See related commentary on pg 2492

CD133 Is a Marker for Long-Term Repopulating Murine Epidermal Stem Cells

Alexandra Charruyer[1], Lauren R. Strachan[1], Lili Yue[1], Alexandra S. Toth[1], Gary Cecchini[2,3], Maria L. Mancianti[4] and Ruby Ghadially[1,3]

Maintenance, repair, and renewal of the epidermis are thought to depend on a pool of dedicated epidermal stem cells (EpiSCs). Like for many somatic tissues, isolation of a nearly pure population of stem cells is a primary goal in cutaneous biology. We used a quantitative transplantation assay, using injection of keratinocytes into subcutis combined with limiting dilution analysis, to assess the long-term repopulating ability of putative murine EpiSC populations. Putative EpiSC populations were isolated by FACS sorting. The $CD133^+$ population and the subpopulation of $CD133^+$ cells that exhibits high mitochondrial membrane potential ($D\Psi m^{hi}$) were enriched for long-term repopulating EpiSCs versus unfractionated cells (3.9- and 5.2-fold, respectively). Evidence for self-renewal capacity was obtained by serial transplantation of long-term epidermal repopulating units derived from $CD133^+$ and $CD133^+\Delta\Psi m^{hi}$ keratinocytes. $CD133^+$ keratinocytes were multipotent and produced significantly more hair follicles than $CD133^-$ cells. $CD133^+$ cells were a subset of the previously described integrin $\alpha6^+CD34^+$ bulge cell population, and $28.9 \pm 8.6\%$ were label-retaining cells. Thus, murine keratinocytes within the $CD133^+$ and $CD133^+\Delta\Psi m^{hi}$ populations contain EpiSCs that regenerate the epidermis for the long term, are self-renewing, multipotent, and label-retaining cells.

Journal of Investigative Dermatology (2012) **132,** 2522–2533; doi:10.1038/jid.2012.196; published online 5 July 2012

INTRODUCTION

Murine epidermis is maintained by tissue stem cells that can be defined by their long-term repopulating and self-renewal abilities. These defining features are essential to the identification and characterization of epidermal stem cells (EpiSCs) and their progeny.

Keratinocytes spontaneously form cysts with a differentiated keratinizing epidermis, following traumatic or surgical implantation into the human subcutis (Ohnishi and Watanabe, 1999; Silver and Ho, 2003; Hall *et al.*, 2006). Production of such cysts has been used to study the conjunctival epithelium (Doran *et al.*, 1980; Wei *et al.*, 1997), lung alveolar cells (Yu *et al.*, 2007), and epidermal keratinocytes (Grimwood *et al.*, 1988; Zheng *et al.*, 2005). Epidermal keratinocytes produced stratified squamous structures with keratohyalin granules, stratum corneum, basement membrane, and protein expression, indicating a fully differentiated epidermis (Doran *et al.*, 1980; Grimwood *et al.*, 1988). Thus, subcutaneous injection of keratinocytes into mice results in the phenotype and differentiation pattern of the original epidermis.

$CD133^+$ cells were human progenitors in kidney (Bussolati *et al.*, 2005), nervous system (Uchida *et al.*, 2000), and in epithelial tissues including prostate (Richardson *et al.*, 2004), foreskin (Yu *et al.*, 2002; Mizrak *et al.*, 2008; Guo and Jahoda, 2009), and colorectal adenocarcinoma (Corbeil *et al.*, 2000). CD133 cells were murine progenitors in neural cells (Corti *et al.*, 2007; Coskun *et al.*, 2008), liver (Rountree *et al.*, 2007), kidney (Weigmann *et al.*, 1997), and intestine (Snippert *et al.*, 2009; Zhu *et al.*, 2009). We selected CD133 as a possible marker of murine EpiSCs.

Murine embryonic stem cells with high mitochondrial membrane potential ($\Delta\Psi m$) show decreased differentiation and increased teratoma formation (Schieke *et al.*, 2008). TMRM, a fluorescent derivative of R123, was used to isolate putative EpiSCs based on high $\Delta\Psi m$, as it provides more accurate quantification than the parent compound (Scaduto and Grotyohann, 1999).

In both human and murine epidermis, integrin $\alpha6^{hi}CD71^{lo}$ keratinocytes showed features of stem cells (Li *et al.*, 1998; Kaur and Li, 2000; Tani *et al.*, 2000).

In this study, epidermal allografts were produced by injection of putative EpiSCs into murine subcutis. The frequency of long-term repopulating EpiSCs was determined by limiting

[1]*Department of Dermatology, University of California, San Francisco, San Francisco, California, USA;* [2]*Division of Molecular Biology, Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California, USA;* [3]*VA Medical Center, San Francisco, California, USA and* [4]*Department of Pathology, Alta Bates Medical Center, Berkeley, California, USA*

Correspondence: Ruby Ghadially, Department of Dermatology and Epithelial Section, Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, University of California, San Francisco, 4150 Clement Street MC190, San Francisco, California 94121, USA.
E-mail: ghadiallyr@derm.ucsf.edu

Abbreviations: $\Delta\Psi m$, mitochondrial membrane potential; EpiSC, epidermal stem cell; ERU, epidermal repopulating unit; GFP, green fluorescent protein; TAC, transit amplifying cell; UNF, unfractionated

Received 6 July 2011; revised 25 April 2012; accepted 3 May 2012; published online 5 July 2012

dilution analysis (Schneider *et al.*, 2003; Strachan *et al.*, 2008; Charruyer *et al.*, 2009). Enrichment for murine EpiSCs was 3.9-fold over unfractionated (UNF) keratinocytes in $CD133^+$ keratinocytes and 5.2-fold in $CD133^+\Psi\Delta m^{hi}$ keratinocytes. $CD133^+$ and $CD133^+\Psi\Delta m^{hi}$ keratinocytes displayed superior long-term repopulating and self-renewal ability, multipotency, and label retention.

RESULTS

Generation of murine epidermal repopulating units in an allograft model

Epidermal structures generated from injection of keratinocytes were termed epidermal repopulating units (ERUs) pursuant to hematologic terminology (Szilvassy *et al.*, 1990). After injection of GFP^+ keratinocytes, 100% of 100 ERUs analyzed were GFP^+, confirming derivation from GFP^+ keratinocytes injected (Figure 1a).

Immunohistochemistry showed keratin 14 in the basal layers (Figure 1b) and involucrin in the suprabasal layers (Figure 1c). Linear fluorescence at the ERU periphery of the ERU with anti-laminin antibody (Figure 1d) indicated a basement membrane.

To determine whether ERUs originated from single cells, keratinocytes incubated with Vybrant DiI (565 nm, red) or DiO (501 nm, green) were mixed 1:1. One week after injection of 100,000, 40,000, 20,000, or 6,250 keratinocytes, 17/79 (21%), 5/46 (10%), 0 (0%), or 0 (0%) bicolored ERUs were observed, respectively (Figure 1e–g), indicating that at lower doses ERUs originated from a single cell.

In this *in vivo* repopulation assay, only EpiSCs originally injected (and their progeny) persist after 9 weeks, whereas transit amplifying cells (and their progeny) are no longer present

Long-term repopulation combined with limiting dilution analysis has been used to quantify EpiSCs (Schneider *et al.*, 2003; Strachan *et al.*, 2008; Charruyer *et al.*, 2009). As short-term repopulating cells exhaust their proliferative ability over time, the frequency of ERUs decreases. When only ERUs from long-term repopulating keratinocytes remain, ERU frequency does not change at subsequent time points. Here we used a transplantation assay modified from previous studies (Schneider *et al.*, 2003; Strachan *et al.*, 2008). UNF keratinocytes were injected at a range of doses (1–100,000 cells), and the frequency of ERUs was determined at different repopulation times by limiting dilution analysis (Table 1). The frequency of ERUs decreased between 1 and 6 weeks ($P \leqslant 0.001$; $n=5$) from 1 in 48 (SE = 1 in 35–66) to 1 in 790 (SE = 1 in 576–1,084). After 6 weeks, no significant change in the frequency of ERUs was detected. The overall likelihood ratio test for differences in EpiSC frequencies between weeks 1 and 24 yielded a significant result ($P<0.001$), but between weeks 6 and 24 ($P=0.25$), 9 and 24 ($P=0.63$), 12 and 24 ($P=0.34$), and 18 and 24 ($P=0.88$) yielded nonsignificant results. In previous transplantation studies (Schneider *et al.*, 2003; Strachan *et al.*, 2008), no ERUs were lost after 9 weeks and no significant change was found after 6 weeks in the present model. Therefore, we selected 9 weeks as the time at which we are studying ERUs derived only from EpiSCs.

$CD133^+$ and $CD133^+\Delta\Psi m^{hi}$ keratinocytes are located in the bulge and are enriched for long-term repopulating EpiSCs

CD133 immunostaining was located in the bulge of neonatal and adult hair follicles, as seen in humans (Jiang *et al.*, 2010) (Figure 2a and b).

