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

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Abstract

Maintenance, repair and renewal of the epidermis are thought to depend on a pool of dedicated epidermal stem cells. 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 epidermal stem populations. Putative epidermal stem cell 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 epidermal stem cells vs. 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±8.6% were label retaining cells. Thus, murine keratinocytes within the $CD133^+$ and $CD133^+\Delta\Psi m^{hi}$ populations contain epidermal stem cells that regenerate epidermis for the long-term, are self-renewing, multipotent, and label-retaining cells.

MeSH Keywords

Keratinocyte; stem cell; epidermis; murine; CD133; mitochondrial membrane potential

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CONFLICT OF INTEREST

The authors indicate no potential conflict of interest.

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 (Hall et al., 2006; Silver and Ho, 2003; Ohnishi and Watanabe, 1999). Production of such cysts has been used to study 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.

$CD133^+$ marked 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 marked 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 **(ΔΨm)** 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 ΔΨm, as it provides more accurate quantification than the parent compound (Scaduto et al., 1999).

In both human and murine epidermis, integrin $\alpha6^{hi}CD71^{lo}$ keratinocytes showed features of stem cells (Kaur and Li, 2000; Li et al., 1998; 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 dilution analysis (Schneider et al., 2003; Charruyer et al., 2009; Strachan et al., 2008). 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 (565nm, red) or DiO (501nm, 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%) and 0 (0%) bi-colored ERUs, respectively (Figure 1e, f and 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, while transit amplifying cells (TACs) (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; Charruyer et al., 2009; Strachan et al., 2008). 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 determined at different repopulation times by limiting dilution analysis (Table1). The frequency of ERUs decreased between 1 and 6 weeks ($P \leq 0.001$; 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 6–24 ($P = 0.25$), 9–24 ($P = 0.63$), 12–24 ($P=0.34$), and 18–24 ($P=0.88$) yielded non-significant 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 total (mean=4.2±3.1%, n=7) (Figure 2c). In 10–12 week 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 ($O\Psi m^{hi}$) and low membrane potential ($O\Psi m^{lo}$) were defined as 5% highest and lowest TMRM fluorescence, as previously (Schieke et al., 2008; Schieke et al., 2006). Approximately 2% of total cells were $CD133^+$ $O\Psi m^{hi}$. Therefore, we selected the 2% $CD133^+$ $O\Psi m^{hi}$ and 2% $CD133^-$ $O\Psi m^{lo}$ 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). 1 in 379 (SE 1 in 274–526) $CD133^+$ keratinocytes was an EpiSC capable of long-term repopulation, vs. only 1 in 9,487 (SE 1 in 6,644–13,547) $CD133^-$ cells. 1 in 285 (SE 1 in 220–371) $CD133^+\Delta\Psi m^{hi}$ cells was capable of long-term repopulation vs. only 1 in 5,323 (SE 1 in 3,410–8,308) $CD133^-\Delta\Psi m^{lo}$ cells. The enrichment provided by $CD133^+\Delta\Psi m^{hi}$ vs. $CD133^+$ cells 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 (Figures 2g and h), while 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 (Ehama et al., 2007; Kishimoto et al., 1999). $CD133^+$ keratinocytes isolated from versican-GFP epidermis were GFP^- (Supplementary Figure S1a).

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 co-expressing CD133 and integrin α6 (Li et al., 1998) were located in the hair follicle bulge (Figure 3a). Cells co-expressing CD133 and Delta 1 (Estrach et al., 2008) were located in the bulge (Figure 3b). Integrin $\alpha6^+CD34^+$ keratinocytes exhibited EpiSC properties (Trempus et al., 2003;Trempus et al., 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). Using 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 keratin 14^+ (n=3), consistent with basal keratinocytes. Keratin 15 co-localized with label retaining cells (Lyle et al., 1998; Lyle et al., 1999). Using direct counting 15.5±4% of $CD133^+$ keratinocytes were 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 (Lee et al., 2008; Reinisch et al., 2007; 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) (Figures 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 obtained, followed by re-injection 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, while 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 (Zheng et al., 2005; Morris et al., 2004; Yang and Cotsarelis, 2010). Multipotency was studied using co-injection of 30,000 to 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, 1000 cells counted) and 37.3±5.9% of $CD133^+\Delta\Psi m^{hi}$ cells were label retaining cells (n=3, 1000 cells counted) (Figure 4f and Supplementary Figure S2).

