As Epidermal Stem Cells Age they do not Substantially Change their Characteristics

Luchuan Liang,* Sathivel Chinnathambi,* Matthew Stern,* Ann Tomanek-Chalkley,* Tony D. Manuel,* and Jackie R. Bickenbach*†

*Departments of Anatomy and Cell Biology and †Dermatology, The University of Iowa, Iowa City, Iowa, USA

In this study, we ask the basic question: do stem cells age? We demonstrated that epidermal stem cells isolated from neonatal mice had the capacity to form multiple cell lineages during development. Here we demonstrate the cell lineages are clonal, and that epidermal stem cells isolated from the footpad epithelium of old mice have similar capabilities. Using Hoechst dye exclusion and cell size, we isolated viable homogenous populations of epidermal stem and transit-amplifying (TA) cells from GFP-transgenic mice, and injected these cells into 3.5-d blastocysts. Only the stem-injected blastocysts produced mice with GFP$^+$ cells in their tissues. Furthermore, aged and young stem cells showed similar gene and protein expression profiles that showed some differences from the TA cell profiles. These data suggest that there may be a fundamental difference between somatic stem and TA cells, and that an epidermal stem cell placed in a developmental environment can alter its fate determination no matter what its age.

Key words: plasticity/skin
J Investig Dermatol Symp Proc 9:229–237, 2004

It has been known for decades that the epidermis contains a subpopulation of basal cells that exhibit the properties expected of somatic stem cells: slow cell cycle, high proliferative potential, and capacity to maintain and repair the tissue in which they reside (for examples see Potten and Hendry, 1973; Bickenbach, 1981; Lavker and Sun, 1982, 2000; Morris and Potten, 1999). These somatic stem cells were first identified as label-retaining cells (LRC) by long-term nuclear retention of tritiated thymidine (Bickenbach, 1981). They are found in the interfollicular basal layer of the epidermis (Bickenbach, 1981, 1998; Lavker and Sun, 1982; Bickenbach and Mackenzie, 1984) and in the bulge area of the hair follicle (Cotsarelis *et al*, 1990; Michel *et al*, 1996; Morris and Potten, 1999). All somatic stem cells, including those from the epidermis, are self-renewing and able to produce daughter transit-amplifying (TA) cells that undergo a finite number of cell divisions before they differentiate and leave the proliferative basal compartment (for a review see Lajtha, 1979; Potten, 1997). The specifics of how the stem cell population differs from the TA cell population have long been speculated, but hampered by the lack of isolation methods that yield homogenous populations. We previously reported a technique to isolate a stem cell population (Dunnwald *et al*, 2001), which combined two characteristics—small cell size shown to yield a highly proliferative epidermal population (Barrandon and Green, 1985; Morris *et al*, 1990), and exclusion of Hoechst 33342 dye reported to isolate a side population (SP) of hematopoietic stem cells (Goodell *et al*, 1996, 1997). More recently, we showed that neonatal epidermal stem cells, but not the TA cells, isolated by this method could produce cell lineages from all three germ layers in the developing mouse (Liang and Bickenbach, 2002). Thus this isolation method yielded epidermal cells that showed characteristics of stem cells that appeared to retain an intrinsic property that allowed them to specifically respond to extrinsic signals in the developmental environment.

Another characteristic expected of stem cells is a long life span. It is possible that somatic stem cells are lifelong residents of the tissues they inhabit (for a review see Cairns, 1975; Lajtha, 1979; Potten, 1997). Although most of the examples used to support this hypothesis are from the hematopoietic system where cells from one sex incorporated into the bone marrow of hosts of the opposite sex functioned for several months (Flowers *et al*, 1990; Krause *et al*, 2001), the epidermis also has very long lived cells. LRCs in the basal layer were shown to persist for at least 6 mo in the interfollicular epidermis (Bickenbach, 1981; Bickenbach *et al*, 1986) and for 14 mo in the hair follicle (Morris and Potten, 1999). Although such data suggest that stem cells may have a very long life, it is not clear what happens to stem cells as an organism ages. It could be that they age in a fashion similar to other cells, but it could also be that they are held out of the normal cellular aging process and are used to replace dysfunctional or dead cells, thereby restoring failing tissues.