For FACS isolation of $CD133^+$ keratinocytes, an isotype control was used to set a gate resulting in <1% of total cells in the positive gate. 7AAD was used to exclude dead cells. In day 4 neonates, the $CD133^+$ keratinocytes constituted 2–7.6% of the total (mean = 4.2 ± 3.1%, $n=7$) (Figure 2c). In 10- to 12-week-old adults, the $CD133^+$ keratinocyte population was not significantly different (2–4.2% of total cells, mean = 2.8 ± 1%, $P=0.4$, $n=4$).

High membrane potential ($\Delta\Psi m^{hi}$) and low membrane potential ($\Delta\Psi m^{lo}$) were defined as 5% highest and lowest TMRM fluorescence, as previously described (Schieke *et al.*, 2006, 2008). Approximately 2% of total cells were $CD133^+$ $\Delta\Psi m^{hi}$. Therefore, we selected the 2% $CD133^+$ $\Delta\Psi m^{hi}$ and 2% $CD133^-$ $\Delta\Psi m^{lo}$ populations (Figure 2d).

Integrin $\alpha6^{hi}CD71^{lo/hi}$ keratinocytes (7–10% total) were selected based on appropriate isotype controls (Figure 2e), as previously described (Tani *et al.*, 2000; Li *et al.*, 2004; Youn *et al.*, 2004).

When injected into the subcutis of NOD/SCID mice, $CD133^+$ cells produced ERUs with keratinized epidermis, indistinguishable from those from UNF cells (Figure 2f). One in 379 (SE = 1 in 274–526) $CD133^+$ keratinocytes was an EpiSC capable of long-term repopulation versus only 1 in 9,487 (SE = 1 in 6,644–13,547) $CD133^-$ cells, and 1 in 285 (SE = 1 in 220–371) $CD133^+\Delta\Psi m^{hi}$ cells was capable of long-term repopulation versus only 1 in 5,323 (SE = 1 in 3,410–8,308) $CD133^-\Delta\Psi m^{lo}$ cells. The enrichment provided by $CD133^+\Delta\Psi m^{hi}$ versus $CD133^+$ was not significantly different ($P=0.49$). One in 1,488 (SE = 1 in 1,079–2,052) integrin $\alpha6^{hi}CD71^{lo}$ cells was capable of long-term repopulation, similar to UNF cells (1 in 1,491 (SE = 1 in 1,109–2,002)) (Figure 2g). Thus, the $CD133^+$ population was enriched for long-term repopulating EpiSCs 3.9-fold over the UNF population, and the $CD133^+\Delta\Psi m^{hi}$ population was enriched for long-term repopulating EpiSCs 5.2-fold (Figure 2g and h), whereas the $CD133^-$ and $CD133^-\Delta\Psi m^{lo}$ populations were depleted. The integrin $\alpha6^{hi}CD71^{lo}$ population of keratinocytes was not enriched for long-term repopulating cells in this assay.

To exclude the possibility of contamination of $CD133^+$ keratinocytes by $CD133^+$ dermal papilla cells, we studied versican GFP–tagged mice that express GFP in dermal papilla cells (Kishimoto *et al.*, 1999; Ehama *et al.*, 2007). $CD133^+$ keratinocytes isolated from the versican GFP epidermis were GFP^- (Supplementary Figure S1a online).

In pilot studies, $SSEA1^+$, $CD44^+$, and $CD133^+CD44^+$ populations were not enriched in long-term repopulating EpiSCs (data not shown).

$CD133^+$ and $CD133^+\Delta\Psi m^{hi}$ murine keratinocytes express stem cell markers and display functional stem cell characteristics, including self-renewal, multipotency, and label retention

Overlap between CD133 and previously described EpiSC markers was analyzed. Using histology, cells coexpressing

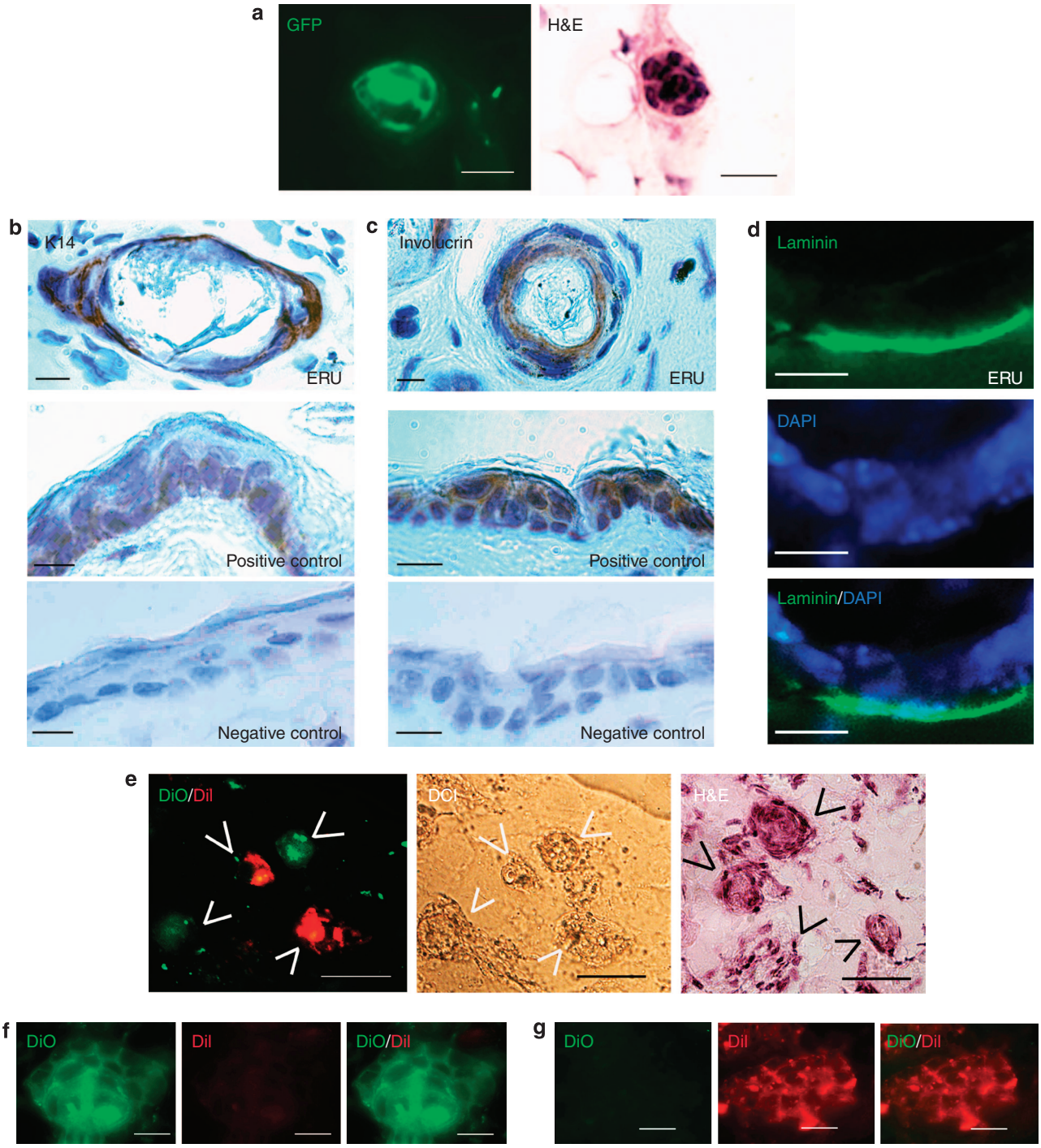


Figure 1. Generation of epidermal repopulating units (ERUs) following subcutaneous injection of murine keratinocytes in an allograft model. (**a**) ERUs formed following injection of green fluorescent protein-positive (GFP^+) murine keratinocytes into a GFP^- NOD/SCID mouse are GFP^+, confirming derivation from the GFP^+ donor keratinocytes. Fluorescence microscopy (488 nm) (left panel). Hematoxylin and eosin (H&E) staining of an adjacent section (right panel). A total of 100 ERUs were analyzed (three experiments). (**b**–**d**) Epidermal differentiation is seen in ERUs. (**b**) Keratin 14 is expressed in the basal layers. (**c**) Involucrin is expressed in the suprabasal epidermis. (**d**) Laminin is present at the basement membrane of the ERU. Positive controls were the intact murine epidermis. Negative controls were performed with omission of the primary antibody. (**e**–**g**) ERUs are formed from single cells. Keratinocytes were labeled with Vybrant DiI or DiO and mixed in a 1:1 ratio before injection into NOD/SCID mice. Resultant ERUs (arrow heads) were green or red, but not mixed. (**e**) Four of the ERUs produced by injection of 20,000 keratinocytes. Fluorescence microscopy (left panel), DCI of the same section (middle panel), and H&E staining of the adjacent section (right panel). (**f**) A DiO-positive ERU and (**g**) a DiI-positive ERU. Bars = 10 μm in **a**–**d**; 25 μm in **e**; and 10 μm in **f** and **g**. DCI, differential contrast of interferences.

