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The CD44^{+}ALDH^{+} Population of Human Keratinocytes Is Enriched for Epidermal Stem Cells with Long-Term Repopulating Ability

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Abstract

Like for other somatic tissues, isolation of a pure population of stem cells has been a primary goal in epidermal biology. We isolated discrete populations of freshly obtained human neonatal keratinocytes (HNKs) using previously untested candidate stem cell markers aldehyde dehydrogenase (ALDH) and CD44 as well as the previously studied combination of integrin α6 and CD71. An in vivo transplantation assay combined with limiting dilution analysis was used to quantify enrichment for long-term repopulating cells in the isolated populations. The ALDH^{+}CD44^{+} population was enriched 12.6-fold for long-term repopulating epidermal stem cells (EpiSCs) and the integrin α6^{hi}CD71^{lo} population was enriched 5.6-fold, over unfractionated cells. In addition to long-term repopulation, CD44^{+}ALDH^{+} keratinocytes exhibited other stem cell properties. CD44^{+}ALDH^{+} keratinocytes had self-renewal ability, demonstrated by increased numbers of cells expressing nuclear Bmi-1, serial transplantation of CD44^{+}ALDH^{+} cells, and holoclone formation in vitro. CD44^{+}ALDH^{+} cells were multipotent, producing greater numbers of hair follicle-like structures than CD44$^{−}$ALDH$^{−}$ cells. Furthermore, 58% ± 7% of CD44^{+}ALDH^{+} cells exhibited label-retention. In vitro, CD44^{+}ALDH^{+} cells showed enhanced colony formation, in both keratinocyte and embryonic stem cell growth media. In summary, the CD44^{+}ALDH^{+} population exhibits stem cell properties including long-term epidermal regeneration, multipotency, label retention, and holoclone formation. This study shows that it is possible to quantify the

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relative number of EpiSCs in human keratinocyte populations using long-term repopulation as a functional test of stem cell nature. Future studies will combine isolation strategies as dictated by the results of quantitative transplantation assays, in order to achieve a nearly pure population of EpiSCs.

Keywords

Keratinocyte; Stem cell; Epidermis; Human; Aldehyde dehydrogenase; CD44

Introduction

Human epidermis comprises a multilayered squamous epithelium. Epidermal stem cells (EpiSCs) are thought to reside in the basal layer and enable the epidermis to undergo massive expansion during development and wound healing. Given the critical role of EpiSCs in maintaining the epidermis and their potential involvement in the development of squamous and basal cell carcinomas, methods for the isolation and quantitative analysis of human EpiSCs are needed.

Human stem cells and their progeny have previously been studied ex vivo using multiple approaches. For example, human foreskin was transplanted onto nude mice and infected with a lentivirus encoding a fluorescent marker protein, in order to trace the progeny of labeled basal cells. After 28 weeks, labeled columns composed of basal cells and their progeny were observed [1]. Also, organotypic culture systems have been established, using human keratinocytes cultured on dermal equivalents [2, 3]. In this manner, human epidermis could be regenerated for months (>10 weeks) [2, 3]. Another group established a transplantation assay based on repopulation of epidermis inside a rat trachea implanted subcutaneously in a severe combined immunodeficient (SCID) mouse [4, 5].

Keratinocytes implanted into the human dermis or subcutis as a result of surgery or trauma spontaneously form epidermal cysts [6, 7] characterized by a stratified squamous epithelium reminiscent of that on the skin surface and the uppermost part of the hair follicle [8–10]. The experimental production of this type of epithelial structure has been used to study multiple epithelia including epidermis [9, 10], lung [11], and mammary epithelium [12], as well as epidermal differentiation of induced pluripotent stem cells [13].

Putative EpiSC markers in human skin include integrin β1 [14, 15], integrin α6 in combination with CD71 [16, 17], AC133 [18], p63 [19], keratin 15 [20], ABCG2 [21, 22], and delta 1 [23]. Integrin $\alpha6^{hi}CD71^{lo}$ is one of the most used EpiSC-enriched populations in the field of epidermal biology [16, 24].

One strategy to find candidate tissue stem cell markers is to use conserved stem and progenitor cell functions that may be applicable to multiple tissues. Aldehyde dehydrogenase 1 (ALDH1) is a detoxifying enzyme that oxidizes intracellular aldehydes [25]. ALDH oxidizes retinol to retinoic acid and may have a role in early stem cell differentiation [26]. It has been shown that hematopoietic [27, 28] [29], neural [30, 31], and

mammary [32] stem cells have high ALDH1 activity. $CD44^+$ is a marker of normal prostate epithelial stem cells [33] and of cancer stem cells from multiple epithelia [34–36].

In addition to long-term repopulation, self-renewal ability is another key defining stem cell property. In previous studies, evidence for self-renewal has included serial transplantations in vivo [37–39], holoclone formation in vitro [40, 41], and expression of nuclear Bmi-1 (B-lymphoma Moloney murine leukemia virus insertion region-1, an epigenetic regulator known to be involved in the self-renewal of stem cells from multiple tissues) [42–45]. Finally, multipotency [46] and label-retaining ability are features of stem cells [47].

In the study presented here, we quantified the frequency of long-term repopulating cells in the $ALDH^+CD44^+$ population of keratinocytes, previously untested in epidermis. Injection of keratinocytes into murine subcutis was combined with limiting dilution analysis to quantitatively assess the frequency of long-term repopulating EpiSCs in fluorescence activated cell sorting (FACS)-selected populations of human keratinocytes. The $ALDH^+CD44^+$ population showed long-term repopulating ability, self-renewal ability, multipotency, and label retention. This study is a step toward the goal of combining EpiSC markers in a strategic fashion in order to select a nearly pure population of EpiSCs.

Materials and Methods

Animals

Non-obese diabetic SCID (NOD/SCID) mice (Jackson Laboratories, Bar Harbor, ME, http://www.jax.org) were maintained and cared for in accordance with an approved Institutional Animal Care and Use Committee Protocol.

Human Skin Samples

A fresh human adult scalp sample and neonatal foreskins were obtained with the appropriate approvals from the UCSF Committee on Human Research and all studies abided by the rules of the Internal Review Board and the tenets of the Declaration of Helsinki.

Generation of Human Epidermis In Vivo

Human neonatal foreskins were incubated in Dispase (25 U/ml, BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) for 24 hours at 4°C. Epidermis was then peeled from dermis and incubated in 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) at 37°C for 12 minutes. Keratinocytes were collected by centrifugation at 1,000 rpm for 5 minutes. Human neonatal keratinocyte (HNK) suspensions in keratinocyte growth medium (KGM) (Invitrogen) were mixed with Matrigel (BD Biosciences) 1:1(vol/vol) and injected into the subcutis of NOD/SCID mice.

Analysis of Epidermal Regeneration

Xenografts were harvested by punch biopsy (Acuderm, Fort Lauderdale, FL, http://www.acuderm.com). Frozen sections were stained with hematoxylin and eosin and examined for presence (positive sample) or absence (negative sample) of human epidermal repopulation units (ERUs).

Flow Cytometry—Keratinocytes were sorted using a FACSAria (BD) and analyzed with CellQuest software. ALDH$^+$ cells were selected using ALDEFLUOR (Stem Cell Technologies, Vancouver, BC, http://www.stemcell.com) according to the manufacturer's protocol. allophycocyanin (APC) conjugated CD44 (IM7, #17-0441, 0.6 μg/ml) was from eBioscience, San Diego, CA, http://ebioscience.-com. FITC-conjugated integrin α6 (GoH3, #555735, 1:5), APC-conjugated CD71 (M-A712, #551374, 1:5) and isotype controls were from BD Pharmingen (San Diego, http://www.bdbiosciences.com/index[lowen]us.shtml).

Assessment of Human Versus Murine Tissue Origin—Hoechst 33258 dye (Invitrogen) was used to confirm the human derivation of the xenograft ERUs. Human nuclei can be distinguished from murine nuclei, as the latter have multiple hyper-chromatic chromocenters, resulting in a characteristic punctate pattern of fluorescence in contrast to the homogeneous staining of human nuclei [48, 49]. In addition, fluorescent in situ hybridization (FISH) was performed using a CY3-day-UTP-labeled human lymphocyte-derived genomic DNA probe [46] or a Spectrum-Orange-labeled Human Y chromosome-specific DNA probe (provided by H.U. Weier, Berkeley, CA) [50, 51].

Assessing the Single Cell Origin of ERUs with Cell Tracker Nontoxic Carbocyanine Dyes, Vybrant DiI and DiO—Freshly isolated, unsorted HNKs were labeled with either Vybrant DiI (565 nm) or Vybrant DiO (501 nm) (Invitrogen) (following the manufacturer's protocol) and then mixed 1:1. Either 6,250 or 1,562 cells from the mixture were transplanted subcutaneously, harvested after 1 week (five experiments, 767 ERUs studied), and frozen sections were examined under an Axio Plan Z fluorescence microscope (Carl Zeiss Inc., Thornwood, NY, http://www.zeiss.de/micro.com).

