that tissue reconstituting epidermal cells are relatively rarely occurring at a frequency of 1/35,000 total epidermal cells or approximately 0.01% of the basal layer [46]. In contrast, in vivo clonogenicity studies of murine IF epidermis following irradiation and morphological analysis of so-called epidermal proliferative units in homeostatic tissue place the SC estimate at a much higher frequency in the range of 1%–10% [9, 11, 48]. Irrespective of the absolute SC frequency, which is likely to vary due to technical considerations in transplantation studies, or recruitment of non-SCs in response to injury, a clear estimation of the relative LT-TRA of slow-cycling/quiescent keratinocytes versus cycling progenitors has not been performed to date leaving open the important question of whether both populations are equipotent or have distinct tissue renewal capacities. This has become even more important given recent claims in the literature that cycling progenitors may have SC-like properties. Therefore, our goal was to determine whether quiescent epidermal SCs could be functionally distinguished from cycling progenitors of human neonatal foreskin, particularly given that both populations have been put forward as stem-cell like with significant LT-TRA and longevity in murine skin [3]. To date, the CD71+ keratinocytes are the most purified quiescent IF epidermal SC population in human skin with a greater competitive repopulation capacity than Hoechst 33342 effluxing cells [47]. We compared Quiescent CD71+ cells with their more actively cycling counterparts, the CD71− cells and the earliest differentiating CD71−

![Figure 5.](https://www.StemCells.com)
keratinocytes, and found that all three fractions possessed impressive intrinsic short-term TRA. However, the quiescent $\alpha_{6}^{+}$CD71$^{\text{dim}}$ subset were the most potent LT-tissue reconstituting population of the IF epidermis, renewing the proliferative compartment even at limit dilution. Although limit dilution assays of competitive tissue reconstitution have been used to calculate SC frequency in the IF epidermis [46], it must be noted that this assay scores any amount of reconstituted tissue as a positive and does not take into account the extent and quality of the tissue regenerated, unless combined with serial transplantability. We subjected the results obtained from our longest term transplants (10 weeks) from all

**Figure 6.** Quiescent $\alpha_{6}^{+}$CD71$^{\text{dim}}$ are the most potent epidermal tissue reconstituting cells at limit dilution. H&E stained sections of rat trachea (RT) lumens showing differential tissue regenerative ability (TRA) of 100 quiescent $\alpha_{6}^{+}$CD71$^{\text{dim}}$ (A, A', B, B'), Cycling $\alpha_{6}^{+}$CD71$^{\text{dim}}$ (C, C', D, D'), or differentiating $\alpha_{6}^{+}$ (E, E', F, F') epidermal fractions harvested at 6 and 10 weeks after transplant. The quiescent cells exhibited a delay in initial TRA at 6 weeks, generating just a small cluster of cells (A, A'); however, by 10 weeks, they were the only fraction capable of regenerating a morphologically normal epithelium with a polarized basal layer (B, B', arrows). In contrast, 100 cycling cells provided good coverage of the RT lumen at 6 weeks (C, C'), but this was not maintained well for more than 10 weeks (D), yielding a differentiated basal layer with flattened nuclei (D', arrows). Notably, no epithelial reconstitution was obtained from 100 differentiating cells (E, E', F, F') at 6 or 10 weeks consistent with their differentiation status ($n = 4$). Scale bar = 200 μm. Abbreviations: C, cartilage; L, rat trachea lumen; E, epithelial sheet; S, squamous cells.
dilutions tested (100, 1000, and 10,000) to analysis with the webtool http://bioinf.wehi.edu.au/software/elda suitable for limit dilution analysis and obtained estimates for the frequency of tissue repopulating units in the Quies CD71<sup>dim</sup> population at 1 of 400 (upper and lower 95% confidence limits of 183 and 878, respectively) and at 1 of 898 in both the Cycl CD71<sup>br</sup> and Diff CD71<sup>dim</sup> populations (upper and lower 95% CLs of 395 and 2041). However, this type of calculation of the mathematical incidence of tissue reconstituting units from transplant data has to be interpreted cautiously considering that there are likely to be technical limitations and that tissue reconstitution in a RT is merely "a surrogate assay" and highly likely to be influenced by adaptation to a heterotypic environment. In contrast, mammary SC, assays where cells are placed in a native environment, that is, the mammary fat pad, are likely to more accurately estimate SC or TRA.