CD133 and integrin α6 (Li *et al.*, 1998) were located in the hair follicle bulge (Figure 3a). Cells coexpressing CD133 and Delta 1 (Estrach *et al.*, 2007) were located in the bulge (Figure 3b). Integrin $\alpha6^+CD34^+$ keratinocytes exhibited EpiSC properties (Trempus *et al.*, 2003, 2007). Using flow cytometry, 97.1 ± 1.8% of $CD133^+$ keratinocytes were integrin $\alpha6^+$ (not shown, $n=4$), 99 ± 1% were $CD34^+$ (Figure 3c, upper panel, $n=3$), and 78.7 ± 14% were integrin $\alpha6^+CD34^+$ (Figure 3c, middle panel, $n=3$). Only 0.8 ± 0.2% of $CD133^+$ keratinocytes were integrin $\alpha6^{hi}CD71^{lo}$ (Figure 3c, lower panel, $n=4$). On direct counting of FACS isolated cells, 94.3 ± 4.2% of $CD133^+$ cells (Figure 3d) and 95.3 ± 1.1% of $CD34^+$ cells (not shown) were observed to be keratin 14^+ ($n=3$), consistent with basal keratinocytes. Keratin 15 colocalized with label-retaining cells (Lyle *et al.*, 1998, 1999). On direct counting, 15.5 ± 4% of $CD133^+$ keratinocytes were observed to be keratin 15^+ (Figure 3e, $n=4$). $CD133^+$ keratinocytes were $Lgr5^-$ (data not shown; Jaks *et al.*, 2008). 4.5 ± 2.1% of $CD34^+$ cells are $CD133^+$, 9.7 ± 7.8% of integrin $\alpha6^+$ are $CD133^+$ and 12.2 ± 2.2% of integrin $\alpha6^+CD34^+$ cells are $CD133^+$. Thus, $CD133^+$ keratinocytes are a subset of the $\alpha6^+CD34^+$ bulge population.

Bmi-1 is associated with stem cell self-renewal (Reinisch *et al.*, 2007; Lee *et al.*, 2008; Lacroix *et al.*, 2010). More $CD133^+$, $\Delta\Psi m^{hi}$, and $CD133^+\Delta\Psi m^{hi}$ cells expressed nuclear Bmi-1 (19.8 ± 7.6%; $P<0.05$, 28.7 ± 5.3%; $P<0.001$ and 45.9 ± 10%; $P<0.001$, respectively) than UNF, $CD133^-$, $\Delta\Psi m^{lo}$, and $CD133^-\Delta\Psi m^{lo}$ cells (5 ± 2.3, 2.8 ± 2.6, 6.4 ± 4.1, and 4.8 ± 4.5%, respectively) (Figure 4a and b). To further study self-renewal ability, 4,000 $CD133^+$ or $CD133^+\Delta\Psi m^{hi}$ and 4,000–25,000 $CD133^-$ or $CD133^-\Delta\Psi m^{lo}$ keratinocytes were injected into NOD/SCID mice. Primary injection sites were harvested at 9 weeks and cell suspensions were obtained, followed by reinjection into secondary recipient mice. Secondary sites (harvested at 9 weeks) were analyzed microscopically. ERUs were detected in both $CD133^+$ and $CD133^+\Delta\Psi m^{hi}$ secondary transplants, indicating self-renewal ability, whereas no ERUs were detected in $CD133^-$ or $CD133^-\Delta\Psi m^{lo}$ secondary transplants (Figure 4c, $n=3$). These results indicate greater self-renewal ability in the $CD133^+$ and $CD133^+\Delta\Psi m^{hi}$ populations.

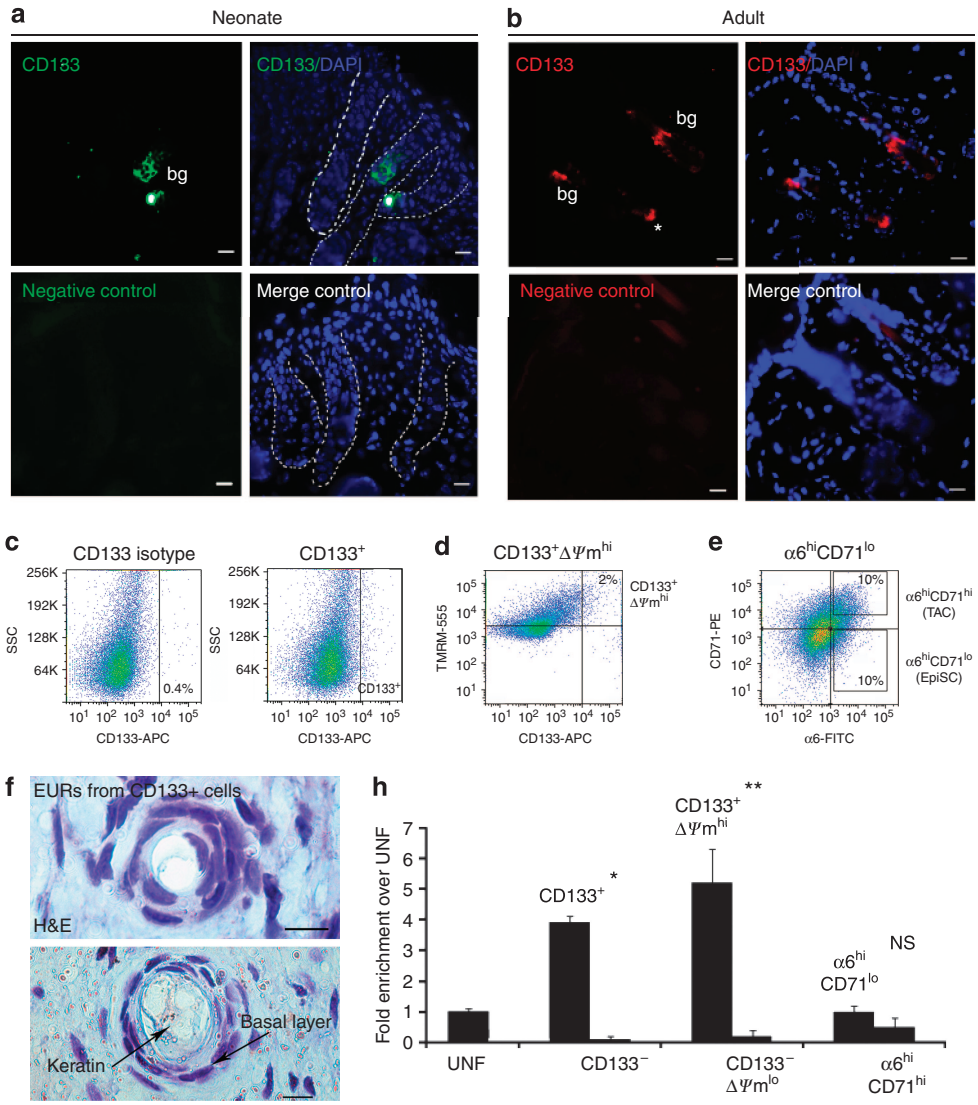
Regeneration of hair follicles *in vivo,* using injection of mixtures of epidermal and dermal cells into immunodeficient mice, is well described (Morris *et al.*, 2004; Zheng *et al.*, 2005; Yang and Cotsarelis, 2010). Multipotency was studied using coinjection of 30,000–90,000 keratinocytes and 100,000 neonatal (day 2) GFP-tagged dermal papilla cells. Eighteen days after injection, $CD133^+$ keratinocytes formed greater numbers of hair follicles than $CD133^-$ keratinocytes (22.3 ± 2.8 vs. 2.7 ± 2.6 hair follicles per 30,000 cells injected, respectively, $P=0.01$, $n=3$) (Figure 4d). These follicles also expressed CD133 in the bulge (Figure 4e). Injection of 100,000 dermal papilla cells did not produce hair follicles ($n=4$).

BrdU incorporation in neonatal mice was used to study label-retaining ability as previously described (Blanpain *et al.*, 2004). After a 30-day chase, 28.9 ± 8.6% of $CD133^+$ cells ($n=3$, 1,000 cells counted) and 37.3 ± 5.9% of $CD133^+\Delta\Psi m^{hi}$ cells were label-retaining cells ($n=3$, 1,000 cells counted) (Figure 4f and Supplementary Figure S2 online).