Immunostaining—For immunofluorescence staining, frozen sections were fixed with acetone and incubated with primary antibodies including cytokeratin 14 (rabbit polyclonal, ab15461, 1:1000), involucrin (SY5, mouse monoclonal, ab68, 1:1000), filaggrin (SPM181, mouse monoclonal, ab17808, 1:300), laminin (LT3, Rat monoclonal, ab3003, 1:10), Bmi-1 (rabbit polyclonal, ab38295, 1:100), CD44 (F10-44-2, mouse monoclonal, ab6124, 1:100) (all from Abcam, Cambridge, MA, http://www.abcam.com), and ALDH1A1 (rabbit polyclonal, HPA002123, 1:1,000, Sigma), then secondary antibodies including Alexa Fluor 488 IgG and Alexa Fluor 594 IgG (Invitrogen). Propidium iodide or 4′,6-diamidino-2-phenylindole was used to counterstain nuclei.

For immunoperoxidase staining, formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated. Slides were incubated with primary antibodies including cytokeratin 14, involucrin, and filaggrin (see above), then secondary antibodies including biotinylated anti-rabbit IgG and anti-mouse IgG (all from Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). Immunolabeled structures were visualized with ABC reagent and ImmPACT NovaRED Peroxidase Substrate (Vector Laboratories). Hematoxylin was used as a counterstain.

For alkaline phosphatase staining, formalin-fixed, paraffin-embedded sections were incubated with primary antibodies including cytokeratin 17 (rabbit polyclonal, ab53707, 1:100, Abcam) and cytokeratin 6 (EPR1603Y, rabbit monoclonal, ab52620, 1:500, Abcam).

The UltraVision Quanto detection system and Permanent Fast Red Quanto substrate system (ThermoFisher Scientific, Fremont, CA, http://www.thermoscientific.comG) were used to detect signal. Hematoxylin was used as a counterstain.

Serial Transplantation—$CD44^+ALDH^+$ or $CD44^-ALDH^-$ keratinocytes were each mixed with 5×10^6 cultured human neonatal fibroblasts and transplanted into recipient NOD/SCID mice. Experiments 1–3 used 700/10,000, 1,000/10,000, and 350/4,000 $CD44^+ALDH^+/CD44^-ALDH^-$ keratinocytes, respectively. Grafts were harvested 9 weeks later. A cell suspension was again generated using 300 collagen digestion U/ml of Collagenase, 370 U/ml of Hyaluronidase, and 100 Kunitz U/ml DNase I (Sigma, St. Louis, MO) in Hank's solution (Cambrex, Walkersville, MD, http://www.cambrex.com) with 10% fetal bovine serum (Invitrogen) for 30 minutes at 37°C. Keratinocytes were then transplanted into a second NOD/SCID recipient for 6–9 weeks and presence or absence of human ERUs was determined by histology.

Holoclone Assay

Single FACS-sorted $CD44^+ALDH^+$, $CD44^-ALDH^-$, or unfractionated (UNF) human keratinocytes were inoculated onto 96-well plates containing Mitomycin C-treated 3T3 cells maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. At 10–12 days, primary colonies were photographed. Each colony was then removed by trypsinization, and cells were transferred to 100 mm dishes with Mitomycin C treated 3T3 cells at 80%–90% confluence. After 12–14 days, secondary colonies were fixed and stained with toluidine blue (Sigma). Each plate was photographed and the identity of the cell that founded each primary colony was determined [holoclone, meroclone, or paraclone based on previously described methods [40]].

Multipotency

$CD44^+ALDH^+$ (2,600–6,000), $CD44^-ALDH^-$ (25,000), or UNF (25,000) HNKs were mixed with freshly sorted green fluorescent protein (GFP+) mouse dermal papilla cells (400 K–1.1 M) and Matrigel (50 μl) and injected subcutaneously into NOD/SCID mice. Biopsies were harvested at 17–21 days and sections were stained with H&E.

Iododeoxyuridine (IdU) Incorporation Studies Using Organotypic Cultures

AlloDerm regenerative tissue matrix (LifeCell Corp., Branchburg, NJ) was placed in 0.4 cm diameter cell culture inserts (Millipore Corp, Billerica, MA, http://www.millipore.com). Human fibroblasts were first inoculated onto AlloDerm (60,000 cells per m^2) for 24 hours [3]. Human keratinocytes were then inoculated at 500,000 cells per cm^2 [3]. Cultures were maintained submerged in 0.07 mM calcium CnT-57 growth medium (ZenBio, Inc., Research Triangle Park, NC) for 8 days and then raised to the air–liquid interface in 1.2 mM calcium CnT-02-3DP1 (ZenBio, Inc.) with 200 Units/ml aprotinin [3]. Cultures were pulse labeled with IdU (1 μM) every 12 hours for 2 weeks. At the end of a 4-week chase, cultures were harvested and incubated in 25 Units/ml Dispase at 37°C for 2 hours. Live $CD44^+ALDH^+$ cells were then FACS-sorted and inoculated onto microscope slides. IdU was detected with 5-bromo-2'-deoxyuridine (BrdU)/IdU antibody (B44, #347580, 1:10, BD Biosciences).

Colony Formation In Vitro

FACS-sorted cells were plated at 1,000 cells per well in uncoated six-well plates (clonal density) and cultured for 3–4 weeks in either embryonic stem cell medium, ESC-Sure (Applied StemCell, Sunnyvale, CA, http://www.appliedstemcell.com), or KGM (Invitrogen). Plates were stained with toluidine blue.

Statistical Analysis

L-Calc (Stemsoft, Vancouver, BC, http://stemsoft.com) was used to determine the frequency of ERUs in the selected cell populations. Limiting dilution analysis was subjected to a χ^2 test. Lack of a significant p value in the χ^2 test was used to demonstrate internal consistency in the distribution of results. Stem cell frequencies from limiting dilution experiments at different weeks (1, 2, 4, 6, 9, and 12) were compared using standard "single-hit" Poisson models for limiting dilution experiments [52]. Results for these analyses were obtained using R statistical software, version 2.9.0 (R Development Core Team, 2009). For comparison of the number of $CD44^+ALDH^+$ versus integrin $\alpha6^{hi}CD71^{lo}$ versus UNF HNKs with nuclear Bmi-1 expression, a one-way ANOVA was used. For comparison of the percent holoclones in $CD44^+ALDH^+$ versus UNF populations and the number of hair follicle-like structures produced by $CD44^+ALDH^+$ versus $CD44^-ALDH^-$ populations, a paired Student's t test was used. In vitro colony forming ability of the five different subpopulations was analyzed using the Kruksal Wallis test, with post hoc pair-wise comparisons using the Bonferroni Dunn test.

Results

Generation of Epidermis/ERUs in a Xenograft model, by Injection of Human Keratinocytes

Freshly obtained HNKs transplanted into NOD/SCID subcutis formed human keratinizing epidermis (Fig. 1A; supporting information Fig. 1), as seen in previous studies [9, 10]. Epidermal cysts generated in this fashion were termed ERUs pursuant to longstanding terminology in hematopoiesis [53]. As in normal human epidermis, keratin 14 antibody immunostained the basal layers of ERUs [54, 55] (Fig. 1B), involucrin antibody stained all suprabasal epidermal layers [56, 57] (Fig. 1C), and filaggrin antibody stained the uppermost epidermal layers [56] (Fig. 1D). Immunofluorescence with FITC-conjugated laminin antibody produced a linear pattern at the basement membrane [58] (Fig. 1E). These findings confirm the production of a differentiated keratinizing epidermis, as seen previously by others [8–10].

To determine whether ERUs originate from single cells, freshly isolated HNKs were labeled with Vybrant DiI (red) or Vybrant DiO (green). DiI and DiO labeled HNKs were mixed in equal numbers (1:1) and then transplanted into NOD/SCID mice. Using doses of 1,562 or 6,250 cells (five separate experiments), 766 out of a total of 767 ERUs were found to be either red or green but not mixed (Fig. 1F), indicating that at these doses ERUs almost always derive from a single cell, rather than resulting from cell aggregation. The lipid staining dyes are diluted as cells multiply and because of this ERU formation was assessed

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at 2 weeks, when label was still visible. Because of this, we cannot be sure that on occasion some cysts do not merge at later time points, due to their close proximity.

The human origin of ERU keratinocytes was confirmed using Hoechst 33258 staining [48, 49]. Nuclei in the human ERUs (Fig. 1G, left panel) showed the expected homogenous nuclear staining with Hoechst 33258 (Fig. 1G, middle panel), while murine nuclei displayed the characteristic punctate staining of hyperchromatic chromocenters (Fig. 1G, right panel). Additionally, FISH with a CY3-day-UTP-labeled human lymphocyte derived genomic DNA probe also stained the ERUs (Fig. 1H, middle panel) but not murine epidermis (Fig. 1H, right panel), confirming their human derivation.

In summary, H&E, keratin 14, involucrin, filaggrin, and laminin staining confirmed epidermal differentiation reminiscent of that in vivo, while Hoechst 33258 and FISH confirmed the human derivation of ERUs in this model. Additionally, DiI and DiO staining of keratinocytes revealed a single cell origin of most ERUs at the doses examined.

