Murine Epidermal Label-Retaining Cells Isolated by Flow Cytometry do not Express the Stem Cell Markers CD34, Sca-1, or Flk-1

Michael R. Albert, Ruth-Ann Foster, and Jonathan C. Vogel
Dermatology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

Keratinocyte stem cells are present in the murine epidermis, based on both *in vitro* and *in vivo* evidence, and better characterization of these cells remains an active goal. Because keratinocyte stem cells are believed to cycle slowly, a good method for identification is based on their ability to retain nucleoside analog, such as bromodeoxyuridine. Adult stem cells have been identified in other tissues, including hematopoietic, neural, and skeletal muscle, and stem cell surface markers have been characterized. We wanted to determine if cell-surface markers present on both hematopoietic and skeletal muscle stem cells (CD34, Sca-1, and Flk-1) were also present on keratinocyte stem cells, and could be used to identify them. The cell-surface expression of cells that retained bromodeoxyuridine label for at least 21 d was compared with that of nonlabel-retaining cells. Double-labeling for flow cytometric analysis was employed, and label-retaining cells were found to lack expression of the tested markers. β1 integrin levels were also evaluated, and although high expression was found on label-retaining cells, it was not specific for these cells. ***Key words: differentiation/mouse/skin/stem cells. J Invest Dermatol 117:943–948, 2001***

The epidermis is a rapidly self-regenerating epithelial tissue, and both rodent and human epidermis have been shown to consist of discrete units of proliferation in which cell production activity is balanced with cell loss (Mackenzie, 1969; Christophers, 1971; Allen and Potten, 1974; Mackenzie *et al*, 1981; Potten, 1981). A stem cell theory for squamous epithelia was proposed that holds that a proliferative hierarchy exists at the basal layer, analogous to that of the hematopoietic system (Potten, 1974; Lajtha, 1979; Lavker and Sun, 1982). The basal layer is believed to consist of distinct subpopulations: stem cells that are undifferentiated, slow-cycling cells with unlimited regenerative potential; transit amplifying cells that undergo a more rapid but finite number of cell divisions; and postmitotic, early differentiating cells.

Several lines of evidence support the existence of epidermal stem cells. Following γ-irradiation (depletion) of epidermis, a small percentage of radioresistant cells are capable of repopulating the epidermis (Potten and Hendry, 1973). Keratinocytes transduced with retroviral vectors expressing an indicator gene (β-galactosidase) and then grafted onto immunocompromised mice have also demonstrated long-term expression (40 wk postgraft period) (Kolodka *et al*, 1998). In addition, individual keratinocytes show heterogeneity with regard to colony-forming ability when grown in culture, and clones have been classified into three types: paraclones, meroclones, and holoclones (Barrandon and Green, 1987). Holoclones, which have the greatest proliferative potential, are believed to represent colonies derived from epidermal stem cells.

Further evidence for the existence of keratinocyte stem cells comes from *in vivo* studies that have demonstrated variation in the rate of cell cycling of epidermal cells. Experiments in which young mice were labeled with tritiated thymidine or BrdU revealed that a small percentage of basal keratinocytes retained nuclear label after 30 d and for up to 240 d (Bickenbach, 1981; Morris *et al*, 1985; Bickenbach *et al*, 1986; Cotsarelis *et al*, 1990; Bickenbach and Chism, 1998). These slowly cycling cells are referred to as label-retaining cells (LRC) and are thought to represent the epidermal stem cell subpopulation, which are quiescent or slowly dividing *in vivo*. LRC were capable of forming colonies *in vitro* (Morris and Potten, 1994) and were found to be undifferentiated, based on the expression of known differentiation markers (cytokeratins and bullous pemphigoid antigen) (Mackenzie *et al*, 1989). Label retention is presently one of the best available indicators of epidermal stem cells.