Table 1. Frequency of epidermal repopulating units at different durations of repopulation

Duration of repopulation	1 week	6 weeks	9 weeks	12 weeks	18 weeks	24 weeks
Cell dose						
	Response ratio: positive samples/total number of injections					
100,000	1/1	5/5	3/4	5/5	4/4	5/6
25,000	4/4	4/4	3/3	7/7	3/3	4/4
6,250	4/4	9/9	6/7	9/10	2/2	3/3
1,562	4/4	8/11	10/13	7/11	3/7	4/11
391	4/4	3/6	3/7	3/7	3/5	6/12
98	7/9	2/4	0/4	0/5	1/3	2/7
24	6/11	1/4	0/1	0/1		2/3
5	0/1					
1	0/2					
ERU frequency	1 in 48**	1 in 790	1 in 1,491	1 in 1,802	1 in 1,373	1 in 1,242
SE[1,2]	35–66	576–1,084	1,109–2,002	1,342–2,420	917–2,054	921–1,674

Abbreviation: ERU, epidermal repopulating unit.
**$P\leqslant0.001$ between 1 week and all the other time points.
[1]SE expressed as 1 in….
[2]Tests for inconsistency (χ^2 Pearson, deviance) were nonsignificant (NS).



g

Isolation strategy / Cell dose	UNF	CD133+	CD133−	CD133+ ΔΨmhi	CD133− ΔΨ mlo	α6hiCD71lo	α6hiCD71hi
100,000	3/4	Response ratio: positive samples/total number of injections					
25,000	3/3		2/2				
6,250	6/7		4/11		1/3		7/8
1,562	10/13		2/9		2/9	8/11	3/9
391	3/7	7/11	1/8	7/12	3/12	1/11	1/7
98	0/4	3/11		8/19	0/4	1/8	1/6
24	0/1	0/7		2/17		0/8	
EpiSC frequency	1 in 1,491	1 in 379	1 in 9,487	1 in 285	1 in 5,323	1 in 1,488	1 in 2,961
SE[a]	1,109–2,002	274–526	6,644–13,547	220–371	3,410–8,308	1,079–2,052	2,143–4,090

$P \leqslant 0.05$

$P \leqslant 0.001$

[a] Tests for inconsistency (χ^2 Pearson, deviance) were NS

CD133$^+$ and CD133$^+\Delta\Psi m^{hi}$ murine keratinocytes have less colony-forming ability *in vitro* than CD133$^-$ and CD133$^-\Delta\Psi m^{lo}$ keratinocytes

It has been assumed that colony-forming efficiency (colonies per 100 cells plated) reflects EpiSC number. However, the greatest *in vitro* short-term proliferative ability is not associated with the greatest long-term repopulating ability *in vivo* (Strachan *et al.*, 2008). A total of 4,000 cells of each population (CD133$^+$, CD133$^+\Delta\Psi m^{hi}$, CD133$^-$, CD133$^-\Delta\Psi m^{lo}$, and UNF) were plated in 35-mm dishes. The CD133$^-$ and CD133$^-\Delta\Psi m^{lo}$ populations showed significantly greater relative clonogenic ability (1.11 ± 0.1 and 0.47 ± 0.06 fold) compared with CD133$^+$ and CD133$^+\Delta\Psi m^{hi}$ populations (0.23 ± 0.07- and 0.07 ± 0.12-fold, respectively) (Figure 5a). Given this *in vitro* result, we examined short-term repopulation *in vivo*. UNF and CD133$^-$ populations also had greater short-term repopulating ability *in vivo* at 1 week compared with CD133$^+$ and CD133$^+\Delta\Psi m^{hi}$ populations (1 in 48 (SE = 1 in 35–66) and 1 in 77 (SE = 1 in 52–144) compared with 1 in 712 (SE = 1 in 492–1032) and 1 in 495 (SE = 1 in 364–671), respectively) (Figure 5b). Thus, the CD133$^+$ population was enriched for keratinocytes with long-term (*in vivo*), but not short-term (*in vivo* or *in vitro*), repopulating ability. Conversely, the CD133$^-$ population showed minimal long-term repopulating ability (*in vivo*), but contained keratinocytes with short-term repopulation ability (*in vivo* and *in vitro*).

DISCUSSION

These studies show that murine CD133$^+$ keratinocytes (a subset of integrin $\alpha6^+$CD34$^+$ keratinocytes) and CD133$^+\Delta\Psi m^{hi}$ keratinocytes contain long-term repopulating, self-renewing, multipotent EpiSCs containing increased proportions of cells with nuclear Bmi-1 expression and label-retaining ability. The CD133$^-$ population contains the clonogenic cells *in vitro* and the short-term repopulating cells *in vivo*. The CD133$^+$ and CD133$^+\Delta\Psi m^{hi}$ populations, although containing long-term repopulating cells, are not clonogenic *in vitro* nor short-term repopulating cells *in vivo*.

Although it was believed that 10% of basal cells were EpiSCs, many studies have found a frequency on a lower order (Bickenbach, 1981; Bickenbach and Chism, 1998; Schneider *et al.*, 2003; Triel *et al.*, 2004; Redvers *et al.*, 2006; Charruyer *et al.*, 2009; Winter and Bickenbach, 2009). Here the frequency of EpiSCs was 1 in 1,491 (SE = 1 in 1,109–2,002). This is expected to be an underestimate, as it is most probable that not all EpiSCs achieve proliferation in this model. In this study, keratinocytes were isolated from freshly obtained day 2–4 neonatal murine epidermis and analyzed by FACS for integrin α6 expression. Integrin α6+ (CD49f)–expressing basal cells constituted $44 \pm 7\%$ of total keratinocytes ($n = 5$), in keeping with previous findings (Schneider *et al.*, 2003). Therefore, in this study, on the order of 0.01% of murine basal cells are EpiSCs. Although our studies were conducted using neonatal murine dorsal epidermis, our result is on the same order as that found using unperturbed adult murine epidermis *in vivo* (Clayton *et al.*, 2007).

CD133$^+\Delta\Psi m^{hi}$ cells were studied for long-term repopulating ability, nuclear Bmi-1 expression, and label retention. Although nuclear Bmi-1 expression was increased in the CD133$^+\Delta\Psi m^{hi}$ versus CD133$^+$ population ($P = 0.02$), the EpiSC enrichment (5.2- vs. 3.9-fold, $P = 0.49$) and the number of label-retaining cells (37.3 ± 5.9 vs. $28.9 \pm 8.6\%$, $P = 0.23$) in CD133$^+\Delta\Psi m^{hi}$ keratinocytes was not significantly different. In addition, although there is strong evidence for integrin $\alpha6^{hi}$CD71^{lo} as a marker of human EpiSCs (Li *et al.*, 1998, 2004; Kaur and Li, 2000; Li and Kaur, 2005) and *in vitro* studies showed that integrin $\alpha6^{hi}$CD71^{lo} murine keratinocytes are quiescent and small, with high nuclear/cytoplasmic ratio (Tani *et al.*, 2000; Yano *et al.*, 2005), our studies indicated that murine integrin $\alpha6^{hi}$CD71^{lo} keratinocytes are not enriched for long-term repopulating cells *in vivo*, in this assay. Other functional characteristics of integrin $\alpha6^{hi}$CD71^{lo} cells were not tested.

CD133$^+$ cells were bulge cells and were CD34$^+$ (AC133 antibody was produced by inoculation of CD34$^+$ human cells (Yin *et al.*, 1997; Bidlingmaier *et al.*, 2008)). Although $94.3 \pm 4.2\%$ of CD133$^+$ keratinocytes were keratin 14$^+$, there may exist a more primitive K14$^-$ stem cell. Although side population cells were integrin $\alpha6^{hi}$CD71^{lo} (Redvers *et al.*, 2006), CD34$^-$, and distinct from the bulge population (Redvers *et al.*, 2006), $99 \pm 1\%$ of CD133$^+$ keratinocytes were distinct from integrin $\alpha6^{hi}$CD71^{lo} cells and CD34$^+$ cells and localized in the hair follicle bulge. CD133$^+$ cells thus appear to be distinct from both integrin $\alpha6^{hi}$CD71^{lo} cells and side population cells.