$CD133^+$ 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/100 cells plated) reflects EpiSC number. However, greatest *in vitro* short-term proliferative ability is not associated with greatest long-term repopulating ability *in vivo* (Strachan et al., 2008). 4,000 cells of each population ($CD133^+$, $CD133^+\Delta\Psi m^{hi}$, $CD133^-$, $CD133^-\Delta\Psi m^{lo}$ and UNF) were plated in 35mm 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) vs. $CD133^+$ 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 one week vs. $CD133^+$ and $CD133^+\Delta\Psi m^{hi}$ populations, [1 in 48 (SE 1 in 35–66) and 1 in 77 (SE 1 in 52–144) vs. 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, while containing long-term repopulating cells, are not clonogenic *in vitro* nor short-term repopulating cells *in vivo*.

While it was believed 10% of basal cells were EpiSCs, many studies have found a frequency on a lower order (Schneider et al., 2003; Charruyer et al., 2009; Bickenbach, 1981; Bickenbach and Chism, 1998; Triel et al, 2004; Redvers et al, 2006; 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 ± 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. While our studies were performed 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 $CD133^+\Delta\Psi m^{hi}$ vs. $CD133^+$ population (p=0.02), the EpiSC enrichment (5.2 fold vs. 3.9 fold, P=0.49) and the number of label retaining cells (37.3±5.9% vs. 28.9±8.6%, P=0.23) in $CD133^+\Delta\Psi m^{hi}$ keratinocytes was not significantly different. Also, while there is strong evidence for integrin $\alpha6^{hi}CD71^{lo}$ as a marker of human EpiSCs (Kaur and Li, 2000; Li et al., 1998; Li et al., 2004; Li, 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)]. While 94.3±4.2% of $CD133^+$ keratinocytes were keratin 14^+, there may exist a more primitive $K14^-$ stem cell. While 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±1% of $CD133^+$ keratinocytes were distinct from integrin $\alpha6^{hi}CD71^{lo}$ cells, $CD34^+$ 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 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 *in vitro* and were short-term repopulating cells *in vivo*. $CD133^+$ cells didn't 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 TACs 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 (Pavlovitch et al., 1991; Budak et al., 2005; Li et al., 1998; Selver et al., 2011; Strachan et al., 2008; Schofield, 1978; Loutit et al., 1981; Louis et al., 2008).

Lineage tracing indicates that follicular cells do not contribute to interfollicular epidermis during homeostasis (Gazizadeh et al, 2001; Morris et al, 2004; Tumbar et al, 2004; Levy et al, 2005; Ito 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, while 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 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 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 interfollicular epidermis (Levy et al, 2007). Our studies show that bulge-derived $CD133^+$ cells (85% keratin 15^-) form both interfollicular epidermis and hair follicles in transplantation assays and form interfollicular epidermis for the long-term (9 weeks). Future lineage tracing is indicated to determine how $CD133^+$ cells and their progeny contribute to follicular and interfollicular epidermis *in vivo*, during homeostasis as well as 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 performed in adherence to 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) (Ehama et al., 2007; Kishimoto et al., 1999) were used.

Keratinocyte and Fibroblast isolation

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

Flow Cytometry

Keratinocytes were sorted using a FACSAria (BD Biosciences) and analyzed with CellQuest™ software. 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 25nM for 15min (Schieke et al., 2008; Schieke et al., 2006). Cells with high ΔΨm accumulate more of this potentiometric dye. (Invitrogen).