Our goal is to determine whether epidermal stem cells change with increasing age. Herein we report that epidermal stem cells isolated by Hoechst dye exclusion and small size from old mice appear to have capabilities similar to the

Abbreviations: BrdU, bromodeoxyuridine; LRC, label-retaining cells; RT, reverse transcription; TA, transit amplifying

neonatal epidermal stem cells when placed in a developmental environment. We also show that neonatal murine epidermal stem cells isolated in this manner have a gene expression profile distinct from that of neonatal murine TA cells, and that human epidermal cells show similar protein expression profiles no matter what their age, suggesting that somatic stem cells may indeed have a very long survival, and that this characteristic is conserved.

Results

Injecting of GFP$^+$ neonatal murine epidermal stem or TA cells into developing blastocysts caused no abnormal mice To ensure that injecting neonatal or aged epidermal cells into developing blastocysts caused no detrimental phenomena, we allowed a few mice who had been injected with GFP$^+$ neonatal stem or TA cells to grow to old age. All of the mice lived an apparently normal life span for C57BL/6 mice. At 22 mo of age, these mice had no outward defects and appeared normal with typical aging phenomena: males had enlarged prostates and females had fragile bones (data not shown). Mice that were derived from TA cell-injected blastocysts showed no GFP expression, whereas the mice derived from stem cell-injected blastocysts showed a mosaic expression of GFP in their tissues even at old age (data not shown). This is similar to our previously published findings for neonatal epidermal stem cells (Liang and Bickenbach, 2002).

Neonatal murine stem cells formed clonal units in lung bronchioles after exposure to a developmental environment To determine whether epidermal stem cells could be induced to form clonal units of structure after transdifferentiation, we analyzed the bronchioles in lung tissues from 60-d-old adult mice derived from blastocysts injected with neonatal GFP$^+$ stem or TA cells (Liang and Bickenbach, 2002). Mice derived from TA-injected blastocysts showed no GFP expression in any of their tissues, including the lung. Mice derived from stem cell-injected blastocysts showed a mosaic expression of GFP in their tissues. When GFP$^+$ cells were found in the bronchiole epithelia of the lung, all epithelial cells in that bronchiole were GFP$^+$. Thus, the transdifferentiated stem cells formed clonal units of structure (Fig 1).

Genes expressed in neonatal murine epidermal stem cells differ from those expressed in TA cells To test whether cells isolated by our procedure corresponded to cells reported by others to be stem cells, we examined these cells for expression of genes reported to identify stem cells. We isolated RNA from freshly sorted (not cultured) cells and performed real-time PCR on the reverse transcribed RNA. We chose six genes to examine in the stem and TA cells: ABC transporter was reported to be higher in cells that could actively exclude the vital Hoechst dye (Goodell *et al*, 1996, 1997); p63 was reported to identify stem cells in epithelial appendages and in various other epithelial cells (Mills *et al*, 1999; Yang *et al*, 1999; Pellegrini *et al*, 2001); integrins α6 and β1 were reported to be more highly expressed in epidermal stem cells (Jones and Watt, 1993; Li *et al*, 1998; Tani *et al*, 2000); CD71 (transferrin receptor) was reported to identify epidermal TA cells (Li *et al*, 1998; Tani *et al*, 2000); and it was suggested that c-myc may drive epidermal stem cells to differentiate (Waikel *et al*, 2001; Watt, 2002). Our data agree with most of these studies (Table I). Although we saw no difference in expression level of integrin β1 between the stem cells and the TA cells isolated by our method, we did find that the stem cells showed 2-fold higher expression level of the ABC transporter ABCG2 (also called Bcrp-1), and a $\sim$1.5-fold higher expression of the ΔN isoform of p63, and of integrin α6. We also found that the TA cells showed $>$2-fold higher expression levels of both c-myc and CD71. Thus, the neonatal epidermal cells show an intrinsic difference in gene expression between the stem cell population and the TA cell population.