CD133$^+$ cells are keratinocytes because CD133$^+$ (CD34$^+$) cells are keratin 14$^+$ and integrin $\alpha6^+$, consistent with basal epidermal cells. Possible contamination of CD133$^+$ keratinocytes by CD133$^+$ dermal papilla cells was excluded using versican GFP–tagged mice (CD133$^+$ dermal papilla cells are GFP$^+$). Isolated CD133$^+$ keratinocytes were GFP$^-$. Furthermore, the GFP$^+$/CD133$^+$ dermal

Figure 2. CD133$^+$ and CD133$^+\Delta\Psi m^{hi}$ keratinocytes are located in the murine bulge and are enriched for long-term repopulating epidermal stem cells. (**a**, **b**) CD133 was expressed in the hair follicle bulge of neonatal and adult murine epidermis. In addition, CD133 was expressed in dermal papilla cells (indicated by asterisk) (Ito *et al.*, 2006). Immunofluorescence using confocal microscopy. (**c**) Isolation of CD133$^+$ and (**d**) isolation of CD133$^+\Delta\Psi m^{hi}$ murine keratinocytes based on isotype control. (**e**) Isolation of integrin $\alpha6^{hi}$CD71^{lo} murine keratinocytes. The selected population was based on the 7–10% of cells most α6 integrin^{hi}CD71^{lo} (putative epidermal stem cells (EpiSCs). A second population (right upper quadrant) was also selected based on the 7–10% of cells most α6 integrin^{hi}CD71^{hi} (putative transit amplifying cells (TACs)) (Li *et al.*, 1998, 2004; Kaur and Li, 2000; Li and Kaur, 2005) (based on isotype controls). (**f**) Epidermal repopulating units (ERUs) formed following injection of CD133$^+$ keratinocytes into an NOD/SCID mouse. (**g**) EpiSC frequency, based on *in vivo* transplantation combined with limiting dilution analysis, in isolated keratinocyte populations at 9 weeks. (**h**) Summary of the data in Figure 2g. Bar graph showing the fold enrichment over unfractionated (UNF) keratinocytes for the selected populations. Error bars = mean ± SE (*$P \leq 0.05$; **$P \leq 0.001$; $n = 3$). bg, bulge; H&E, hematoxylin and eosin; NS, nonsignificant; SSC, side scatter. Bars = 10 μm for **a**, **b**, and **f**.

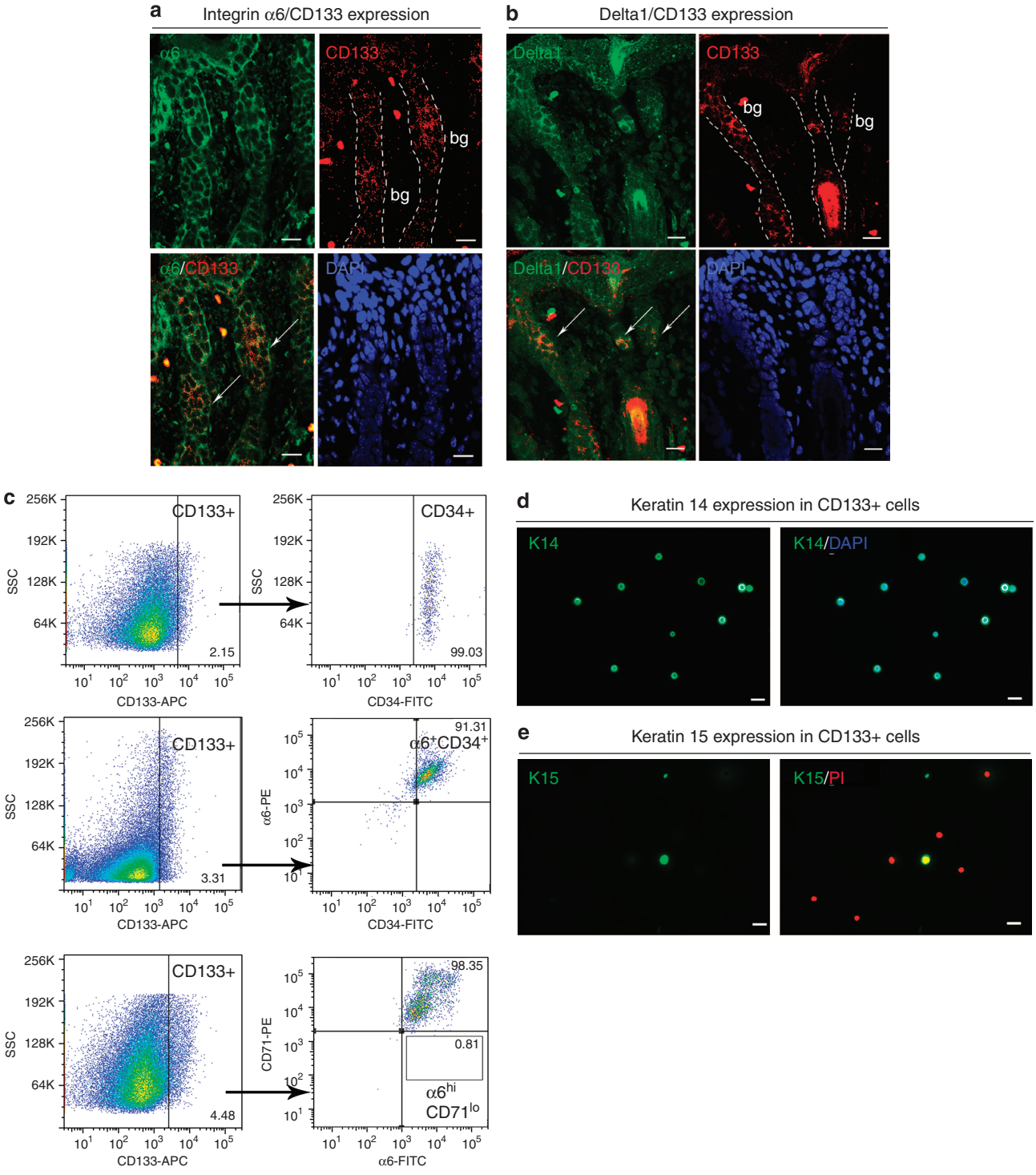


Figure 3. CD133$^+$ keratinocytes colocalize with integrin α6$^+$, Delta1$^+$, and CD34$^+$ keratinocytes. (**a**) CD133 and integrin α6 coexpressing keratinocytes in the bulge (arrows). (**b**) CD133 and Delta-1 coexpressing keratinocytes in the bulge (arrows). (**c**) Overlap between CD133$^+$, CD34$^+$, and integrin α6 populations. CD133$^+$ cells are CD34$^+$ (upper panel), integrin α6$^+$CD34$^+$ (middle panel), and not integrin α6^{hi}CD71^{lo} (lower panel). (**d**) In all, 94.3 ± 4.2% of CD133$^+$ keratinocytes were keratin 14$^+$ (direct counting, 200–500 cells per experiment); (**e**) 15.5 ± 4% of CD133$^+$ cells were keratin 15$^+$ (direct counting, 200–500 cells per experiment). Bars = 10 μm. bg, bulge; SSC, side scatter.

papilla cells were less than 2% of pure dermal papilla cell preparations, so that contaminating CD133$^+$ cells could not explain the 4.2 ± 3.1% CD133$^+$ cells consistently found in the total keratinocyte preparation. Finally, isolated CD133$^+$ cells grow a stratified epithelium *in vivo*, not dermal tissue, when injected into murine subcutis.

The colony-forming efficiency results reflected short-term *in vivo* results. CD133$^-$ keratinocytes were clonogenic