Prior investigations have attempted to characterize epidermal stem cells. Differing expression levels of adhesion molecules have been investigated (reviewed by Watt, 1998). Mouse keratinocytes rapidly adhering to a variety of substrates were enriched for LRC (Bickenbach and Chism, 1998). Keratinocytes with high levels of β1 integrin expression had high colony forming efficiency (Jones and Watt, 1993), suggesting that high expression is a characteristic feature of epidermal stem cells. β1 integrin expression, however, is not unique to stem cells as 40% of the basal cell population showed bright staining for β1 integrin when analyzed by quantitative confocal microscopy (Jones *et al*, 1995). Human keratinocytes with high levels of α6 integrin expression and low CD71 (transferrin receptor) expression were found to have the greatest long-term proliferative capacity amongst basal cells in culture, and cell-cycle

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Reprint requests to: Dr. Jonathan Vogel, Dermatology Branch, National Cancer Institute, National Institutes of Health, Building 10, Room 12 N238, 9000 Rockville Pike, Bethesda, MD 20892-1908. Email: jonvogel@box-j.nih.gov

Abbreviation: LRC, label-retaining cell.

kinetic analysis indicated that they were slow-cycling *in vivo*, consistent with properties expected of epidermal stem cells (Li *et al*, 1998; Kaur and Li, 2000). A study of murine epidermal cells taken from BrdU-labeled mice and isolated by fluorescence-activated cell sorting (FACS) indicated that 70% of LRC were in the high $\alpha 6$ integrin/low CD71 group (Tani *et al*, 2000).

Markers have also been sought for hair follicle stem cells, which reside anatomically in the bulge region of the follicle. Labeling studies with tritiated thymidine in mice (Cotsarelis *et al*, 1990; Taylor *et al*, 2000) and BrdU in a human skin/*scid* mouse model (Lyle *et al*, 1998) have demonstrated that LRC localize to the bulge. Immunostaining for cytokeratin 15 (Lyle *et al*, 1999) and cytokeratin 19 (Michel *et al*, 1996) also localized to the follicular bulge in human skin. Staining for cytokeratin 15 was observed in all basal cells in humans (Waseem *et al*, 1999) and sheep (Whitbread and Powell, 1998), however, and staining for cytokeratin 19 extended beyond the follicular bulge region in humans (Lyle *et al*, 1998). It has been shown that in the skin of normal newborn mice, and adult mice in response to a penetrating wound, follicular keratinocytes migrate to the epidermis (Taylor *et al*, 2000). This suggests the possibility that follicular and epidermal stem cells are not necessarily distinct populations but may represent progenitor cells existing in a single hierarchy.

Recently, the surprisingly multipotent nature of stem cells in various organ systems has been demonstrated. For example, bone marrow contains human mesenchymal stem cells, which can be induced in culture to differentiate to adipocytic, chondrocytic, or osteocytic lineages (Pittenger *et al*, 1999); rat and human mesenchymal stem cells also have the ability to differentiate into neurons in culture (Woodbury *et al*, 2000). The plasticity of stem cells has also been illustrated by animal transplantation studies in which donor and recipient strains differ, so that the cell of origin (i.e., donor *versus* recipient) can be determined. Using this experimental method, it was shown that injected murine neural stem cells (Bjornson *et al*, 1999) and skeletal muscle stem cells (Gussoni *et al*, 1999; Jackson *et al*, 1999) were able to repopulate the hematopoietic compartment in mice that underwent bone marrow ablation. In addition, following bone marrow transplantation, donor-derived cells were found in murine muscle (Ferrari *et al*, 1998; Gussoni *et al*, 1999) and neural cells of the brain (Eglitis and Mezey, 1997; Brazelton *et al*, 2000; Mezey *et al*, 2000) as well as canine vascular endothelial cells (Shi *et al*, 1998). Bone marrow transplantation studies also demonstrated that purified murine hematopoietic stem cells can differentiate into epithelial cells of the skin, lung, and gastrointestinal tract (Krause *et al*, 2001), as well as the liver (Lagasse *et al*, 2000; Krause *et al*, 2001).