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; BD Biosciences) 1:1 (vol:vol) into the subcutis of NOD/SCID mice. CNT-07 is a protein-free fully-defined, calcium 0.07 mM 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 Chi square statistic was used and 5% or less type I error 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, Inc, Santa Cruz, CA, http://www.scbt.com/). AlexaFluor 488-IgF1 (H+L), AlexaFluor 594-IgG and AlexaFluor 488-IgG (Invitrogen) secondaries were used on cell cytospins and paraffin-embedded sections. 300–500 cells were analyzed per sample.

Label retaining cells

Three-day mice were injected twice daily for 3 days with BrdU (75 μg/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) (Strachan et al., 2008; Morris et al., 1988) 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.

Statistical analysis

For comparisons of colony forming efficiency, percentage of cells exhibiting nuclear Bmi-1 and 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 Inc) was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ERU	epidermal repopulating unit
GFP	green fluorescent protein
EpiSC	epidermal stem cell
TAC	transit amplifying cell
ΔΨm	mitochondrial membrane potential
UNF	unfractionated

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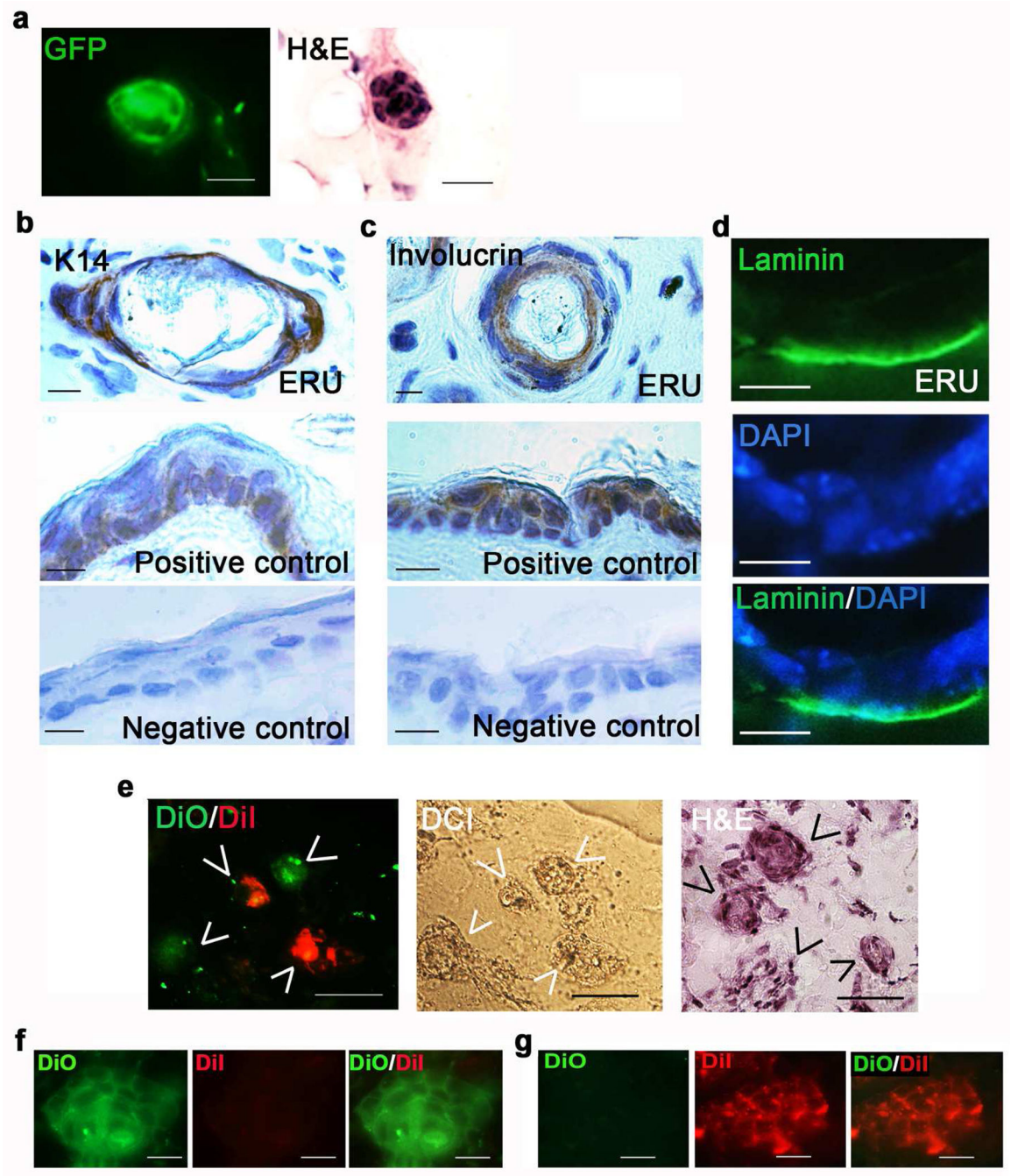
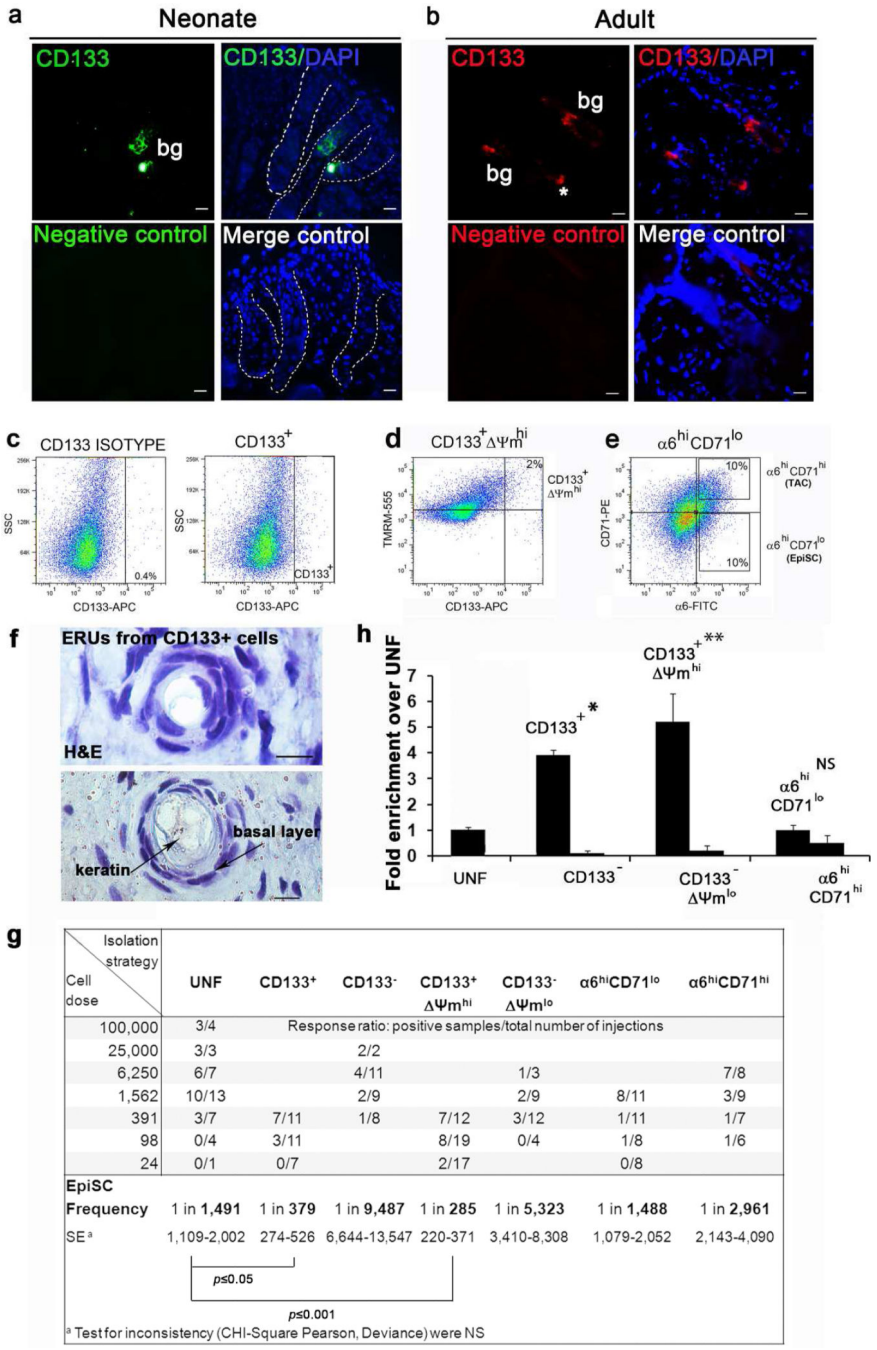