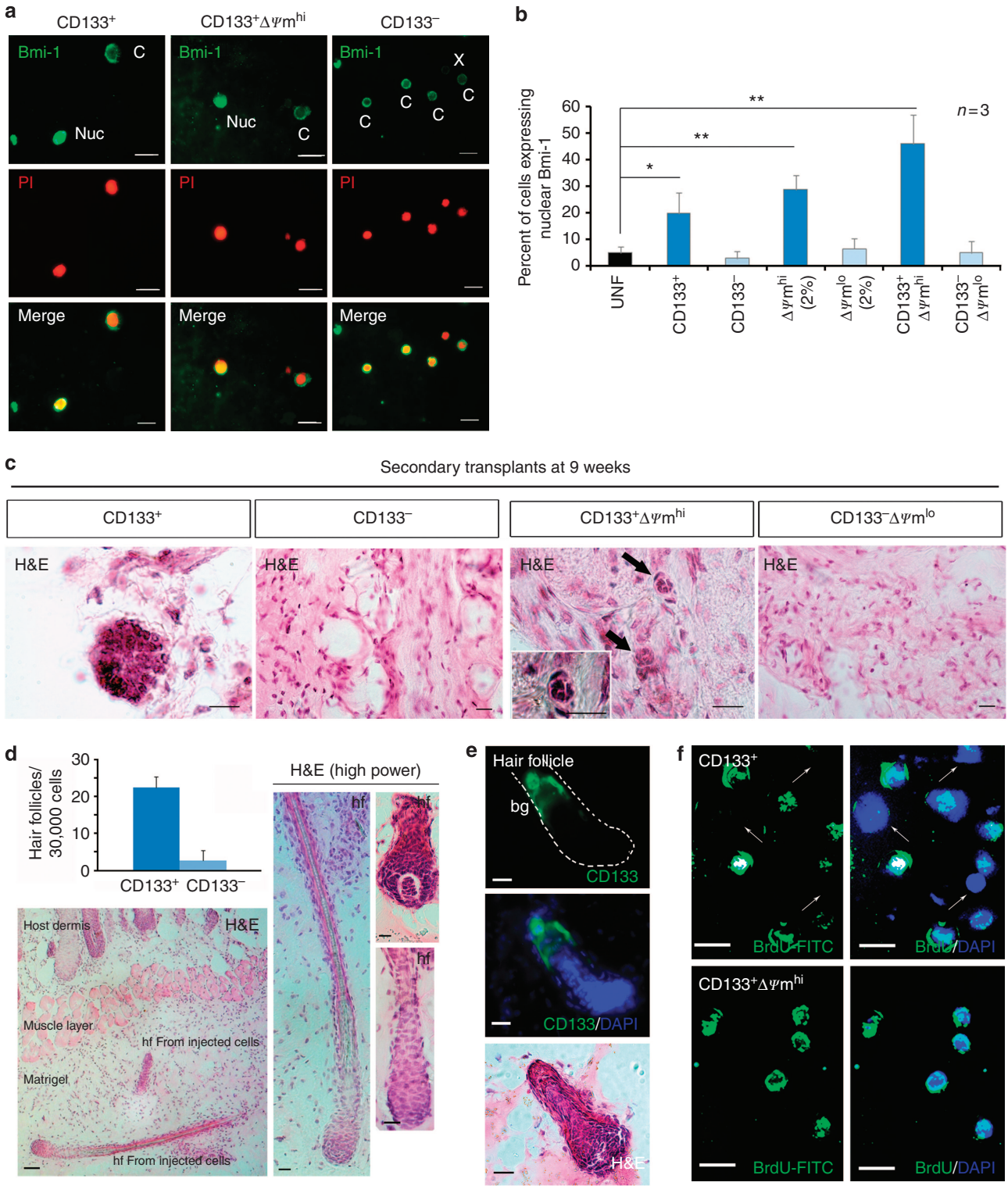
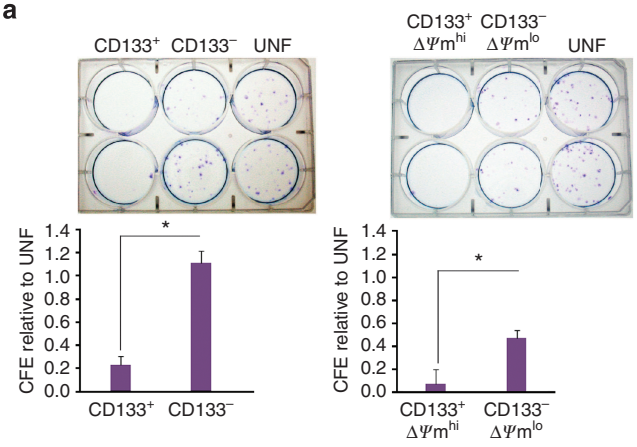


Figure 4. CD133$^+$ and CD133$^+$ΔΨm^{hi} murine keratinocytes display stem cell characteristics. (**a**) Nuclear expression of Bmi-1 in CD133$^+$, ΔΨm^{hi}, and CD133$^+$ΔΨm^{hi} keratinocytes. Cells with nuclear expression (Nuc), cytoplasmic expression (C), or no expression (x) were counted (≥100 cells per population). (**b**) Bar graph of the percentage of cells with nuclear Bmi-1 expression. Error bars = mean ± SD (*P≤0.05; **P≤0.001; n=3). (**c**) Epidermal repopulating units (ERUs) were produced after secondary transplantation of ERUs from 4,000 CD133$^+$ and 4,000 CD133$^+$ΔΨm^{hi} keratinocytes but not after secondary transplantation of ERUs from 4,000 CD133$^-$ and 4,000 CD133$^-$ΔΨm^{lo} keratinocytes. (**d**) Hair follicles derived from CD133$^+$ keratinocytes that were coinjected with day 2 murine dermal papilla cells (hematoxylin and eosin (H&E)). Bar graph: error bars = mean ± SD, P=0.001, n=3. (**e**) CD133 immunostaining of a hair follicle derived from CD133$^+$ keratinocytes coinjected with day 2 murine dermal papilla cells and H&E staining of the adjacent section. (**f**) 28.9 ± 8.6% of CD133$^+$ and 37.3 ± 5.9% of CD133$^+$ΔΨm^{hi} keratinocytes are label-retaining cells (arrows indicate BrdU$^-$ cells; mean ± SD, n=3). bg, bulge; hf, hair follicle. Bars = 10 μm for **a**, **c**, and **f**; 50 μm for **d** (low power images); 10 μm for **d** (high power images); and 20 μm for **e**.

a



b

Cell dose \ Isolation strategy	UNF	CD133−	CD133+	CD133+ΔΨmhi
	Response ratio: positive samples/total number of injections			
6,250	4/4	1/1		
1,562	4/4	1/1	4/4	
391	4/4	6/6	3/8	6/15
98	7/9	5/7	1/10	2/13
24	6/11	1/4	0/9	4/11
5	0/1			
1	0/2			
ERU frequency	1 in 48	1 in 77	1 in 712	1 in 495
SE[a]	35–66	52–144	492–1032	364–671

[a] Tests for inconsistency (χ^2 Pearson, deviance) were NS

Figure 5. In both *in vitro* and *in vivo* studies, the short-term repopulating cells reside in the CD133$^-$ population, rather than in the CD133$^+$ population. (**a**) Murine keratinocyte populations were sorted and 4,000 cells plated in each well. After 3–4 weeks, wells were stained with toluidine blue. Relative colony-forming efficiency of CD133$^+$, CD133$^+$ΔΨm^{hi}, CD133$^-$, and CD133$^-$ΔΨm^{lo} keratinocytes is shown in the bar graph. Error bars = mean ± SD (*$P \leqslant 0.05$; $n = 3$). (**b**) Assessment of epidermal repopulating unit (ERU) frequency using *in vivo* transplantation and limiting dilution analysis at 1 week (short-term repopulation) *in vivo*. CFE, colony forming efficiency; NS, nonsignificant; UNF, unfractionated.

in vitro and were short-term repopulating cells *in vivo*. CD133$^+$ cells did not grow *in vitro* and were not short-term repopulating cells *in vivo*, but were long-term repopulating cells *in vivo*. Culture conditions may favor proliferation of transit amplifying cells and/or reprogram proliferative ability (for review see Cotsarelis, 2006), or *in vitro* analysis may reflect a wounding response, not homeostasis (Kaur, 2006). These results are consistent with the belief that stem cells have minimal clonogenic ability *in vitro* and that not all colony-forming cells constitute stem cells (Schofield, 1978; Loutit *et al.*, 1981; Pavlovitch *et al.*, 1991; Li *et al.*, 1998; Budak *et al.*, 2005; Louis *et al.*, 2008; Strachan *et al.*, 2008; Selver *et al.*, 2011).

Lineage tracing indicates that follicular cells do not contribute to the interfollicular epidermis during homeostasis (Ghazizadeh and Taichman, 2001; Morris *et al.*, 2004; Tumbar *et al.*, 2004; Ito *et al.*, 2005; Levy *et al.*, 2005). Keratin 15$^+$ bulge cells formed interfollicular epidermis in transplantation assays (at 4 weeks) (Morris *et al.*, 2004), but lineage analysis after wounding demonstrated that keratin 15$^+$ bulge cells and their progeny contribute to the interfollicular epidermis only transiently, whereas unlabeled cells persisted (Ito *et al.*, 2005). In another study, lineage tracing after wounding demonstrated that Shh-expressing cells (present throughout the pilosebaceous unit and comprising both keratin 15$^+$ and keratin 15$^-$ cells) and their progeny contributed to the interfollicular epidermis for at least 4 months (Levy *et al.*, 2007). By contrasting and synthesizing the results of these two sets of experiments, Levy *et al.* (2007) suggested the possibility of a distinct cell in the follicle (derived from Shh$^+$ but not keratin 15$^+$ cells) with the ability to become a long-term repopulating stem cell of the interfollicular epidermis. Our studies show that bulge-derived CD133$^+$ cells (85% keratin 15$^-$) form both the interfollicular epidermis and hair follicles in transplantation assays and form the interfollicular epidermis for the long term (9 weeks). Future lineage tracing is indicated to determine how CD133$^+$ cells and their progeny contribute to the follicular and interfollicular epidermis *in vivo,* during homeostasis and wounding.

Finally, our results characterize CD133$^+$ EpiSCs using functional properties and provide a basis for future studies aimed at quantitative comparison of the enrichment in long-term repopulating stem cells and short-term repopulating progenitors provided by different EpiSC isolation strategies.