Interestingly, murine hematopoietic and skeletal muscle stem cells, isolated by their common ability to efflux the vital dye Hoechst 33342, have also been found to share the marker Sca-1 (Gussoni *et al*, 1999; Jackson *et al*, 1999). Another study in which skeletal muscle stem cells from the *mdx* mouse model for Duchenne muscular dystrophy were isolated by culture techniques found that they shared the hematopoietic markers CD34 and Flk-1, in addition to Sca-1 (Lee *et al*, 2000). This led us to postulate that these markers might be similarly shared on epidermal stem cells. We investigated if these markers were expressed on LRC in the mouse epidermis, and thus might represent a potential means of isolating and better understanding the biology of epidermal stem cells.

MATERIALS AND METHODS

BrdU labeling BALB/C mice were labeled during the second week of life. Mice received intraperitoneal injections twice a day for four consecutive days with 0.25 mg of BrdU (Sigma, St. Louis, MO) diluted in 0.1 ml of phosphate-buffered saline (PBS). Mice were then allowed to grow without further labeling.

Isolation of keratinocytes Following a different chase period up to 28 d, truncal skin specimens were obtained and the subcutaneous fat was removed. Specimens were incubated overnight at 4°C in a 1:1 mixture of dispase (50 caseinolytic units per ml, Collaborative Biomedical Products, Bedford, MA) and Hanks' balanced salt solution. Skin samples were transferred to 0.25% trypsin (Gibco/BRL, Paisley, U.K.) for 60 min at 37°C. Epidermal sheets were then removed and dissociated into single cells by pipetting. Completeness of epidermal removal was assessed by hematoxylin and eosin staining.

Monoclonal antibodies The following purchased monoclonal antibodies were used for flow cytometric analysis: anti-BrdU antibodies [clone MOPC-21, mouse IgG1 fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated, or BrdU Flow Kit, Pharmingen, San Diego, CA]; anti-CD34 (clone RAM34, rat IgG2a-FITC, Pharmingen); anti-Sca-1 (clone D7, rat IgG2a-FITC, Pharmingen); anti-Flk1 (clone Avas 12α1, rat IgG2a-PE, Pharmingen); anti-CD29 (integrin β1 chain) (clone Ha2/5, Armenian hamster IgM-FITC, Pharmingen); antipan-cytokeratin (clone C-11, mouse IgG1-FITC, Sigma). The appropriate isotype-matched negative control antibodies were utilized for FACS studies in each experiment.

Immunohistochemistry Anti-BrdU-peroxidase, Fab fragments (clone BMG-6H8, Boehringer, Mannheim, Germany) were employed for immunohistochemistry. BrdU staining was performed on 70% ethanol-fixed, paraffin-embedded sections of truncal skin. Paraffin-embedded sections were dewaxed and rehydrated. Sections were treated with 4 M HCl for 15 min at room temperature, neutralized with 0.1 M sodium borate for 5 min, and washed three times in PBS with 10% fetal bovine serum (5 min each). This was followed by incubation with anti-BrdU-peroxidase Fab fragments (1:50 dilution in PBS with 1% bovine serum albumin) at room temperature for 1 h. Staining was then performed with a peroxidase detection kit (Vector VIP Substrate Kit, Vector Laboratories, Burlingame, CA).

Immunofluorescence staining and FACS of keratinocytes Cells were processed for BrdU staining and FACS analysis as described by Pharmingen either with fixation in 70% ethanol for 20 min, denaturing with 2 M HCl for 20 min, and neutralization with 0.1 M sodium borate for 5 min at room temperature or using the BrdU Flow Kit (Pharmingen), which employs paraformaldehyde fixation and then DNase treatment (30 μg per 10^6 cells) for 1 h at 37°C. Cells were double-stained with separate 1 h incubations at room temperature. Compensation settings were established using positive control antibodies to H-2Dd (clone 34-5-8S, mouse IgG2a-FITC and mouse IgG2a-PE, Pharmingen). Following antibody staining and washing with PBS with 0.5% bovine serum albumin, cells were resuspended in 5% fetal bovine serum in PBS and washed twice, and flow cytometric analysis was performed using FACscan or FACSCalibur and CellQuest software (Becton Dickinson). For each experiment, a minimum of 10,000 cells were counted after gating on forward and side scatter to exclude debris.