Selection of the Endpoint at Which Only EpiSCs Originally Injected (and Their Progeny) Persist

In order to study stem cell frequency quantitatively using long-term repopulation assays it is necessary to determine the time point at which ERUs can be identified as arising from long-term repopulating stem cells versus short-term repopulating transit amplifying cells (TACs) [37, 53, 59, 60]. At the time point at which short-term proliferating TAC-derived ERUs have exhausted their replicative ability and no longer exist, the frequency of ERUs stops decreasing and thereafter remains constant [37, 53, 59–61], allowing the assessment of EpiSCs originally injected and their progeny. For HNKs, the ERU frequency at weeks 1, 2, 4, 6, 9, and 12 was determined by limiting dilution analysis (Fig. 1I). It can be seen that the ERU frequency decreases from 1 in 96 keratinocytes at 1 week to 1 in 792 keratinocytes at 2 weeks and then to 1 in 1,939 keratinocytes by 6 weeks, following which the frequency appears to remain stable. The overall likelihood ratio test for differences in stem cell frequencies between weeks 1 and 12 was significant ($p < .001$). Likelihood ratio tests for adequacy of the single-hit Poisson model were nonsignificant for all analyses, indicating an acceptable fit to the data. Inspection of the week-specific results indicated that observed differences in frequencies were due primarily to changes occurring in the first 4 weeks, with relatively stable results in weeks 6–12. This observation was confirmed by a separate likelihood ratio test for differences between results in weeks 6–12, which was not significant ($p = .95$). A similar analysis limited to weeks 9–12 also yielded nonsignificant results ($p = .99$). The relatively large p-values obtained in analyses limited to later time points, coupled with the relatively small differences in observed frequencies and large overlap in 95% confidence intervals for these time points supported the use of 9 weeks as the point after which ERU frequency remains stable. We therefore used 9 weeks in subsequent experiments as the endpoint at which to study long-term repopulating EpiSCs.

The CD44$^+$ALDH$^+$ and Integrin α6^{hi}CD71^{lo} Populations of Human Keratinocytes Are Enriched for Long-Term Repopulating EpiSCs

To quantify long-term repopulating EpiSCs, putative human EpiSCs were selected by FACS, transplanted into the subcutis of NOD/SCID mice, and limiting dilution analysis of ERU frequency was performed at week 9. ALDH$^+$ cells comprised 1.6%–13.5% of total HNKs and CD44$^+$ALDH$^+$ cells comprised 0.03%–0.2% of total HNKs (Fig. 2A). Integrin α6^{hi}CD71 ^{lo} cells (7%–10% of total, Fig. 2B) were selected using published methods [16, 24].

In neonatal human foreskin epidermis, immunohistochemistry for ALDH1 and CD44 each showed staining in the basal layers and occasional costained cells were detected (Fig. 2C). In adult epidermis, the coexpression of CD44 and ALDH was found in the depths of the rete ridges (deepest part of rete peg) (Fig. 2D). In the adult hair follicle, the bulge region and the opening of the sebaceous gland showed coexpression of CD44 and ALDH (Fig. 2E). 82% ± 2% of CD44$^+$ALDH$^+$ cells were keratin 14+, evidence that CD44$^+$ALDH$^+$ cells are mostly basal cells (supporting information Fig. 2).

The frequency of long-term repopulating cells was 1 in 586 cells (SE 783-438) in the ALDH$^+$ population versus 1 in 69,918 (SE 283,641-17,235) in the ALDH$^-$ population (not shown), 1 in 170 cells (SE 240-120) in the CD44$^+$ALDH$^+$ population versus 1 in 218,510 (SE 591,130-80,770) in the CD44$^-$ALDH$^-$ population, and 1 in 390 cells (SE 520-290) in the integrin α6^{hi}CD71^{lo} population versus 1 in 24,030 (SE 36,860-15,660) in the integrin α6^{lo}CD71^{hi} population, while the frequency of long-term repopulating cells in the UNF population was 2,160 (SE 2,610-1,790) (Fig. 3A). When compared to UNF keratinocytes, ERUs were enriched 3.7-fold in the ALDH$^+$ population (not shown), 12.6-fold in the CD44$^+$ALDH$^+$ population, and 5.6-fold in the integrin α6^{hi}CD71 ^{lo} population (Fig. 3A, 3B). Thus there was significant enrichment for EpiSCs in both the CD44$^+$ALDH$^+$ (1 in 170) and integrin α6^{hi}CD71^{lo} populations (1 in 390) over UNF. However, the enrichment for CD44$^+$ALDH$^+$ versus integrin α6^{hi}CD71^{lo} was not significantly different (p = .06).

CD44$^+$ALDH$^+$ cells produced ERUs with a normal pattern of epidermal differentiation (Fig. 3C) and CD44 and ALDH were both present in the basal layer of ERUs (Fig. 3D).

To further investigate the longevity of these cells using an alternative methodology [60, 61], 5,000 CD44$^+$ALDH$^+$ or 50,000 UNF HNKs, mixed with one million human neonatal fibroblasts were implanted in chambers on the backs of NOD/SCID mice. Transplants from four out of four CD44$^+$ALDH$^+$ samples and three out of four UNF samples showed a fully differentiated epidermis at 9 weeks (Fig. 3E). Staining for laminin, keratin 14, and filaggrin showed a fully differentiated epidermis. Epidermis from CD44$^+$ALDH$^+$ cells was indistinguishable from that from UNF cells by H&E.

The CD44$^+$ALDH$^+$ Population of Keratinocytes Exhibits Self-Renewal Ability In Vivo and In Vitro

Bmi-1 is involved in the self-renewal of stem cells from multiple tissues [42–45]. CD44$^+$ALDH$^+$ HNKs and integrin α6^{hi}CD71^{lo} HNKs contained significantly greater

numbers of cells expressing nuclear Bmi-1 (36.1% and 35.8%, respectively) versus UNF HNKs (12.7%, $p = .004$ and 0.024, respectively, Fig. 4A).

To study self-renewal in vivo, $CD44^+ALDH^+$ keratinocytes mixed with 5×10^6 cultured human neonatal fibroblasts or $CD44^-ALDH^-$ keratinocytes mixed with 5×10^6 cultured human neonatal fibroblasts were injected into recipient NOD/SCID mice. Experiments 1–3 used 700/10,000, 1,000/10,000, and 350/4,000 $CD44^+ALDH^+/CD44^-ALDH^-$ keratinocytes (dependent on the quantity of tissue available). Grafts were harvested 9 weeks later. A cell suspension was again generated from the harvested graft, transplanted into a second NOD/SCID recipient, and the presence or absence of human epidermal ERUs was determined by histology. Secondary transplants derived from human $CD44^+ALDH^+$ keratinocytes once again regenerated ERUs, providing evidence for self-renewal capacity of the $CD44^+ALDH^+$ keratinocytes (Fig. 4B). ERUs were not seen in any of the $CD44^-ALDH^-$transplants. It was noted that, unlike in the primary transplants (Fig. 3C), ERUs in secondary transplants were clusters of basaloid cells lacking stratification (Fig. 4B), suggestive of undifferentiated keratinocytes. Finally, because more than one cell was transplanted in the experiments above, we cannot rule out the possibility that the primary and secondary ERUs were derived from distinct $CD44^+ALDH^+$ cells.

Holoclone formation in vitro was studied as further evidence for self-renewal ability. For each sample, 108–288 single $CD44^+ALDH^+$, $CD44^-ALDH^-$, or UNF cells were plated, one cell per well, in 96-well plates. After 10–13 days, colonies were counted and secondarily plated. No colonies were produced from over 160 $CD44^-ALDH^-$ individually plated cells (three independent experiments). After 13–16 days, secondary colonies were stained and assessed for large smooth colonies with central differentiation (holoclones) using the classification of Barrandon and Green [40] (Fig. 4C). The number of holoclones in the $CD44^+ALDH^+$ population of keratinocytes was twice that in the UNF population; 1.4% ± 0.46% vs. 0.7% ± 0.35%, $n = 3$, $p = .01$, (Fig. 4D).

The $CD44^+ALDH^+$ Population of Keratinocytes Is Multipotent

To determine whether $CD44^+ALDH^+$ HNKs are multipotent, HNKs were implanted with trichogenic murine dermal cells, using previously described methods to generate chimeric hair follicles from human keratinocytes [46, 62]. Doses of 2,500–25,000 $CD44^+ALDH^+$ or $CD44^-ALDH^-$ HNKs were implanted with 7×10^5 GFP+ dermal papilla cells and grafts were studied for the formation of chimeric hair follicles at 18–21 days (four independent experiments). 100,000–700,000 dermal papilla cells alone did not produce hair follicle-like structures ($n = 3$). $CD44^+ALDH^+$ cells produced significantly greater numbers of hair follicle-like structures than did $CD44^-ALDH^-$ cells (9.0 ± 2.1 vs.1.9 + 0.9 per 25,000 HNKs injected, $p = .007$; Fig. 5A). CD44 and ALDH staining was present in a location consistent with regeneration of a bulge region (Fig. 5B). H&E, keratin 6, and keratin 17 staining (Fig. 5C–5E) confirmed the hair follicle-like nature of the structures observed, and FISH using a human Y chromosome-specific Spectrum Orange-labeled DNA-probe confirmed the presence of human cells in the follicle-like structures (Fig. 5F).

The CD44$^+$ALDH$^+$ Population of Keratinocytes Contains Label-Retaining Cells

Stem cells are expected to cycle slowly and therefore retain label. Organotypic cultures were prepared using 500,000 CD44$^+$ALDH$^+$ HNKs per cm^2, as previously described [3]. After 2 weeks, cultures were pulse-labeled with IdU every 12 hours for 2 further weeks. After the 2-week pulse, IdU-treated cultures showed almost 100% IdU$^+$ basal cells (Fig. 6A). After a 4-week chase, the cultures were harvested, a cell suspension was isolated, and CD44$^+$ALDH$^+$ keratinocytes were FACS-sorted onto microscope slides for immunofluorescence studies. 58% ± 7% of the CD44$^+$ALDH$^+$ keratinocytes were IdU-expressing label-retaining cells at 4 weeks of chase (n = 3, Fig. 6B).