MATERIALS AND METHODS

Mice

IACUC approval (VAMC San Francisco, CA) was obtained and work was performed in accordance with institutional guidelines. C57BL/6TgN(ACTbEGFP1Osb), NOD/SCID (Jackson Laboratory, http://www.jax.org), and versican GFP–tagged transgenic mice (kindly provided by Jiro Kishimoto as a gift to Daniel Bikle) (Kishimoto *et al.*, 1999; Ehama *et al.*, 2007) were used.

Keratinocyte and fibroblast isolation

Excised skin was incubated in dispase and then trypsin (Schneider *et al.*, 2003), and a cell suspension was obtained. Fibroblasts were isolated by incubating the dermis in 0.25% collagenase IA (Sigma-Aldrich, St Louis, MO, http://www.sigmaaldrich.com) at 37°C for 1 hour.

Flow cytometry

Keratinocytes were sorted using a FACSAria (BD Biosciences, San Jose, CA) and analyzed with the CellQuest software (Becton Dickinson, San Jose, CA). Antibodies included APC-CD133 (AC133 (Corti *et al.*, 2007; Rountree *et al.*, 2007; Snippert *et al.*, 2009)), APC-IgG1K isotype control, Alexa Fluor 488-SSEA-1, PE-CD44 (all from eBioscience, San Diego, CA, http://www.ebioscience.com), and FITC-integrin α6, FITC-IgG2a isotype control, PE-CD71, PE-IgG1K isotype control, and FITC-CD34 (all from BD Pharmingen, San Diego, CA). PE-integrin α6 antibody was from Abcam (Cambridge, MA, http://www.abcam.com).

To sort for keratinocytes with high ΔΨm, cells were treated with TMRM 25 nM for 15 minutes (Schieke *et al.*, 2006, 2008). Cells with high ΔΨm accumulate more of this potentiometric dye (Invitrogen, Grand Island, NY).

Epidermal regeneration *in vivo*

Keratinocytes in Progenitor Cell Technology Epidermal Keratinocyte Medium Complete (CNT07, Chemicon, Temecula, CA, http://www.chemicon.com) were injected with Matrigel (0.5 mg ml^{-1}; BD Biosciences) 1:1 (vol/vol) into the subcutis of NOD/SCID mice. CNT-07 is a protein-free fully defined, 0.07 mM calcium formulation with no antibiotics/antimycotics, containing amino acids, minerals, vitamins, and organic compounds. Grafts were harvested and examined for the presence of ERUs histologically.

Limiting dilution analysis of ERU frequency

For each time point, injection sites were scored as positive if at least one ERU was observed microscopically (Schneider *et al.*, 2003) and the ratio of positive/total sites determined for each dose. Statistical software for limiting dilution analysis (L-CALC, Stemsoft, Vancouver, BC, Canada, http://www.stemsoft.com) was used. The χ^2 statistic was used and 5% or less type I error was considered significant.

Immunohistochemistry

Involucrin and keratin 14 primary antibodies (Abcam) and VECTASTAIN Elite ABC reagent followed by ImmPACT NovaRED Peroxidase Substrate (Vector, Burlingame, CA) were used on paraffin-embedded sections.

Immunofluorescence

Antibodies included CD133 (AC133) (eBioscience), laminin, Delta1, Lgr5, Keratin15, Keratin14 and Bmi-1 (all from Abcam), and integrin α6 (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com/). AlexaFluor 488-IgF1 (H + L), AlexaFluor 594-IgG, and AlexaFluor 488-IgG (Invitrogen) secondary antibodies were used on cell cytospins and paraffin-embedded sections. In all, 300–500 cells were analyzed per sample.

Label-retaining cells

Three-day-old mice were injected twice daily for 3 days with BrdU (75 μg per dose). Skin was collected 30 days later. FITC-conjugated BrdU antibody (Abcam) was used to localize label-retaining cells using microscopy.

Multipotency

CD133$^+$ or CD133$^-$ keratinocytes and versican GFP–tagged dermal papilla cells (day 2) mixed with Matrigel 1:1 (vol/vol) were injected into NOD/SCID subcutis. Grafts were harvested 18 days after injection and examined histologically.

Colony-forming efficiency

Keratinocytes were seeded at clonal density (100–500 cells per cm^2) (Morris *et al.*, 1988; Strachan *et al.*, 2008) onto six-well plates with CNT07 medium. After 3–4 weeks, cells were fixed, stained with toluidine blue (Sigma-Aldrich), and colony-forming efficiency (number of colonies per 100 cells seeded) was expressed relative to UNF cells.

Statistical analysis

For comparisons of colony-forming efficiency, the percentage of cells exhibiting nuclear Bmi-1, and the percentage of ERUs labeled with both DiI and DiO, a two-tailed Student's *t*-test was used. For comparisons of the frequency of repopulating units in each subpopulation at each time point, a two-tailed *t*-test within the L-CALC program (Stemsoft Software) was used.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by an NIH AR01 grant (RG), the Department of Veterans Affairs (RG), which were administered by the Northern California Institute for Research and Education, and with resources of the Veterans Affairs Medical Center, San Francisco, California, as well as by gifts from D Gregory, JC McIntosh, and S Reeves. We thank S Fong for his outstanding technical support.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Bickenbach JR (1981) Identification and behavior of label-retaining cells in oral mucosa and skin. *J Dent Res* 60(Spec No C):1611–20

Bickenbach JR, Chism E (1998) Selection and extended growth of murine epidermal stem cells in culture. *Exp Cell Res* 244:184–95

Bidlingmaier S, Zhu X, Liu B (2008) The utility and limitations of glycosylated human CD133 epitopes in defining cancer stem cells. *J Mol Med* 86:1025–32

Blanpain C, Lowry EW, Geoghegan A *et al.* (2004) Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* 118:635–48

Budak MT, Alpdogan OS, Zhou M *et al.* (2005) Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. *J Cell Sci* 118(Part 8):1715–24

Bussolati B, Bruno S, Grange C *et al.* (2005) Isolation of renal progenitor cells from adult human kidney. *Am J Pathol* 166:545–55

Charruyer A, Barland CO, Yue L *et al.* (2009) Transit-amplifying cell frequency and cell cycle kinetics are altered in aged epidermis. *J Invest Dermatol* 129:2574–83

Clayton E, Doupe DP, Klein AM *et al.* (2007) A single type of progenitor cell maintains normal epidermis. *Nature* 446:185–9

Corbeil D, Roper K, Hellwig A *et al.* (2000) The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. *J Biol Chem* 275:5512–20

Corti S, Nizzardo M, Nardini M *et al.* (2007) Isolation and characterization of murine neural stem/progenitor cells based on Prominin-1 expression. *Exp Neurol* 205:547–62

Coskun V, Wu H, Blanchi B *et al.* (2008) CD133+ neural stem cells in the ependyma of mammalian postnatal forebrain. *Proc Natl Acad Sci USA* 105:1026–31

Cotsarelis G (2006) Epithelial stem cells: a folliculocentric view. *J Invest Dermatol* 126:1459–68

Doran TI, Vidrich A, Sun TT (1980) Intrinsic and extrinsic regulation of the differentiation of skin, corneal and esophageal epithelial cells. *Cell* 22:17–25

Ehama R, Ishimatsu-Tsuji Y, Iriyama S *et al.* (2007) Hair follicle regeneration using grafted rodent and human cells. *J Invest Dermatol* 127:2106–15

Estrach S, Cordes R, Hozumi K *et al.* (2007) Role of the Notch ligand Delta1 in embryonic and adult mouse epidermis. *J Invest Dermatol* 128:825–32

Ghazizadeh S, Taichman LB (2001) Multiple classes of stem cells in cutaneous epithelium: a lineage analysis of adult mouse skin. *EMBO J* 20:1215–22

Grimwood RE, Baskin JB, Nielsen LD *et al.* (1988) Fibronectin extracellular matrix assembly by human epidermal cells implanted into athymic mice. *J Invest Dermatol* 90:434–40

Guo A, Jahoda CA (2009) An improved method of human keratinocyte culture from skin explants: cell expansion is linked to markers of activated progenitor cells. *Exp Dermatol* 18:720–6

Hall JP, Sheffey RJ, Chagares WE *et al.* (2006) Epidermal inclusion cyst in the foot of a Vietnam Veteran. *J Am Podiatr Med Assoc* 96:445–7

Ito M, Liu Y, Yang Z *et al.* (2005) Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nat Med* 11:1351–4

Ito Y, Hamazaki TS, Ohnuma K *et al.* (2006) Isolation of murine hair-inducing cells using the cell surface marker prominin-1/CD133. *J Invest Dermatol* 127:1052–60

Jaks V, Barker N, Kasper M *et al.* (2008) Lgr5 marks cycling, yet long-lived, hair follicle stem cells. *Nat Genet* 40:1291–9