RESULTS

Identification of LRC by immunohistochemistry Immunohistochemistry was conducted to determine if a subset of epidermal and hair follicle cells retained BrdU label 3–4 wk after intraperitoneal labeling was completed and to confirm that LRC were present in the expected anatomical location. Immediately after the completion of labeling in our mice, most basal cells showed staining for BrdU (**Fig 1*a***). Marked nuclear staining was apparent in a linear pattern along the basal layer of the epidermis, as well as in hair follicles and numerous fibroblasts in the dermis. At 1 wk, label was detected diffusely in the epidermis, including prominent staining in postmitotic suprabasilar cells (**Fig 1*b***). Two weeks after labeling, far fewer basal and suprabasilar cells showed staining (**Fig 1*c***), although strong staining was present in the bulge region of hair follicles (**Fig 1*d***). By 3–4 wk, epidermal staining was detectable only in rare, scattered basal cells (**Fig 1*e***). These data confirm that BrdU labeling was successful and that LRC were present in the expected anatomical locations of epithelial stem cells.

Identification of LRC by flow cytometry Subsequently, we evaluated if cells labeled with BrdU could be detected by using flow cytometry. Immediately after labeling, 25%–40% of total epidermal cells showed staining for BrdU compared to isotype control by flow cytometric analysis (**Fig 2*a*, *b***). This labeling index is consistent with the initial presence of labeled keratinocytes in the basal keratinocyte population and not the suprabasal population of epidermal suspensions (reported to contain 30% basal cells, 55%