Aged murine epidermal stem cells show similar plasticity to neonatal stem cells when placed in a developmental environment We had previously shown that neonatal epidermal stem cells could be reprogrammed to

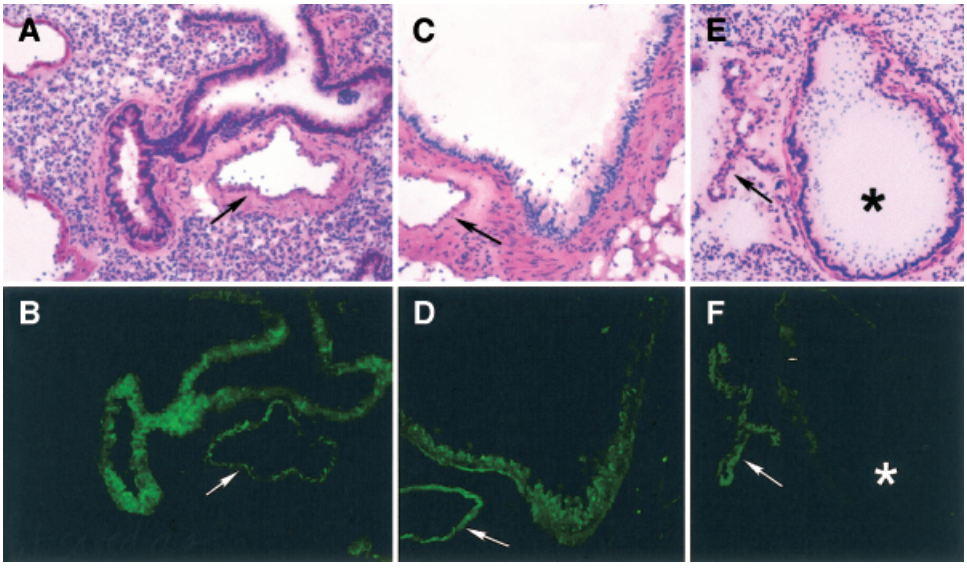

Figure 1
GFP$^+$ cells are found as clonal units in lung bronchioles of adult mice derived from neonatal stem cell-injected blastocysts. GFP$^+$ stem or transit-amplifying (TA) cells isolated from neonate mouse backskin were injected into blastocysts, then the tissues from the resulting offspring were sampled at 60 d after birth. GFP$^+$ cells were found in all tissues derived from stem-injected blastocysts, but not in tissues derived from TA-injected blastocysts. Examples of lung tissue are shown here. (*A–D*) Sections of bronchioles in lung tissue from 60-d-old mice derived from stem cell-injected blastocysts. (*E*, *F*) Sections of bronchiole in lung tissue from 60-d-old mouse derived from TA cell-injected blastocyst. *A*, *C*, and *E* are H&E stained sections adjacent to sections *B*, *D*, and *F* that are viewed for GFP fluorescence. Stars denote lumen of bronchioles. Arrows point to arteries with autofluorescing actin.

Table I. Genes expressed in sorted neonatal mouse epidermal stem and TA cells

Genes expressed higher in stem[a]		Genes expressed higher in TA[a]		No difference[a]
ABC transporter (ABCG2, Bcrp-1)	2.1 ± 0.02	CD71 (transferrin receptor)	2.2 ± 0.2	Integrin β1
p63 (ΔN isoform)	1.3 ± 0.1	c-Myc	2.0 ± 0.4	
Integrin α6	1.5 ± 0.2			

[a]n = 5, mean ± SD. TA, transit amplifying.