Jiang S, Zhao L, Purandare B *et al.* (2010) Differential expression of stem cell markers in human follicular bulge and interfollicular epidermal compartments. *Histochem Cell Biol* 133:455–65

Kaur P (2006) Interfollicular epidermal stem cells: identification, challenges, potential. *J Invest Dermatol* 126:1450–8

Kaur P, Li A (2000) Adhesive properties of human basal epidermal cells: an analysis of keratinocyte stem cells, transit amplifying cells, and postmitotic differentiating cells. *J Invest Dermatol* 114:413–20

Kishimoto J, Ehama R, Wu L *et al.* (1999) Selective activation of the versican promoter by epithelial-mesenchymal interactions during hair follicle development. *Proc Natl Acad Sci USA* 96:7336–41

Lacroix M, Caramel J, Goguet-Rubio P *et al.* (2010) Transcription factor E4F1 is essential for epidermal stem cell maintenance and skin homeostasis. *Proc Natl Acad Sci USA* 107:21076–81

Lee K, Adhikary G, Balasubramanian S *et al.* (2008) Expression of Bmi-1 in epidermis enhances cell survival by altering cell cycle regulatory protein expression and inhibiting apoptosis. *J Invest Dermatol* 128:9–17

Levy V, Lindon C, Harfe BD *et al.* (2005) Distinct stem cell populations regenerate the follicle and interfollicular epidermis. *Dev Cell* 9:855–61

Levy V, Lindon C, Zheng Y *et al.* (2007) Epidermal stem cells arise from the hair follicle after wounding. *FASEB J* 21:1358–66

Li A, Pouliot N, Redvers R *et al.* (2004) Extensive tissue-regenerative capacity of neonatal human keratinocyte stem cells and their progeny. *J Clin Invest* 113:390–400

Li A, Simmons PJ, Kaur P (1998) Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. *Proc Natl Acad Sci USA* 95:3902–7

Li A, Kaur P (2005) FACS enrichment of human keratinocyte stem cells. *Methods Mol Biol* 289:87–96

Louis SA, Rietze RL, Deleyrolle L *et al.* (2008) Enumeration of neural stem and progenitor cells in the neural colony-forming cell assay. *Stem Cells* 26:988–96

Loutit JF, Peters J, Marshall MJ (1981) Colony forming units and haematopoietic stem cells in osteoclastopoiesis. *Metab Bone Dis Relat Res* 3:131–3

Lyle S, Christofidou-Solomidou M, Liu Y *et al.* (1998) The C8/144B monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. *J Cell Sci* 111(Part 21):3179–88

Lyle S, Christofidou-Solomidou M, Liu Y *et al.* (1999) Human hair follicle bulge cells are biochemically distinct and possess an epithelial stem cell phenotype. *J Investig Dermatol Symp Proc* 4:296–301

Mizrak D, Brittan M, Alison MR (2008) CD133: molecule of the moment. *J Pathol* 214:3–9

Morris RJ, Liu Y, Marles L *et al.* (2004) Capturing and profiling adult hair follicle stem cells. *Nat Biotech* 22:411–7

Morris RJ, Tacker KC, Fischer SM *et al.* (1988) Quantitation of primary *in vitro* clonogenic keratinocytes from normal adult murine epidermis, following initiation, and during promotion of epidermal tumors. *Cancer Res* 48:6285–90

Ohnishi T, Watanabe S (1999) Immunohistochemical observation of cytokeratins in keratinous cysts including plantar epidermoid cyst. *J Cutan Pathol* 26:424–9

Pavlovitch JH, Rizk-Rabin M, Jaffray P *et al.* (1991) Characteristics of homogeneously samll keratinocytes from newborn rat skin: possible epidermal stem cells. *Am J Physiol* 261(Part 1):C964–72

Redvers RP, Li A, Kaur P (2006) Side population in adult murine epidermis exhibits phenotypic and functional characteristics of keratinocyte stem cells. *Proc Natl Acad Sci USA* 103:13168–73

Reinisch CM, Uthman A, Erovic BM *et al.* (2007) Expression of BMI-1 in normal skin and inflammatory and neoplastic skin lesions. *J Cutan Pathol* 34:174–80

Richardson GD, Robson CN, Lang SH *et al.* (2004) CD133, a novel marker for human prostatic epithelial stem cells. *J Cell Sci* 117:3539–45

Rountree CB, Barsky L, Ge S *et al.* (2007) A CD133-expressing murine liver oval cell population with bilineage potential. *Stem Cells* 25:2419–29

Scaduto RC, Grotyohann LW (1999) Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys J* 76:469–77

Schieke SM, Ma M, Cao L *et al.* (2008) Mitochondrial metabolism modulates differentiation and teratoma formation capacity in mouse embryonic stem cells. *J Biol Chem* 283:28506–12

Schieke SM, Phillips D, McCoy JP Jr *et al.* (2006) The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *J Biol Chem* 281:27643–52

Schneider TE, Barland C, Alex AM *et al.* (2003) Measuring stem cell frequency in epidermis: a quantitative *in vivo* functional assay for long-term repopulating cells. *Proc Natl Acad Sci USA* 100:11412–7

Schofield R (1978) The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* 4:7–25

Selver OB, Barash A, Ahmed M *et al.* (2011) ABCG2-dependent dye exclusion activity and clonal potential in epithelial cells continuously growing for 1 month from limbal explants. *Invest Ophtalmol Vis Sci* 53:4330–7

Silver SG, Ho VCY (2003) Benign epithelial tumors. In: Irwin Freedberg AE, Klaus Wolff, (eds). *Fitzpatrick's Dermatology in General Medicine*. McGraw-Hill: New York p 778

Snippert HJ, van Es JH, van den Born M *et al.* (2009) Prominin-1/CD133 marks stem cells and early progenitors in mouse small intestine. *Gastroenterology* 136:2187–94 e2181

Strachan LR, Scalapino KJ, Lawrence HJ *et al.* (2008) Rapid adhesion to collagen isolates murine keratinocytes with limited long-term repopulating ability *in vivo* despite high clonogenicity *in vitro*. *Stem Cells* 26:235–43

Szilvassy SJ, Humphries RK, Lansdorp PM *et al.* (1990) Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. *Proc Natl Acad Sci USA* 87:8736–40

Tani H, Morris RJ, Kaur P (2000) Enrichment for murine keratinocyte stem cells based on cell surface phenotype. *Proc Natl Acad Sci USA* 97:10960–5

Trempus CS, Morris RJ, Bortner CD *et al.* (2003) Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. *J Invest Dermatol* 120:501–11

Trempus CS, Morris RJ, Ehinger M *et al.* (2007) CD34 expression by hair follicle stem cells is required for skin tumor development in mice. *Cancer Res* 67:4173–81

Triel C, Vestergaard ME, Bolund L *et al.* (2004) Side population cells in human and mouse epidermis lack stem cell characteristics. *Exp Cell Res* 295:79–90

Tumbar T, Guasch G, Greco V *et al.* (2004) Defining the epithelial stem cell niche in skin. *Science* 303:359–63

Uchida N, Buck DW, He D *et al.* (2000) Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci USA* 97:14720–5

Wei ZG, Lin T, Sun TT *et al.* (1997) Clonal analysis of the *in vivo* differentiation potential of keratinocytes. *Invest Ophthalmol Vis Sci* 38:753–61

Weigmann A, Corbeil D, Hellwig A *et al.* (1997) Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. *Proc Natl Acad Sci USA* 94:12425–30

Winter MC, Bickenbach JR (2009) Aging epidermis is maintained by changes in transit-amplifying cell kinetics, not stem cell kinetics. *J Invest Dermatol* 129:2541–3

Yang C, Cotsarelis G (2010) Review of hair follicle dermal cells. *J Dermatol Sci* 57:2–11

Yano S, Ito Y, Fujimoto M *et al.* (2005) Characterization and localization of side population cells in mouse skin. *Stem Cells* 23:834–41

Yin AH, Miraglia S, Zanjani ED *et al.* (1997) AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* 90:5002–12

Youn SW, Kim DS, Cho HJ *et al.* (2004) Cellular senescence induced loss of stem cell proportion in the skin *in vitro*. *J Dermatol Sci* 35:113–23

Yu W, Fang X, Ewald A *et al.* (2007) Formation of cysts by alveolar type II cells in three-dimensional culture reveals a novel mechanism for epithelial morphogenesis. *Mol Biol Cell* 18:1693–700

Yu Y, Flint A, Dvorin EL *et al.* (2002) AC133-2, a novel isoform of human AC133 stem cell antigen. *J Biol Chem* 277:20711–6

Zheng Y, Du X, Wang W *et al.* (2005) Organogenesis from dissociated cells: generation of mature cycling hair follicles from skin-derived cells. *J Invest Dermatol* 124:867–76

Zhu L, Gibson P, Currle DS *et al.* (2009) Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. *Nature* 457: 603–7