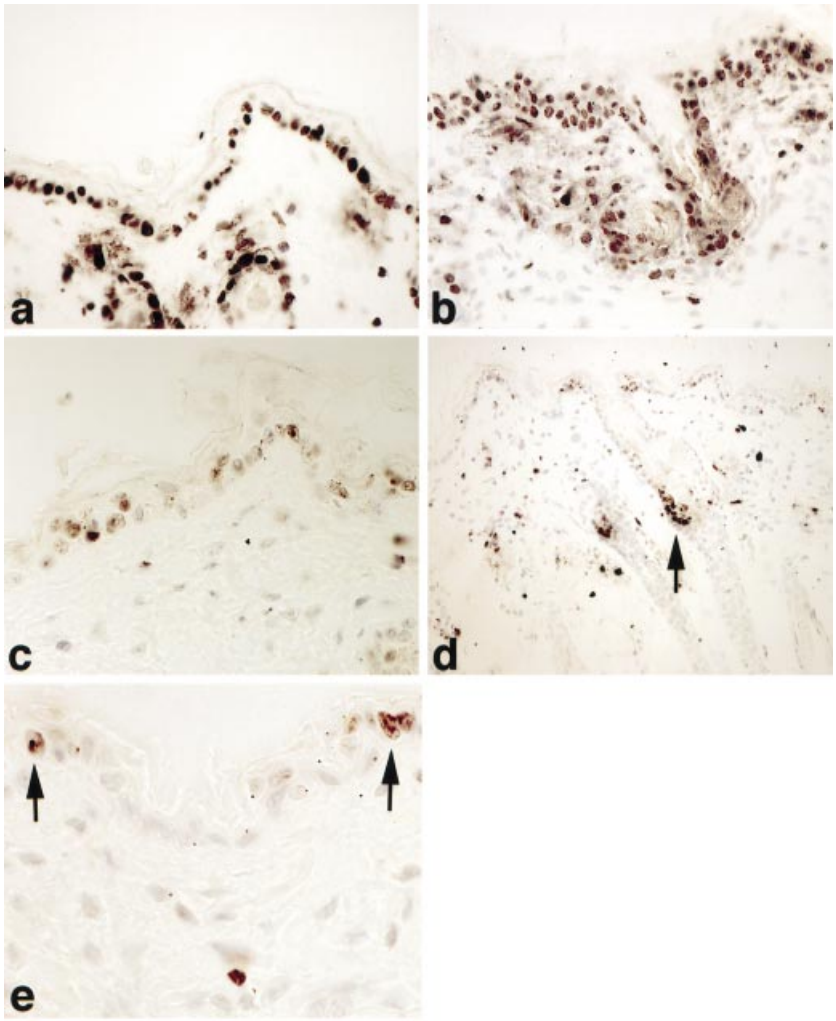

Figure 1. Immunohistochemical analysis of BrdU retention in keratinocytes at various time points after labeling. Immunoperoxidase staining for BrdU in ethanol-fixed mouse skin sections (*a*) day 0, (*b*) 7 d, (*c*, *d*) 14 d, and (*e*) 28 d after *in vivo* labeling. *Arrows* show (*d*) strong staining in the bulge of the hair follicle at 14 d and (*e*) scattered LRC present in the epidermis at 28 d after labeling. (*a*, *b*) 40×; (*c*) 60×; (*d*) 20×; (*e*) 80×. Representative results from three experiments shown.

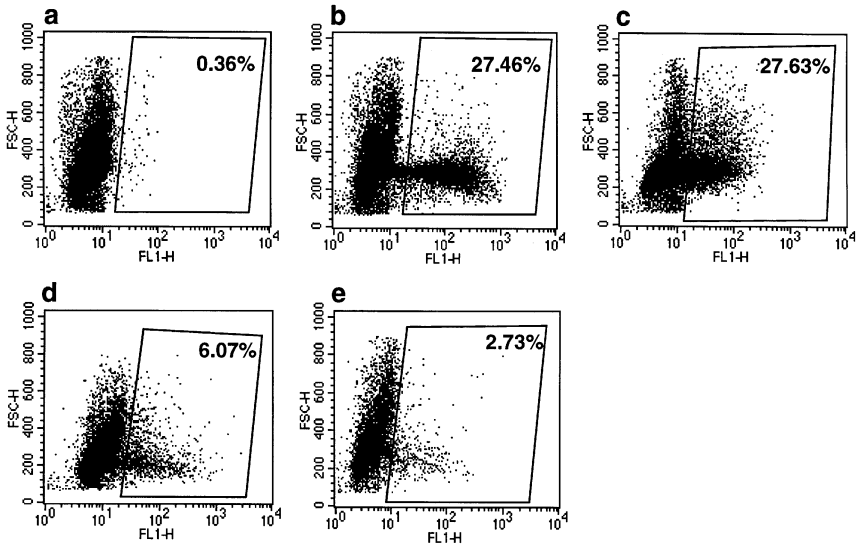

Figure 2. Flow cytometric analysis of epidermal cells from labeled mice at various time points after labeling. Suspensions of epidermal cells stained with (*a*) isotype control (FITC) or (*b* –*e*)monoclonal antibody against BrdU (FITC). Cells were isolated from mice (*a*, *b*) immediately, (*c*) 7 d, (*d*) 19 d, or (*e*) 28 d after *in vivo* labeling. Results are representative of at least three experiments per time point.

suprabasal cells, and 15% nonepithelial cells). The percentage of labeled cells that we observe by FACS analysis also correlates well with the histologic staining in the basal layer at time 0. Labeled cells were predominantly small in size, as reflected by low forward and side scatter, consistent with immunohistochemical staining showing prominent staining along the basal layer. A marked separation in fluorescence intensity was apparent for labeled and unlabeled cells. At 1 wk after labeling (**Fig 2*c***), there was still a substantial labeled population, which now included a greater proportion of larger cells, probably representing suprabasilar cells that were labeled on