The CD44$^+$ALDH$^+$ Population of Keratinocytes Possesses Enhanced In Vitro Colony Forming Ability

To test CD44$^+$ALDH$^+$ keratinocytes for their growth properties in vitro, HNK subpopulations were plated at 1,000 cells per well (six-well plates) and cultivated for 21–28 days in either KGM (Invitrogen) or embryonic stem cell media (ESC-Sure, Applied StemCell).

In KGM, there was a significant difference in the number of colonies produced in the five groups (CD44$^+$ALDH$^+$, CD44$^-$ALDH$^-$, integrin α6^{hi}CD71^{lo}, integrin α6^{lo}CD71^{hi}, and UNF, p = .002). CD44$^+$ALDH$^+$ HNKs formed colonies whereas CD44$^-$ALDH$^-$ HNKs did not and colony forming ability of CD44$^+$ALDH$^+$ HNKs was significantly greater than CD44$^-$ALDH$^-$ HNKs (3.4 ± 0.7 vs. 0 ± 0, p < .05), using a post hoc pairwise comparison (Bonferroni-Dunn) (Fig. 7A left panel; supporting information Fig. 3). Colony forming ability of integrin α6^{hi}CD71^{lo} HNKs was also significantly greater than integrin α6^{lo}CD71^{hi} HNKs (5.8 ± 2.8 vs. 0.01 ± 0.01, p < .05 Fig. 7B left panel). No significant difference was detected in the colony forming ability of CD44$^+$ALDH$^+$ versus integrin α6^{hi}CD71^{lo} HNKs (n = 4, Fig. 7).

We then tested whether these cells would grow in a medium designed to support the growth of primitive stem cells. In embryonic stem cell medium, there was a significant difference in the five groups (CD44$^+$ALDH$^+$, CD44$^-$ALDH$^-$, integrin α6^{hi}CD71^{lo}, integrin α6^{lo}CD71^{hi}, and UNF, p = .003) and colony forming ability of CD44$^+$ALDH$^+$ HNKs was significantly greater than CD44$^-$ALDH$^-$ HNKs (3.5 ± 1.2 vs. 0.01 ± 0.01, p = <.05) and α6^{hi}CD71^{lo} HNKs (3.5 ± 1.2 vs. 0.0 ± 0, p = <.01) using a post hoc pairwise comparison (Bonferroni-Dunn) Fig. 7A). While neither integrin α6^{hi}CD71^{lo} nor α6^{hi}CD71^{hi} HNKs (putative EpiSCs and TACs, respectively [16, 63] grew in embryonic stem cell medium, integrin α6^{lo}CD71^{hi} HNKs did, although a statistically significant difference was not detected between α6^{hi}CD71^{lo} and integrin α6^{lo}CD71^{hi} HNKs (0 ± 0 vs. 0.39 ± 0.18) (Fig. 7B).

Discussion

The assay presented here provides a quantitative assessment of the ability of putative stem cell markers to enrich for cells capable of long-term regeneration of the epidermis. This study quantitatively demonstrates a 12.6-fold enrichment for EpiSCs in CD44$^+$ALDH$^+$ HNKs, previously untested in epidermis. While the CD44$^+$ALDH$^+$ population is enriched so

that 1 in 170 cells is an EpiSC, the ultimate goal is to obtain a nearly pure population of EpiSCs, as has been accomplished in hematopoiesis. Isolation of a nearly pure population of EpiSCs is of high importance for regenerative medicine applications, targeting EpiSCs for genetic manipulations and for more basic studies of EpiSC regulation and differentiation.

In these studies, $CD44^{+}ALDH^{+}$ and integrin $\alpha6^{hi}CD71^{lo}$ human keratinocytes regenerated epidermis for the long-term when injected subcutaneously into NOD/SCID mice. It has been demonstrated that skin, corneal, and esophageal epithelial each maintained their distinctive in vivo phenotype when injected subcutaneously and reacquire their distinctive in vivo keratin patterns [9]. In cell culture, using fibroblast support, this was not the case indicating that these differences do not depend on specific mesenchymal instruction but on permissive factors present in vivo but not in vitro [9]. Injected keratinocytes reacquired a normal ultrastructural appearance with keratohyalin granules, lamellar bodies, and enucleated horny cells [10] and expressed bullous pemphigoid antigen and laminin at the epithelial–stromal interface [64]. Our studies showed normal expression of keratin 14, involucrin, fillagrin, and laminin.

The absolute frequency of repopulating cells detected in this assay is almost certainly affected by the methods. FACS places significant stress on cells and engagement of cell surface receptors may modulate cell function and behavior. However, the assay provides a quantitative comparison of the relative frequency of cells capable of long-term repopulation present in different keratinocyte populations. Consistent with the studies done using the subcutaneous transplantation assay, longevity studies using keratinocytes transplanted with neonatal human fibroblasts onto the backs of NOD/SCID mice also showed that $CD44^{+}ALDH^{+}$ keratinocytes produced epidermis for the long-term.

We performed FACS analysis to determine whether the $CD44^{+}ALDH^{+}$ and integrin $\alpha6^{hi}CD71^{lo}$ populations of keratinocytes were the same population. We found that only 1.1%–2.3% of integrin $\alpha6^{hi}CD71^{lo}$ keratinocytes were $ALDH^{+}$, while there was no overlap with the $CD44^{+}ALDH^{+}$ keratinocytes. This is interesting in view of the in vitro findings where $CD44^{+}ALDH^{+}$ but not integrin $\alpha6^{hi}CD71^{lo}$ keratinocytes were able to grow in embryonic stem cell medium. Thus, both the FACS analysis and the growth in embryonic stem cell medium studies suggest two distinct populations of progenitors.

While in neonatal foreskin $CD44^{+}ALDH^{+}$ cells were found along the entire basal layer, in adult epidermis $CD44^{+}ALDH^{+}$ cells were found in known stem cell niches, including both the bulge [65] and sebaceous gland [66]. $CD44^{+}ALDH^{+}$ interfollicular cells were predominantly in the depths of the rete ridges (tips of rete pegs). This location is similar to that of $K15^{+}$ integrin $\alpha6^{hi}$ cells [17] and desmoglein-3^{dim} cells [67] but different from the location of integrin $\beta1^{hi}$ [15] and delta 1^{+} cells [23] that are located in the shallowest parts of the rete ridges (suprapapillary plate). The reason for these different findings is not clear but may possibly involve previously described methodological issues [17].

$CD44^{+}ALDH^{+}$ HNKs produced ERUs when serially transplanted. ERUs in secondary transplants were clusters of basaloid cells lacking stratification. This may reflect the primitive nature of the cell that has the capability for serial transplantation.

$CD44^+ALDH^+$ cells displayed enhanced multipotency versus $CD44^-ALDH^-$ cells. However, while hairs did not grow from dermal papilla cells injected alone, some hair-like structures did grow from $CD44^-ALDH^-$ cells injected in combination with dermal papilla cells. This is may be due to the fact that there are groups of progenitor cells distinct from the $CD44^+ALDH^+$ cells (e.g., integrin $\alpha6^{hi}CD71^{lo}$) that are also capable of hair production. Also, it is possible that in this assay, progenitor cells that are not as primitive as stem cells may produce hair-like structures, as is the case in hematopoesis where multipotent progenitors can be either longterm repopulating cells or less primitive progenitors [68, 69].

Holoclone formation was increased 2-fold in $CD44^+ALDH^+$ over UNF HNKs, whereas, in vivo, long-term repopulating cells were enriched 12-fold. This apparent discrepancy may be explained by evidence that primitive stem cells do not have clonal capacity in vitro. Hematopoietic stem cells with long-term bone marrow repopulating capacity have been shown to have minimal to no clonal capacity in vitro [70, 71], and there is prior evidence that stem cell-rich fractions exhibit equal or substantially lower colony forming efficiency than the source total population in human neonatal foreskin [16], human limbal epithelium, [72] and rat epidermis [73].

A previous study found that integrin $\alpha6^{lo}$ keratinocytes (not stem cells) had poor regenerative capacity in vitro (over 14 days) but could produce an in vivo epidermis in rat trachea (over 6 weeks) [2]. In the studies presented here, likewise, integrin $\alpha6^{lo}$ ($CD71^{hi}$) keratinocytes did not grow in KGM but had a small number of long-term repopulating cells and did grow in embryonic stem cell medium. Altogether, the above studies suggest that integrin $\alpha6^{lo}$ keratinocytes contain a small population of primitive cells. In addition, in the studies presented here, integrin $\alpha6^{hi}CD71^{lo}$ keratinocytes grew only in KGM, not embryonic stem cell medium, but contained long-term repopulating cells. Thus some long-term repopulating cells appear to grow preferentially in KGM and some in embryonic stem cell medium, suggesting that not all long-term repopulating cells behave similarly in vitro.

While classically the epidermis has been thought to be sustained by a EpiSC/TAC hierarchy of progenitors, some observations contradict the EpiSC/TAC model [for review see [74, 75]]. Using inducible genetic labeling, clone-size distributions were consistent with a model of homeostasis involving only one type of progenitor cell [76]. Thus in the study presented here, the loss of repopulating units over time may be wrongly attributed to the loss of TAC-derived units and could conceivably represent either the disappearance of multiple tiers of progenitors (as in other tissues) or the chance loss of ERUs from a single type of epithelial progenitor.