Figure 1. Generation of ERUs following subcutaneous injection of murine keratinocytes in an allograft model

(a) ERUs formed following injection of GFP^+ murine keratinocytes into a GFP^- NOD/SCID mouse are GFP^+ confirming derivation from the GFP^+ donor keratinocytes. Fluorescence microscopy (488 nm) (left panel). H&E of an adjacent section (right panel). 100 ERUs were analyzed (3 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 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 of the adjacent section (right panel). **(f)** A DiO positive ERU and **(g)** a DiI positive ERU. Scale bars = 10 μm in **a–d**; 25 μm in **e**; and 10 μm in **f** and **g**.



g

Isolation strategy / Cell dose	UNF	CD133+	CD133-	CD133+ ΔΨmhi	CD133- ΔΨmlo	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

$p \leq 0.05$

$p \leq 0.001$

[a] Test for inconsistency (CHI-Square Pearson, Deviance) were NS

Figure 2. CD133$^+$ and CD133$^+$ΔΨm^{hi} keratinocytes are located in the murine bulge and are enriched for long-term repopulating epidermal stem cells

(a, b) CD133 expression in the hair follicle bulge of neonatal and adult murine epidermis. In addition, CD133 was expressed in the derma papilla cells (asterisk*) (Ito et al., 2006). Immunofluorescence using confocal microscopy. **(c)** Isolation of CD133$^+$ and **(d)** isolation of CD133$^+$ΔΨm^{hi} murine keratinocytes based on isotype control. **(e)** Isolation of integrin α6^{hi}CD71^{lo} murine keratinocytes. The selected population was based on the 7–10% of cells most α6 integrin^{hi}CD71^{lo} (putative EpiSCs). A second population (right upper quadrant)

was also selected based on the 7–10% of cells most α6 integrin^{hi}CD71^{hi} (putative TACs) (Kaur and Li, 2000; Li et al., 2004; Li et al., 1998; Li, 2005) (based on isotype controls). **(f)** ERUs formed following injection of CD133^{+} keratinocytes into a 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 UNF keratinocytes for the selected populations. Error bars = mean±SE (* $P \leq 0.05$; ** $P \leq 0.001$; n=3). Scale bars= 10 μm for **a, b, f.** bg= bulge