alter their cell fate by exposure to this environment (Liang and Bickenbach, 2002). Thus to begin to determine how epidermal stem cells age, we compared the response of aged epidermal stem cells to the neonatal cells placed in the blastocyst environment. GFP^+ epidermal stem or TA cells, isolated from footpads of 21-mo-old GFP^+ mice were injected into 3.5-d-old C57BL/6 blastocysts. Both TA cell-injected blastocysts and stem cell-injected blastocysts yielded viable, apparently normal, mice with no visible abnormalities. Samples of skin, heart, and liver were examined 4 mo after birth for the presence of GFP^+ cells. We found GFP^+ cells only in the mice derived from stem cell-injected blastocysts, not in mice from TA cell-injected blastocysts (Fig 2). All tissues that had GFP^+ cells showed a varying mosaic pattern of GFP^+ cells intermixed with GFP^- cells (Fig 2) similar to what we had observed when we injected neonatal stem cells (Liang and Bickenbach, 2002). Thus, the aged epidermal stem cells retained the ability to respond to the developmental environment, suggesting that stem cells may not age as other cells do. Furthermore, the aged stem

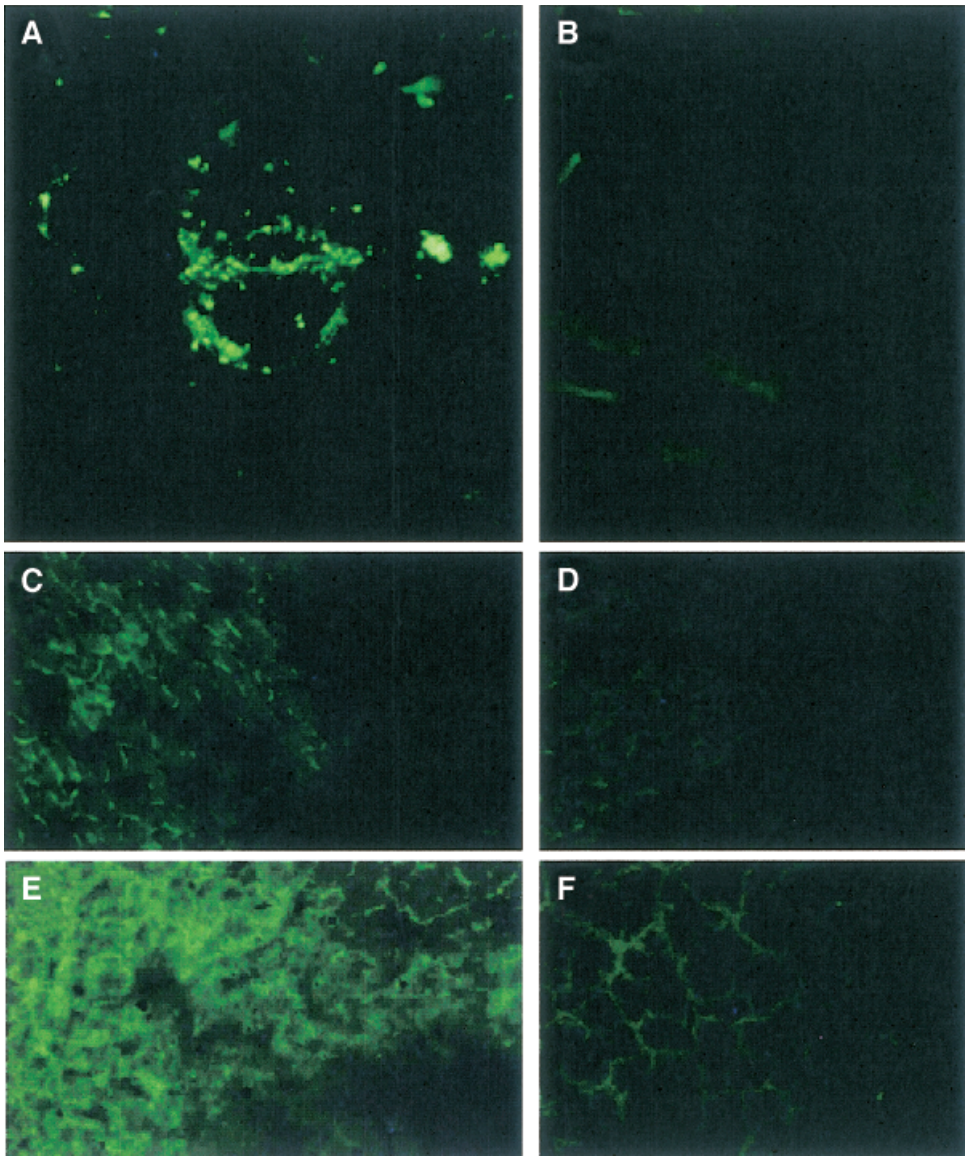

Figure 2
GFP^+ cells are found in various tissues of mice derived from blastocysts injected with old epidermal stem cells. Epidermal stem and transit-amplifying (TA) cells were isolated from footpads of 21-mo-old GFP^+ mice, injected into E3.5 d blastocysts, and tissues from resulting offspring sampled 4 mo after birth. Examples of dermis, heart, and liver are shown here. (*A*) Dermis from mouse derived from stem-injected blastocyst; (*B*) dermis from mouse derived from TA-injected blastocyst; (*C*) heart from mouse derived from stem-injected blastocyst; (*D*) heart from mouse derived from TA-injected blastocyst; (*E*) liver from mouse derived from stem-injected blastocyst; (*F*) liver from mouse derived from TA-injected blastocyst. Note, the actin in the cortical web of the liver autofluoreces and can be clearly seen in the liver from TA-injected blasotcysts; however, since no cytosolic fluorescence is seen in these liver sections, we can conclude that no GFP is present.

cells and their progeny survived into adulthood after the blastocyst injection, suggesting that the developmental environment in some way will be able to reprogram the stem cells to alter their cell fate, and to perhaps alter their rate of aging. These experiments are based upon only one generation and to fully explore altered rates of aging several more generations need to be examined.