While the properties of the integrin $\alpha6^{hi}CD71^{lo}$ keratinocyte population have been well documented, here we evaluated stem cell properties of the $CD44^+ALDH^+$ keratinocytes, previously untested as EpiSC markers. In future studies there is a need to systematically quantitate the ability of individual potential stem cell markers to select for cells with the functional property of long-term tissue regeneration, with the goal of combining markers to select a nearly pure population of EpiSCs. Different progenitor populations can then be correlated with specific phenotypes and in vitro growth behaviors.

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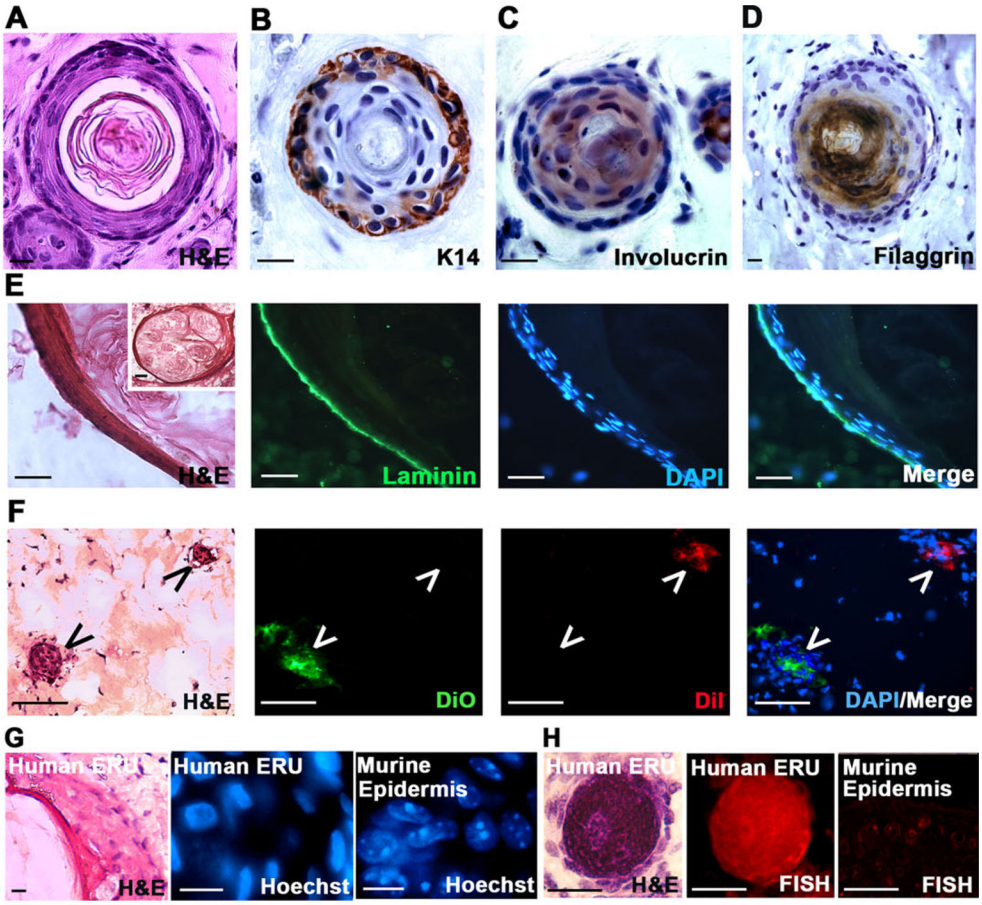

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Duration of repopulation / Cell dose	1 week	2 weeks	4 weeks	6 weeks	9 weeks	12 weeks
	Response ratio: Positive samples / total number of injections					
100,000					1/1	1/1
25,000			2/2	6/6	5/5	2/2
6,250			3/3	7/7	10/11	10/12
1,562	9/9	2/3	2/4	4/10	6/10	9/17
391	11/12	3/5	3/6	3/10	1/7	6/15
96	6/9					
24	5/10					
6	0/6					
ERU Frequency	1 in **96**	1 in **792**	1 in **1,196**	1 in **1,939**	1 in **2,191**	1 in **2,149**
SE[a]	54-173	474-1,324	752-1,903	1,415-2,656	1,632-2,942	1,683-2,743

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

Figure 1.

ERUs in the xenograft model show epidermal differentiation and human derivation. (**A**): H&E staining of a human ERU, produced by injecting human neonatal keratinocyte (HNKs) into murine subcutis (9 weeks) and showing keratinizing epidermis. (**B**): Keratin 14 is present in the basal layers of human ERUs. (**C**): Involucrin is present in the suprabasal layers. (**D**): Filaggrin is present in the uppermost layers of the epidermis. Appropriate positive and negative controls were performed (supporting information Fig. 1). (**E**): Linear pattern of fluorescence of laminin expression along the basement membrane of the ERU

(Inset, lower power view of ERU). (**F**): To determine whether ERUs from implanted HNKs were derived from single cells, HNKs were labeled with Vybrant DiI or Vybrant DiO and mixed in a 1:1 ratio before injection into nonobese diabetic severe combined immunodeficient mice. Resultant ERUs were either green or red. DAPI was used to counter stain nuclei. (**G**): Hoechst 33258 staining confirmed that characteristic homogeneous staining was seen in human ERU nuclei (middle panel). In contrast the characteristic bright punctate pattern of hyperchromatic chromocenters was seen in the murine epidermal nuclei (right panel). (**H**): FISH against human genomic DNA labels the human ERU (middle panel) but not murine epidermis (right panel). (**I**): The frequency of cells capable of forming ERUs at weeks 1, 2, 4, 6, 9, and 12 was determined by limiting dilution analysis. At 9 weeks the frequency of ERUs stops decreasing significantly and thereafter remains constant. (A, B, C, D, G) scale bars = 10 μm. (E, F, H) scale bars = 50 μm. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; ERU, epidermal repopulating units; FISH, fluorescent in situ hybridization.

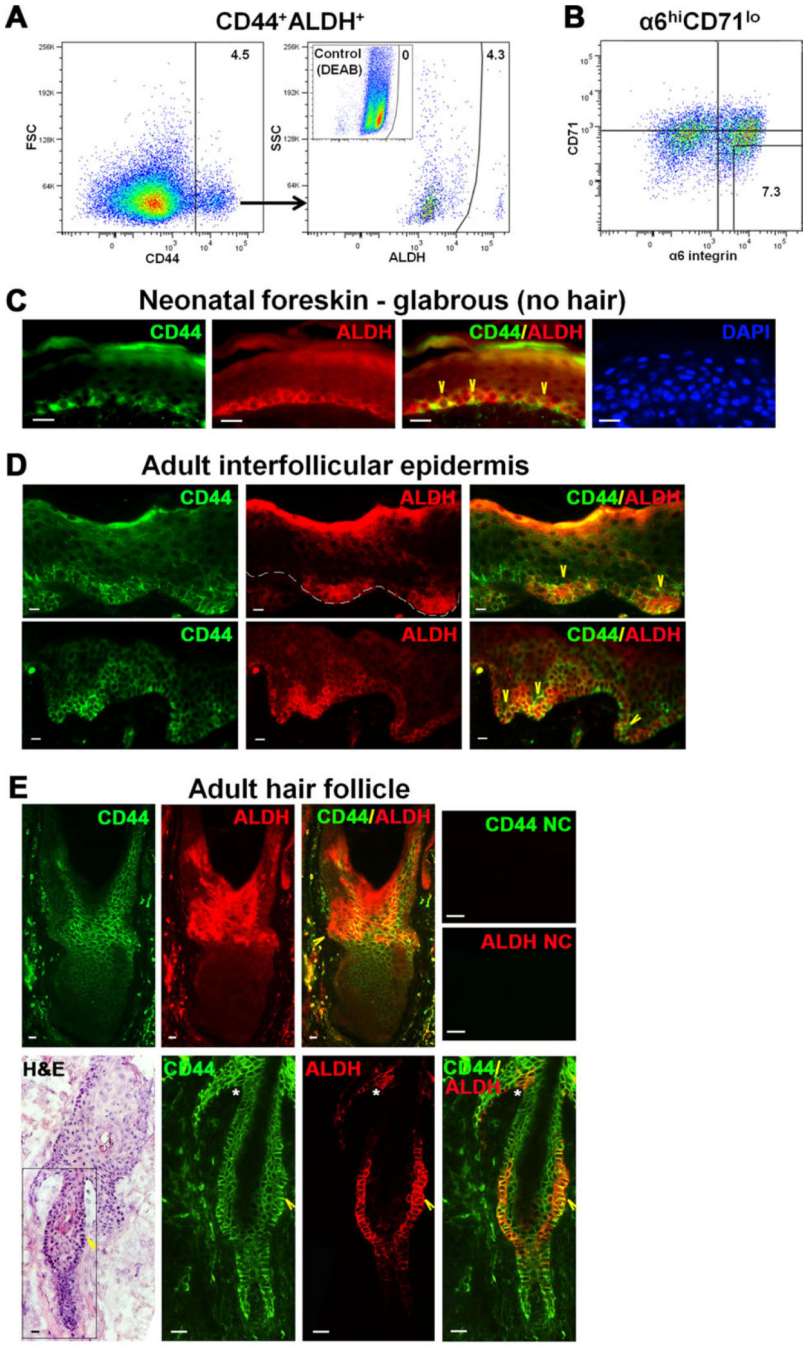

Figure 2.