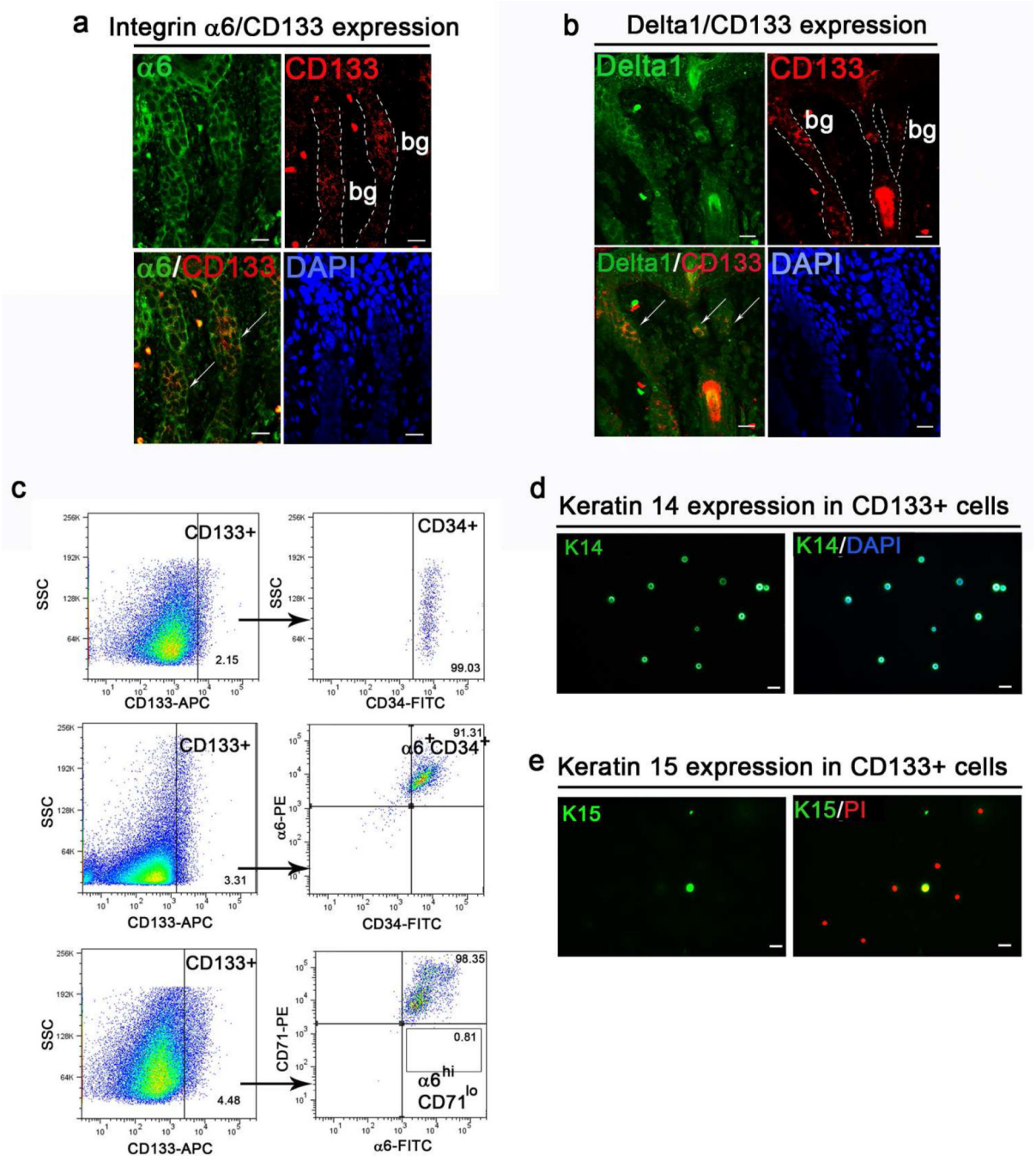


Figure 3. CD133$^+$ keratinocytes co-localize with integrin α6$^+$, Delta1$^+$ and CD34$^+$ keratinocytes

(a) CD133 and α6 integrin co-expressing keratinocytes in the bulge (arrows). **(b)** CD133 and Delta-1 co-expressing 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)** 94.3±4.2% of CD133$^+$ keratinocytes were keratin 14$^+$ (direct counting, 200–500 cells/experiment). **(e)** 15.5±4% of CD133$^+$ cells were keratin 15$^+$ (direct counting, 200–500 cells/experiment). Scale bars= 10 μm. bg= bulge