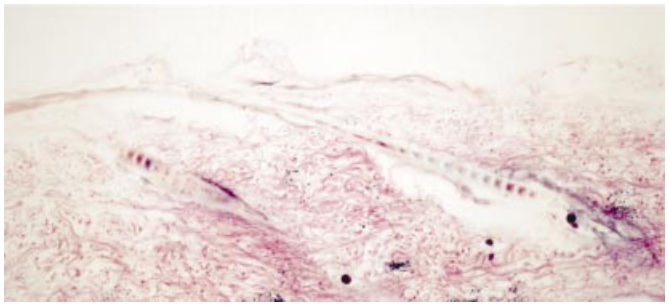

Figure 3. Histology of skin following separation of epidermal sheet. Hematoxylin and eosin staining of the dermis (20×) shows no remaining basal cells and few residual follicular keratinocytes, indicating these cells were included in the epidermal suspension.

immunohistologic sections (**Fig 1*b***). A continuum of fluorescence intensity (less distinct separation) was observed because ongoing cell division dilutes the BrdU label in daughter keratinocytes. By 19 d, labeled cells constituted a still distinct population by FACS, making up less than 10% of total cells (**Fig 2*d***). At 4 wk an LRC population of approximately 1%–3% of cells was consistently detected (**Fig 2*e***). The percentage of BrdU-labeled keratinocytes we detected at both day 0 and day 28 is comparable to a previous report of tritiated thymidine LRC detected in mouse truncal skin by *in situ* autoradiography (Bickenbach *et al*, 1986).

Epidermis contains many cell types including Langerhans cells and melanocytes. To demonstrate that LRC were predominantly keratinocytes, we employed double-staining with a pan-cytokeratin antibody. Of the total cell population, more than 80% of cells were positive for keratin; among the LRC populations, more than 70% were positive for keratin (data not shown). To assess the completeness of keratinocyte isolation, dermal specimens remaining after separation of epidermal sheets were analyzed by staining with hematoxylin and eosin (**Fig 3**). There was no evidence of residual interfollicular keratinocytes, and the cell suspension included the majority of follicular epithelial cells. This indicates that follicular keratinocytes, including LRC of the bulge region, were included in our analysis.

Evaluation of cell-surface markers In order to ascertain if BrdU positive LRC expressed known stem cell markers of other tissues, the presence of CD34, Sca-1, and Flk-1 on LRC was analyzed by double-staining. A representative cytofluorometric analysis of cells double-stained with monoclonal antibodies directed at CD34 and BrdU is shown in **Fig 4(*a*)–(*c*)**. A small population of cells retaining detectable BrdU is evident, but they do not exhibit CD34 expression. Similarly, separate experiments revealed that LRC populations do not express Sca-1 or Flk-1, respectively (**Fig 4*d*–*i***). This suggests that these stem cell markers are not expressed on keratinocyte stem cells.

To ensure that the fixation and DNA denaturing treatment necessary to detect BrdU did not adversely affect the detection of stem cell surface markers, flow cytometric analysis of treated and untreated cells was compared (data not shown). A similar pattern of cell-surface expression was observed whether the cells were treated or untreated, with the incidence of positive cells remaining approximately the same (CD34 2.37% *vs* 2.16%; Sca-1 6.62% *vs* 6.21%; Flk-1 0.39% *vs* 0.50%).

FACS analysis was performed with monoclonal antibodies directed against BrdU and β1 integrin to evaluate the relationship between LRC and β1 expression. A spectrum of β1 integrin expression was detected with LRC containing both high and low levels of β1 expression (**Fig 4*j*–*l***). These data confirm that high expression of β1 integrin is present on some LRC, but is not specific for these cells (0.61% of cells are β1 integrin bright and LRC+ 13.11% of cells are β1 integrin bright and LRC–).

DISCUSSION

Cutaneous gene therapy is a promising method of treatment for genetic skin diseases and certain systemic diseases caused by the deficiency of a factor or enzyme (Gerrard *et al*, 1993; Hengge *et al*, 1995; Choate *et al*, 1996; White *et al*, 1998). Among the advantages of targeting the skin are its accessibility for *in vivo* and *ex vivo* manipulation, and the ease with which it can be monitored (Vogel *et al*, 1996). Although attempts to introduce transgenes into the skin have been successful, robust long-term expression remains elusive. One approach to achieving this goal is to isolate and target stem cells (Bickenbach and Roop, 1999). Increased efficiency of gene transfer to these cells may result in long-term expression in a significant percentage of keratinocytes. Despite some success in defining potential keratinocyte stem cell markers, more work needs to be done to further characterize these cells.