Fluorescence-activated cell sorting (FACS) isolation and immunohistochemistry of $CD44^{+}ALDH^{+}$ keratinocytes. (**A**): FACS isolation of $CD44^{+}ALDH^{+}$ keratinocytes. DEAB, an ALDH inhibitor, was used as a negative control. CD44 isotype control performed but not shown. (**B**): FACS isolation of 7%–10% of integrin $\alpha6^{hi}CD71^{lo}$ keratinocytes. Isolation performed as previously described [20]. (isotype controls performed but not shown). (**C**): In neonatal human foreskin epidermis, immunohistochemistry for ALDH1 showed staining in the basal layers. CD44 staining was also located in the basal layers. There were occasional

double positive cells in the basal layer. (**D**): In adult epidermis, cells coexpressing CD44 and ALDH were found in the depths of the rete ridges (tips of the rete pegs) (arrowheads). (**E**): In the adult hair follicle (facial skin) cells that coexpressed CD44 and ALDH were located in the bulge region (arrowheads) and at the opening of the sebaceous gland (asterisk). Negative controls were performed with no primary antibody. Scale bars = 10 μm. Abbreviation: ALDH, aldehyde dehydrogenase.

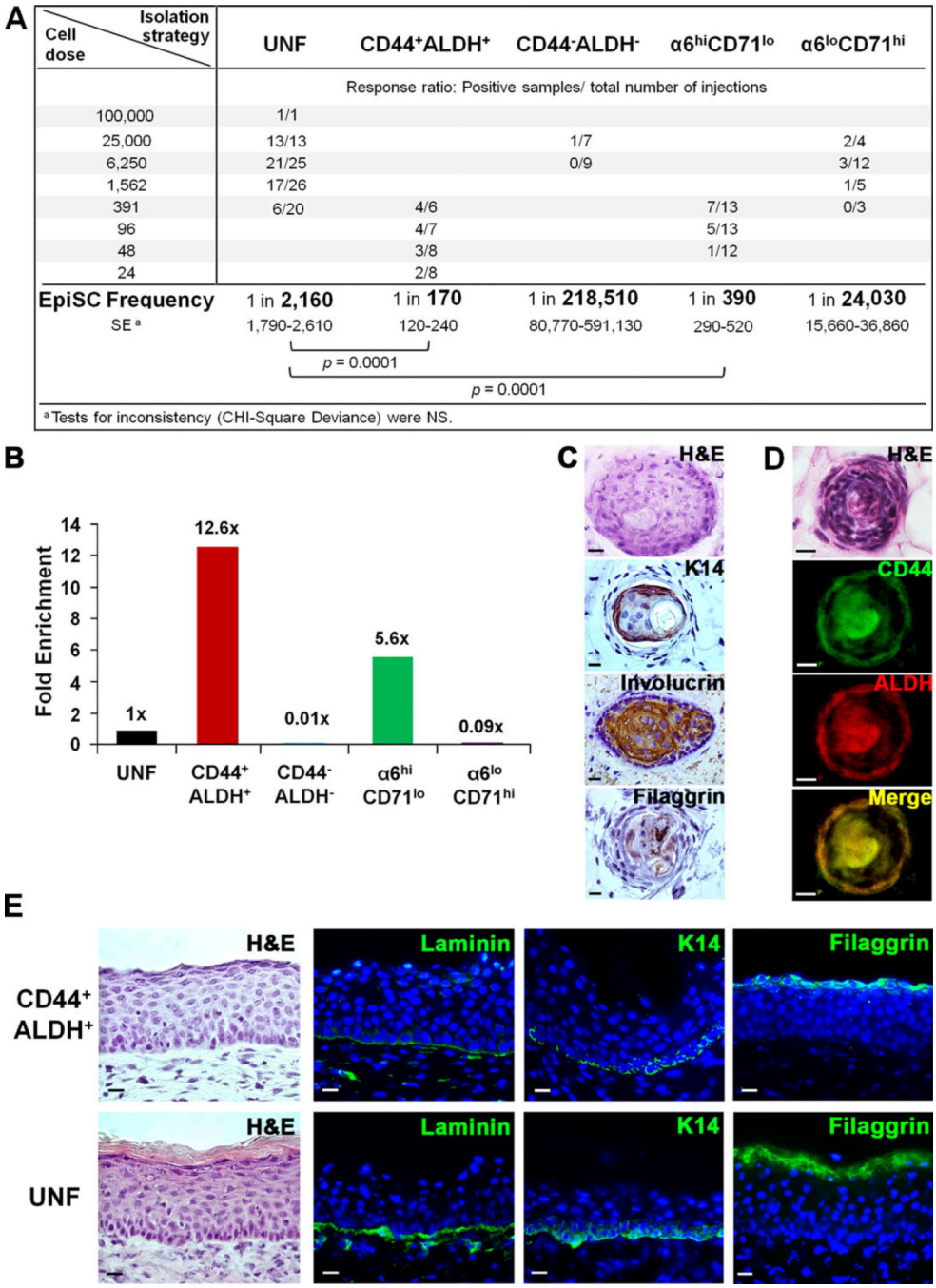

Figure 3.

The CD44^{+}ALDH^{+} and integrin α6^{hi}CD71^{lo} populations of keratinocytes are enriched in cells with long-term repopulating ability. (**A**): Limiting dilution analysis of EpiSC frequency in selected keratinocyte populations at 9 weeks. There is a significant increase in EpiSC frequency in the CD44^{+}ALDH^{+} and integrin α6^{hi}CD71^{lo} populations versus the UNF population. For each cell dose the number of positive responses (injection sites with at least one ERU) over the total number of injection sites is the response ratio. (**B**): Histogram showing the fold enrichment of EpiSCs in selected populations over the UNF HNK

population. The $CD44^{+}ALDH^{+}$ and integrin $\alpha6^{hi}CD71^{lo}$ populations are enriched for long-term repopulating EpiSCs over UNF cells. (**C**): Epidermal repopulating units (ERUs) produced by injection of $CD44^{+}ALDH^{+}$ keratinocytes exhibited a fully differentiated epidermis. Keratin 14 was present in the basal layer, involucrin in the suprabasal layers and filaggrin in the uppermost layers. (**D**): ERUs produced by injection of $CD44^{+}ALDH^{+}$ keratinocytes showed expression of CD44 (fluorescein isothiocyanate/green) and ALDH (phycoerythrin/red) in the basal layer (there was also nonspecific staining of keratin in the center of the ERU). (**E**): $CD44^{+}ALDH^{+}$ keratinocytes, in chambers on the dorsum of mice, produced a well-differentiated epidermis that persisted for at least 9 weeks. Expression of laminin was present along the basement membrane, keratin 14 in the basal layer and filaggrin in the uppermost layer. Scale bars = 10 μm. Abbreviations: ALDH, aldehyde dehydrogenase; EpiSC, epidermal stem cell; UNF, unfractionated population.