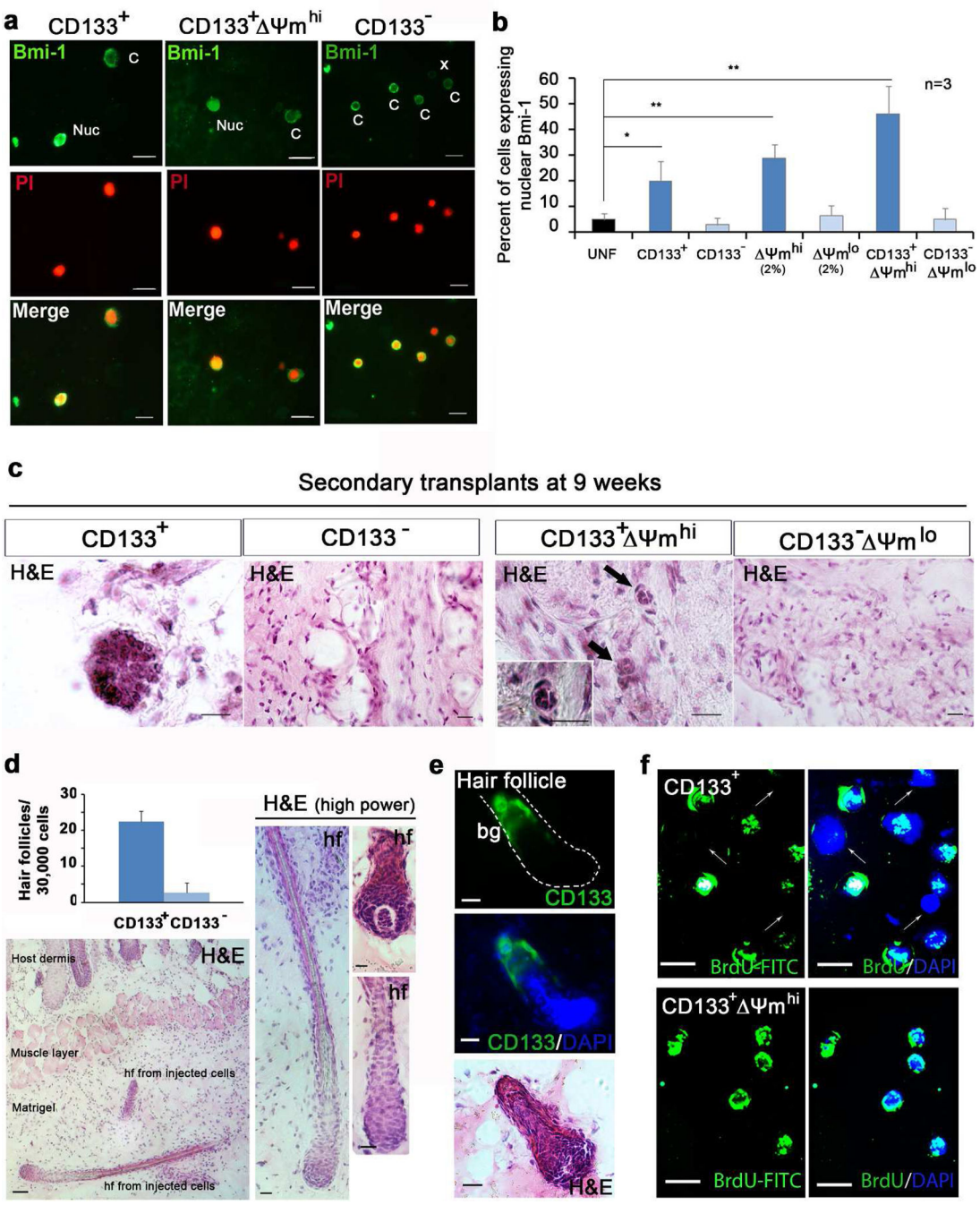
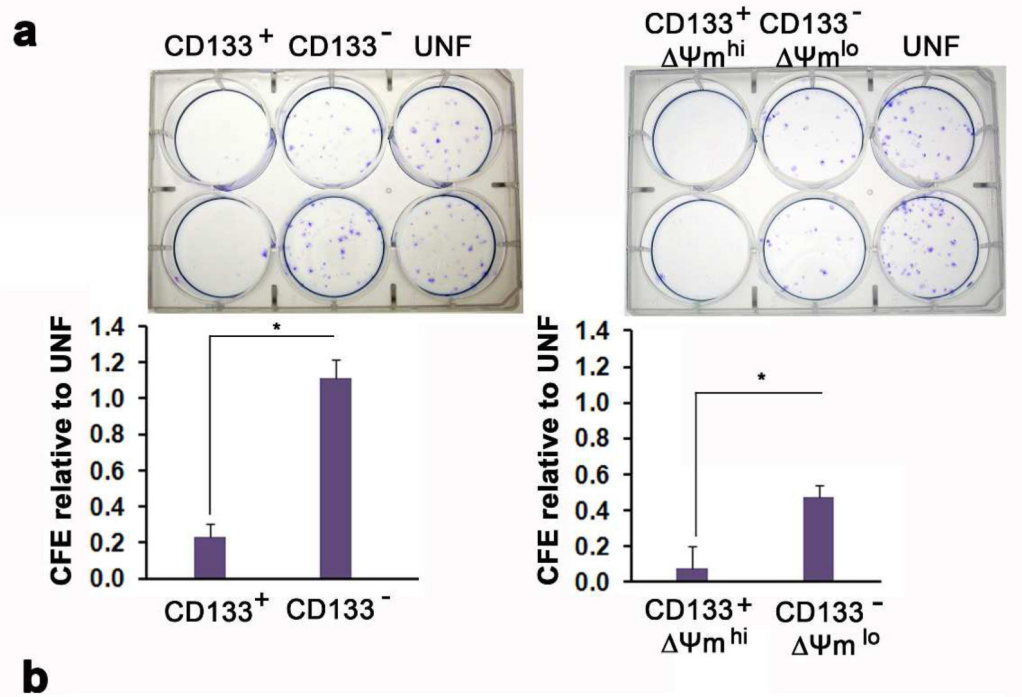