Because label retention is thought to be one of the best methods to identify epidermal and follicular stem cells, we investigated potential cell-surface markers on LRC, which might serve as a means of epidermal stem cell targeting. We evaluated CD34, Sca-1, and Flk-1, known stem cell antigens that were first described on hematopoietic stem cells. CD34 is a saliomucin-like adhesion molecule, found on 1%–3% of bone marrow cells as well as endothelial cells, that has been used as a selection marker for hematopoietic stem cells in mice and humans (Strauss *et al*, 1991). Although CD34 negative hematopoietic stem cells have now been described (Osawa *et al*, 1996; Goodell *et al*, 1997), enrichment of CD34 positive bone marrow cells remains a highly utilized technique in clinical gene therapy trials (Halene and Kohn, 2000). Sca-1 (Ly-6 A/E or stem cell antigen 1) is a phosphatidyl-inositol-anchored protein of unknown function, but possibly an adhesion molecule, commonly used for the purification of murine hematopoietic stem cells (Patterson *et al*, 2000). Flk-1 is the mouse homolog of the human vascular endothelial growth factor receptor 2 (VEGFR2 or KDR), a recently described marker for purifying hematopoietic stem cells, in conjunction with CD34 (Ziegler *et al*, 1999). These markers were chosen because skeletal muscle stem cells have been reported to share one or all of these markers (see above), raising the possibility that other tissue stem cells may also possess these markers (Gussoni *et al*, 1999; Jackson *et al*, 1999; Lee *et al*, 2000).

In this study we were able to consistently find a small population of slowly cycling cells that retained BrdU label at 3–4 wk. These LRC were primarily keratinocytes, as more than 70% stained with a pan-cytokeratin antibody. Flow cytometry indicated that LRC were predominantly small sized, as has been previously demonstrated using density gradient sedimentation (Morris *et al*, 1990; Tani *et al*, 2000). Using two-color flow cytometry we did not find cell-surface CD34, Sca-1, and Flk-1 expression in LRC. Therefore, our data do not support that these markers are expressed on epidermal stem cells. As we define stem cells by the property of long-term label retention, however, we cannot exclude the possibility that non-label-retaining keratinocytes containing the markers CD34, Sca-1, or Flk-1 represent important progenitor cells.

Previously, a 2-fold increase in β1 integrin levels in cells with high colony forming efficiency has been reported (Jones and Watt, 1993), and we evaluated its expression on LRC. β1 integrin is found on all basal cells and, as expected, a spectrum of expression on cells in an epidermal cell suspension was observed by flow cytometry. Specificity of high expression was not seen for LRC, however.

Although CD34, Sca-1, and Flk-1 were felt to be the most likely candidate markers for epidermal stem cells, it is possible that other markers will be described that are shared on stem cells from many organ systems, including the skin. In addition to cell-surface markers, other common characteristics such as efflux of vital dye may be shared between epidermal stem cells and other types of stem cells. As our understanding of stem cell plasticity increases and investigations are carried out to determine if, like neural, hematopoietic, and skeletal muscle stem cells, epidermal stem