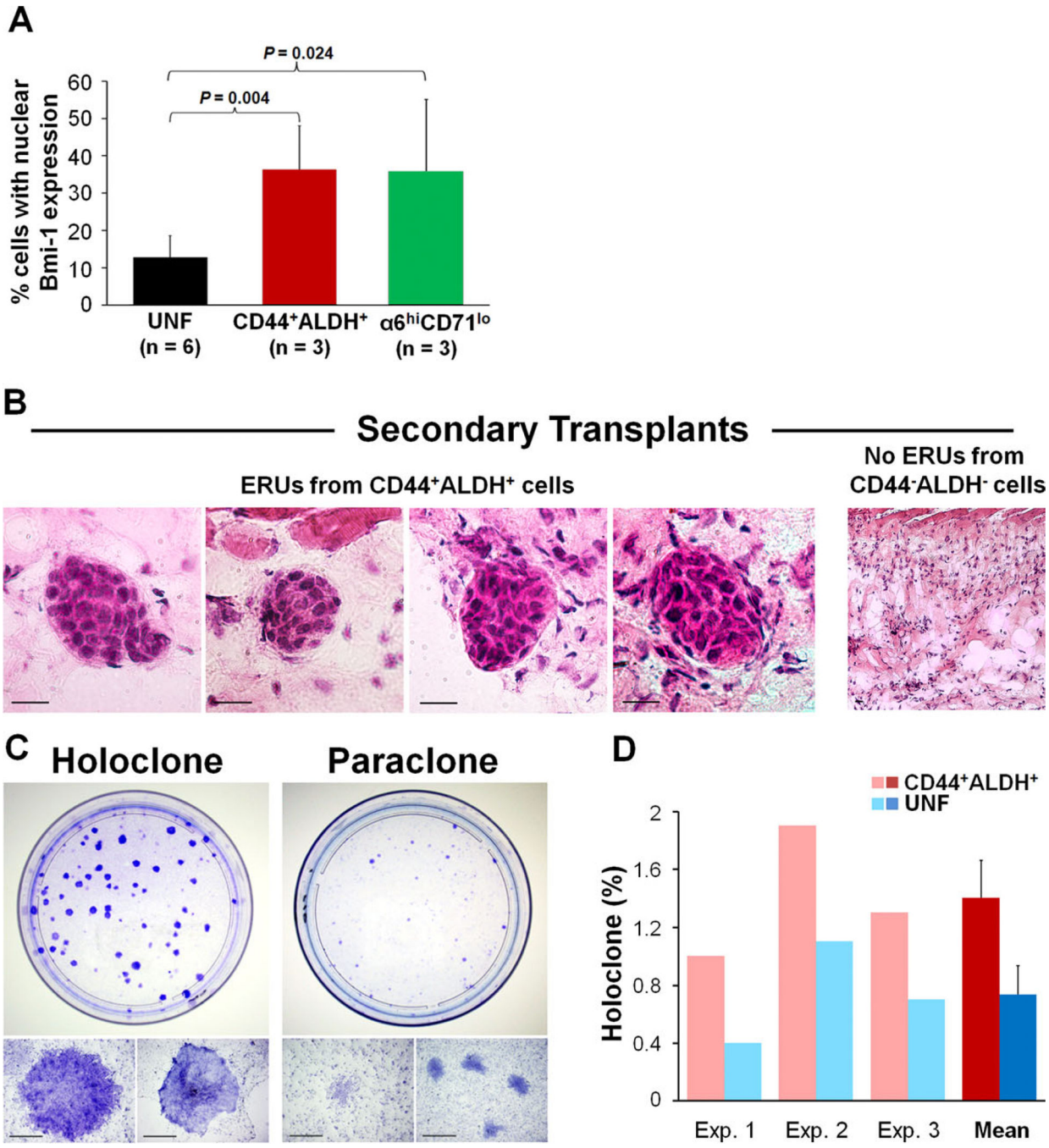

Figure 4.

$CD44^+ALDH^+$ keratinocytes display the stem cell property of self-renewal. (**A**): Bmi-1 expression in $CD44^+ALDH^+$ keratinocytes. The $CD44^+ALDH^+$ and integrin $\alpha6^{hi}CD71^{lo}$ populations of keratinocytes have greater numbers of cells expressing nuclear Bmi-1 than the UNF population. (**B**): $CD44^+ALDH^+$ keratinocytes display the stem cell property of self-renewal. Injection of 700 $CD44^+ALDH^+$ and 10,000 $CD44^-ALDH^-$ cells, followed by secondary transplantation of the resultant grafts (harvested at 9 weeks). Secondary ERUs were seen in $CD44^+ALDH^+$ derived secondary transplants. No ERUs were seen in

$CD44^{-}ALDH^{-}$ secondary transplants ($n = 3$). Scale bar = 20 μm. (**C**): Holoclone formation in $CD44^{+}ALDH^{+}$ versus UNF HNKs. Single cells were plated in 96-well plates. Colonies formed were each secondarily plated into a single dish. In secondary plating, holoclones formed large smooth colonies with central differentiation (left panels, low and high power) and paraclones formed small terminal colonies (right panels, low and high power) (meroclones were intermediate between the two). (**D**): The number of holoclones in $CD44^{+}ALDH^{+}$ keratinocytes was twice that in UNF keratinocytes; 1.4% ± 0.46% versus 0.7% ± 0.35%, $n = 3$, $p = .01$. (B) scale bars = 10 μm. (C) scale bar = 1 mm. Abbreviations: ALDH, aldehyde dehydrogenase; ERUs, Epidermal repopulating units; UNF, unfractionated.

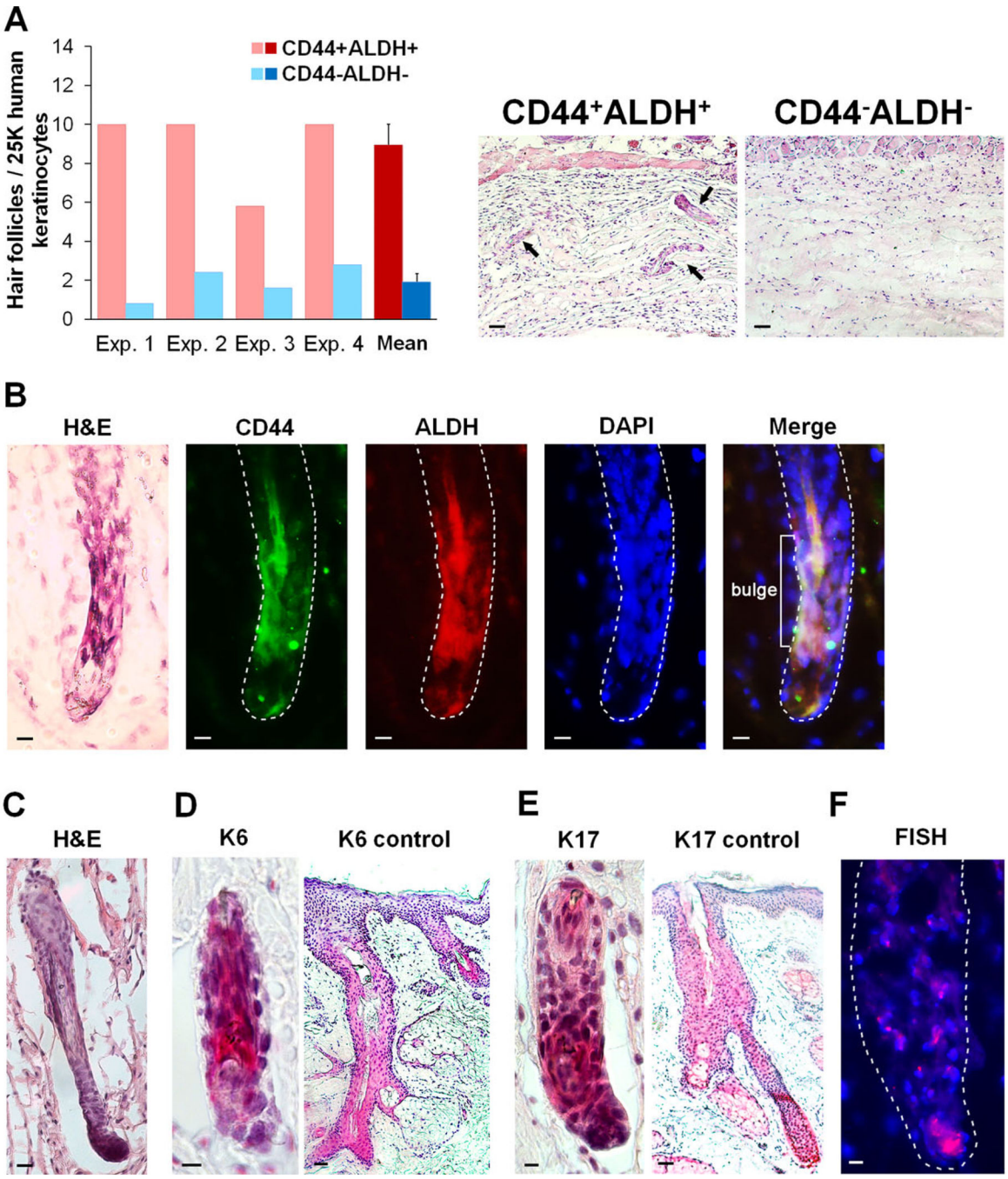

Figure 5.

The CD44$^+$ALDH$^+$ population of keratinocytes is multipotent. (**A**): The histogram illustrates how CD44$^+$ALDH$^+$ keratinocytes showed significantly greater production of hair follicle-like structures than CD44$^-$ALDH$^-$ keratinocytes. H&E staining of CD44$^+$ALDH$^+$ derived hair follicle-like structures (arrows) is shown in the panels on the right. (**B**): The hair follicle-like structures produced from CD44$^+$ALDH$^+$ keratinocytes contain CD44$^+$ALDH$^+$ keratinocytes in a location consistent with regeneration of a bulge region. (**C**): Higher power view of hair follicle-like structure (H&E). (**D**): Immunohistochemistry of hair-follicle-like

structures for hair-associated proteins keratin 6 (alkaline phosphatase-red) and (**E**) keratin 17 (alkaline phosphatase-red) (for (D) and (E) hematoxylin alone was used as counterstain). Controls are adult human hair follicles showing follicular staining (red). (**F**): FISH using a human specific y-chromosome DNA probe showed the presence of human cells (bright pink staining in human nuclei) in the hair follicle-like structures. (B, D-right panel) (E-right panel) scale bars = 50 μm. (D-left panel) (E-left panel) (F) scale bars = 10 μm. (C) scale bar = 20 μm. Abbreviations: ALDH, aldehyde dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence in situ hybridization.