Figure 4. CD133$^+$ and CD133$^+$ΔΨm^{hi} murine keratinocytes display stem cell characteristics

(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 (ε100 cells per population). **(b)** Bar graph of the percentage of cells with nuclear Bmi-1 expression. Error bars = mean±SD (* $P \leq 0.05$; ** $P \leq 0.001$; n=3). **(c)** 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 co-injected with day 2 murine dermal papilla cells (H&E). Bar graph: error bars = mean±SD, *P*=0.001, n=3). **(e)** CD133 immunostaining of hair follicle derived from $CD133^+$ keratinocytes co-injected with day 2 murine dermal papilla cells and H&E of the adjacent section. **(f)** 28.9±8.6% of $CD133^+$ and 37.3±5.9% of $CD133^+\Delta\Psi m^{hi}$ keratinocytes are label retaining cells (arrows indicate $BrdU^-$ cells; mean ± SD, n=3). Scale bars= 10 μm for **a, c, f;** 50 μm for **d** (low power images); 10 μm for **d** (high power images); 20 μm for **e**. bg=bulge; hf=hair follicle



b

Isolation strategy / Cell dose	UNF	CD133⁻	CD133⁺	CD133⁺ΔΨm^hi
	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] Test for inconsistency (CHI-Square 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 the CD133⁺

(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 (*$P \leq 0.05$; n=3) **(b)** Assessment of ERU frequency using *in vivo* transplantation and limiting dilution analysis at 1 week (short-term repopulation) *in vivo*.

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 SE[1, 2]	1 in **48**[**] 35–66	1 in **790** 576–1,084	1 in **1,491** 1,109–2,002	1 in **1,802** 1,342–2,420	1 in **1,373** 917–2,054	1 in **1,242** 921–1,674

[**] $p \leq 0.001$ between 1 week and all the other time points

[1] SE expressed as 1 in…

[2] Tests for inconsistency (CHI-Square Pearson, Deviance) were NS