Figure 7. Expression of epidermal markers shows that the quiescent CD71<sup>dim</sup> fraction is the most potent tissue reconstitutive subset of the human interfollicular epidermis, maintaining a self-renewing K<sup>+</sup>/K14<sup>+</sup>/K15<sup>+</sup> proliferative basal layer. Immunostaining of epithelium derived from 100 quiescent (A, B, D) or cycling (C, E-H) harvested at 6 (A, C, E, G) or 10 weeks (B, D, F, H) for K67 (red) and K14 (green) (B, E, F) or K15 (red) and involucrin (green) (D, G, H), revealing that 100 quiescent cells reconstitute a K14<sup>+</sup>/K67<sup>+</sup> (B), K15<sup>+</sup> basal layer, and involucrin<sup>+</sup> suprabasal layers (D) at 10 weeks that is still proliferating and generating differentiated cells. Pan-cytokeratin<sup>+</sup> cell clusters obtained from 100 quiescent cells at 6 weeks (A, green) confirm their identity as transplanted keratinocytes. In contrast, 100 cycling cells yield a well formed epidermis at 6 weeks with a K15<sup>+</sup>/K67<sup>+</sup> proliferative compartment (E, G), which is terminally differentiated by 10 weeks with flattened and infrequent K67<sup>+</sup> nuclei (F), and fewer cells expressing the basal layer specific K15 (H), indicates loss of the proliferative compartment. Nuclei are stained blue with 4',6-diamidino-2-phenylindole. (C): Negative control antibody. Scale bar = 50 μm.
The study by Schneider et al. [46] and ours are not directly comparable in that we did not perform a competitive TRA assay—our study used irradiated keratinocytes as support cells, whereas live keratinocytes were used in their study; moreover, we used purified populations of stem and progenitors from human skin of variable genetic background, whereas Schneider et al. used murine keratinocytes from a single genetic background (C57Bl/6). In our assay, qualitative analysis of reconstituted tissue was critical, given that in cases where a positive score was obtained, that is, presence of epithelial tissue, significant differences in the self-renewal properties of the reconstituted tissue with respect to maintenance of a proliferating basal layer, and sequentially differentiated epithelial layers were evident. Finally, equating LT TRA to SC activity is misleading and assumes that only SCs can reform tissue—something our data and published work from other laboratories clearly contradict [3, 4, 48]. Nowhere is this more evident than in the hematopoietic system, where the difference between SCs and CPs is a matter of subtle differences in the number of rounds of serial transplantability rather than positive or negative tissue reconstitution [49]. Therefore, our study did not seek to establish the absolute number of tissue-reconstituting cells among fractionated keratinocyte subsets, but rather sought to evaluate comparative TRA potency relative to each other.

We conclude that the proliferative compartment in epithelial tissues is not made up of equipotent keratinocytes, but is organized in a hierarchical fashion with the slow-cycling or quiescent keratinocyte stem cell (KSC) population at its apex. Given that the majority of the basal layer is made up of cycling cells, we infer that our experiments with 1,000 cells per transplant probably reflect the significant role of CPs in maintaining epidermal renewal during homeostasis, consistent with recent published murine data on TA, or more accurately put CP cells [3]. However, the CP population of the epidermis did not possess unlimited self- and tissue-renewal capacity as shown by the 100 cell transplant results, suggesting that they are ultimately dependent on SCs for continual replacement. Our data also indicate that the CP most likely leads the charge for tissue replacement early upon damage—an observation reminiscent of reports showing that the hair germ, although primed to burst into proliferation, is short-lived when compared with the slower cycling/quiescent HF bulge SCs [50, 51]. Gene expression patterns between the three epidermal compartments were also indicative of a hierarchically organized biological continuum showing repression of growth factor signaling in the quiescent population, onset of cell cycle progression and metabolic activity in the cycling population, and onset of differentiation in the $x_{cyc}$ compart-ment, tying in well with the functional properties, we observed with respect to rate and extent of short-term-TRA. Interestingly, we noted that Bmi-1—a gene known to have a functional role in self-renewal—continued to be expressed in both the Quies and Cycl fractions, but was clearly downregulated in Diff cells (Supporting Information Table S1 and Fig. S1). Differences in real-time PCR expression levels: Quies vs. Cycl $p = .265$; Quies vs. Diff $p = .0003)$. This is consistent with the significant TRA measurable in the Cycl/CP population.