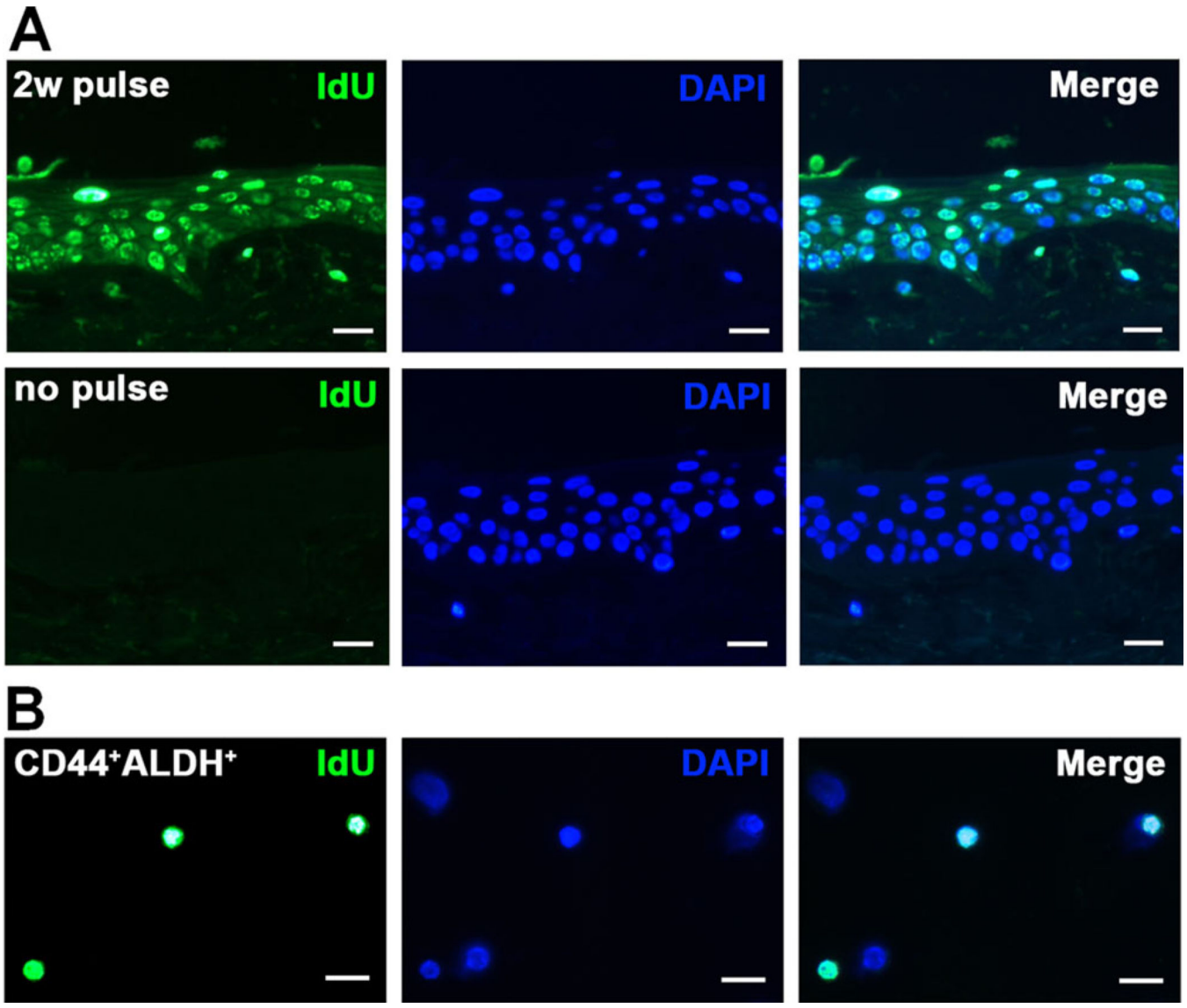

Figure 6.

The $CD44^+ALDH^+$ population of keratinocytes contains label-retaining cells. (**A**): After iododeoxyuridine administration twice daily for 2 weeks, almost 100% of keratinocytes in the basal layer of an organotypic epidermis (from unfractionated keratinocytes) were labeled. Negative controls included untreated keratinocytes (no iododeoxyuridine) (shown) as well as iododeoxyuridine-treated keratinocytes (2.5 μM twice a day for 2 days) with no primary antibody (not shown). Iododeoxyuridine-treated keratinocytes with primary antibody added were used as a positive control (not shown). (**B**): After a 4-week chase, keratinocytes from organotypic epidermis were fluorescence-activated cell-sorted to collect $CD44^+ALDH^+$ keratinocytes and stained for iododeoxyuridine. 58% ± 7% of $CD44^+ALDH^+$ keratinocytes were label retaining cells. Scale bar = 10 μm. Abbreviations: ALDH, aldehyde dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole; IdU, iododeoxyuridine.

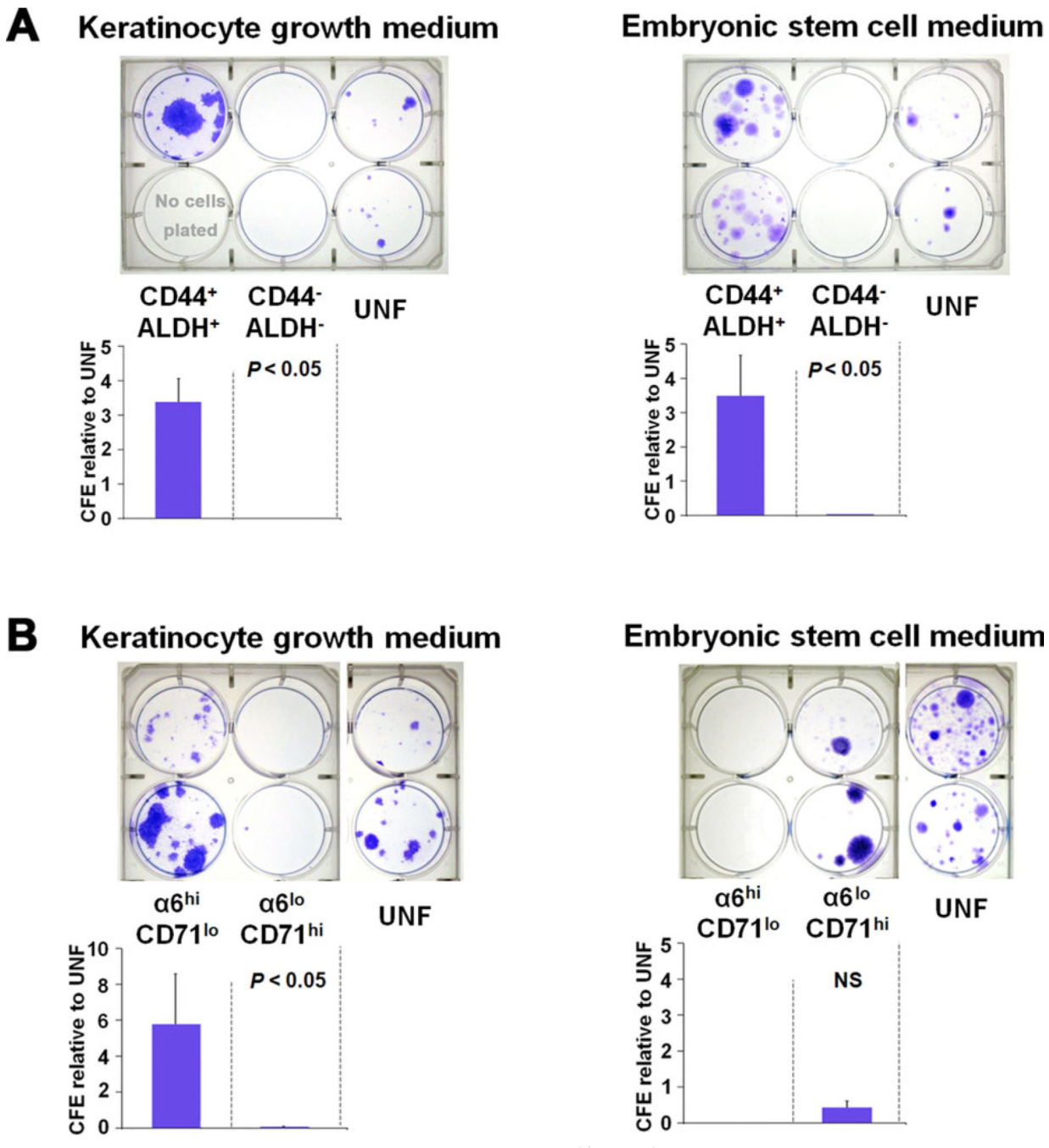

Figure 7.

Colony forming efficiency of $CD44^{+}ALDH^{+}$ and integrin $\alpha6^{hi}CD71^{lo}$ keratinocytes. (**A**): The $CD44^{+}ALDH^{+}$ population has enhanced colony-forming efficiency versus the $CD44^{-}ALDH^{-}$ population in both keratinocyte growth medium and embryonic stem cell medium. To determine the in vitro colony forming efficiency of the selected cells, $CD44^{+}ALDH^{+}$, $CD44^{-}ALDH^{-}$, and UNF keratinocytes were plated in six-well plates, 1,000 cells per well, in either keratinocyte growth medium (left) or embryonic stem cell medium (right). One representative plate from each experiment is shown (see also supporting

information Fig. 3). Bar graphs show mean ± SE (four experiments). (**B**): The integrin $\alpha6^{hi}CD71^{lo}$ population of cells has enhanced colony forming efficiency in keratinocyte growth medium but not in embryonic stem cell medium. The difference in colony forming efficiency between $CD44^{+}ALDH^{+}$ and integrin $\alpha6^{hi}CD71^{lo}$ HNKs in KGM was not significant, but in embryonic stem cell medium $CD44^{+}ALDH^{+}$ human neonatal keratinocytes had significantly greater colony forming efficiency ($p < .01$). One representative plate for each experiment is shown (see also supporting information Fig. 3). Bar graphs show mean ± SE (1,000 cells per well, four experiments). Abbreviations: ALDH, aldehyde dehydrogenase; CFE, colony forming efficiency; UNF, unfractionated.