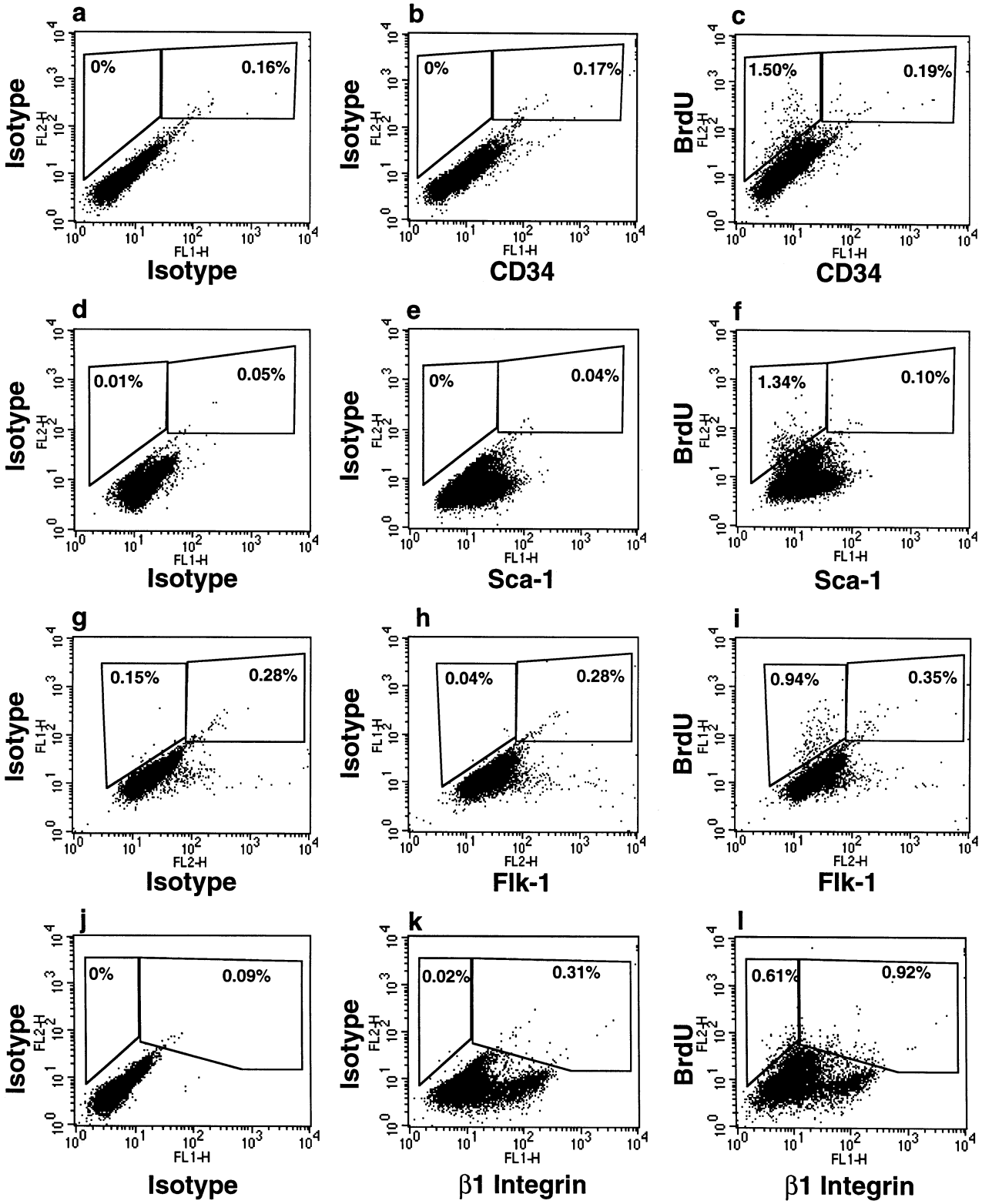

Figure 4. Flow cytometric analysis of cells double stained for BrdU and stem cell markers. Epidermal cells were stained with monoclonal antibody against BrdU and stem cell markers along with appropriate isotype controls. Staining is shown for (*a–c*) BrdU (PE) and CD34 (FITC), (*d–f*) BrdU (PE) and Sca-1(FITC), (*g–i*) BrdU (FITC) and Flk-1 (PE), and (*j–l*) BrdU (PE) and β1 integrin (FITC). Cells were isolated from mice (*a–c*, *g–l*) 21 d and (*d–f*) 24 d after labeling. Results are representative of at least three experiments per cell-surface marker tested.

cells are capable of multipotential differentiation, the likelihood of finding shared and even universal stem cell features should be better determined.

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