Whether SCs routinely lie dormant during homeostasis in human skin as suggested recently for murine tail epidermis [13] remains undetermined. Thus, an important question not addressed by our study is how frequently SCs undergo cell division during homeostasis, particularly as our experimental design requires liberation of SCs and progenitors from the epithelial sheet and the dermal microenvironment to assess their tissue renewal capacity. However, our data suggest that it is likely that SCs probably do not constitute the first-line of defense in repairing epithelial tissue but rather are a back-up for extreme tissue damage with significant reserves of tissue renewing capability useful in these circumstances. We surmise that the removal of slow-cycling or quiescent SCs from their environment leads to their activation and entry into cycle as illustrated by our transplantation experiments. Consistent with our previous observations [26, 27], the $x_{cyc}$ population possessed measurable TRA although it was the least potent of all epidermal subsets. Presumably, the $x_{cyc}$ fraction is capable of some tissue repair, although given its low incidence in the epidermis, it is most likely not routinely called upon for homeostatic tissue renewal. However, severe depletion of the epidermis may recruit these differentiating cells to regenerate tissue transiently until the SCs are activated. We infer that in vivo, during homeostatic tissue renewal, the quiescent $x_{cyc}$ keratinocytes behave somewhat like a “queen bee” dividing only occasionally to replace $x_{cyc}$, whereas the latter population is analogous to “worker bees” industriously doing the bulk of the work of cell replacement to balance cell loss at the surface of skin. This is in contrast to the model put forward recently by Jones and coworkers for the IF epidermis in murine tail whereby progenitors have been suggested to renew the tissue for long periods of time while SCs remain dormant unless called upon by tissue damage [3, 13]. However, the data from the Jones laboratory do not unequivocally exclude the contribution of a minor or quiescent or slowly cycling population—other SCs to routine IF epidermal renewal. Similarly, the data of Clevers and coworkers in small intestinal mucosa [4] and IF epithelium [5] did not specifically determine the role of cycling progenitors, given that Lgr-5 is clearly expressed by cells that are both slow-cycling as determined by label-retention at cell position four in the small intestinal crypt epithelium and within the HF bulge. To support the conclusion that quiescent cells are not actively contributing to epithelial tissue replacement in homeostasis, it is necessary to identify more specific markers that segregate the cycling cells in these tissues from the truly quiescent or slowly cycling label-retaining cells.

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Another implication of the results reported here and by others showing extensive epithelial cell renewal and proliferative ability beyond the SC compartment is that cancer may not necessarily arise solely in SCs. Cancer SCs could reasonably be expected to originate from the stem, progenitor, or the earliest differentiating keratinocytes, given that tremendous proliferative potential resides in all these subsets. The more interesting question is whether more aggressive cancers arise as a consequence of transformation of more primitive SCs, whereas less aggressive cancers may be derived from cycling progenitors. Perhaps more benign, differentiated tumors originate in the earliest differentiating cells taking advantage of their residual proliferative potential. While being more proliferative capacity among all epidermal subsets is consistent with the prime directive of epithelia to provide a barrier to infection and prevent dehydration, particularly in circumstances where significant skin tissue loss is sustained by the organism, the flip side of the coin is that it may also predispose epithelial tissues to the development of cancer.

A comparison of our quiescent human $x_{cyc}$ KSC population’s microarray profile with published gene expression profiling of murine and human hematopoietic SC, murine embryonic SC, and murine neuronal SCs [34, 52] suggests a commonality is IF KSC regulation (Supporting Information Fig. S5), particularly with respect to suppression of proproliferative signals and promotion of adhesion. Analysis of published skin SC profiles particularly from murine and human HF bulge [1, 16, 53] with KSCs also show similarities in...
gene expression with respect to adhesion molecules, and negative regulators of the insulin-like growth factor, Tgf-β, and Wnt pathways (Supporting Information Table S2). However, the underlying mechanisms are likely to be complex given that genetic knockouts of overlapping pathways seem to preferentially deregulate the HF rather than the IF epidermis exemplified by the Bmp receptor 1a knockout mouse [54]. This may be attributed to the necessity to protect barrier function in the organism at all costs to ensure survival, while HF functions such as temperature control and sensory ability are less critical. Future investigation into the interplay of multiple regulators of specific signaling pathways is essential to our understanding of how quiescence and controlled tissue renewal are achieved in vivo. In particular, how specificity of common regulators are decoded by the different skin SC populations needs to be better understood.

CONCLUSION

In conclusion, our data provides direct evidence for a hierarchical organization in the epithelial proliferative compartment and that the CD71dimCD34−CD71intCD34+ cells represent a bona fide epidermal SC population of the IF epidermis. This hierarchy probably exists as a continuum rather than a black and white transition between the SC and the CP compartment typical of many biological systems, particularly the hemopoietic system. Thus, it may be possible to endlessly slice the proliferative compartment into cells with varying degrees of self-renewal capacity with the description of new markers for their separation.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES


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