Aged human epidermal stem cells express proteins similar to those found in young stem cells, but different from those found in TA cells In order to begin to determine whether human and mouse epidermal stem cells are similar and to test whether the altered aging phenomena found in the mouse is conserved in the human stem cells age, we examined the proteins by two-dimensional (2D) gel electrophoresis of human epidermal stem and TA cells isolated from tissues of various ages. We observed many proteins that showed no differences between the stem and TA cells, and no differences among the various ages (see Fig 3 for example). We also observed that some proteins were present in the stem cell populations, no matter what the age, but not in the TA cell populations (Fig 3, Table II). Using MALDI-TOF, we identified some of these differential proteins and made a list of proteins that will require further study (Table II). Two of the proteins that appear interesting and are highly expressed in the stem cells are heat-shock proteins that function in stress-related events. We also found that a myc-associated protein and that cyclin D1 were upregulated in the TA populations, which correlated to our finding of c-myc RNA expression in the mouse TA cells (see Table I) and previously published results on the cell cycle (Dunnwald *et al*, 2003). We also found some age differences in both the stem cells and the TA cells. Epidermal stem cells 65–75-y old expressed a growth arrest specific protein (GAS-7), whereas the older TA cells expressed a cell surface antigen-binding protein, an F-box protein, and a transcription factor. All of these will require further study to understand why these cells show these differences. As controls, we looked for expression of keratin 14 found in all basal keratinocytes, keratin 16 found in cultured keratinocytes, and the actin filament protein. As expected, expression of these proteins was the similar in all cells.

Discussion

We are interested in understanding the basic characteristics of somatic epidermal stem cells and whether the cells change their characteristics as we grow older. We had previously determined that neonatal stem cells, but not neonatal TA cells, could be reprogrammed by the developmental environment to produce multiple cell lineages, and that these altered cell lineages persisted into adulthood (Liang and Bickenbach, 2002). Our previous study suggested that neonatal epidermal stem cells had both an intrinsic stem cell property and an ability to respond to an extrinsic signal that distinguished them from the TA cells. We did not identify, however, specific stem cell genes or proteins, and we did not address whether epidermal stem cells lost these abilities with increasing age of the mouse. In this study, we discovered that epidermal stem cells isolated from neonates or from old adult mice show a similar plasticity response when injected into the developmental blastocyst environment, and that neither age of epidermal TA cells showed this type of plasticity response. We have also shown here that epidermal stem and TA cells express some different genes and show some differential protein expression that do not appear to be age related.

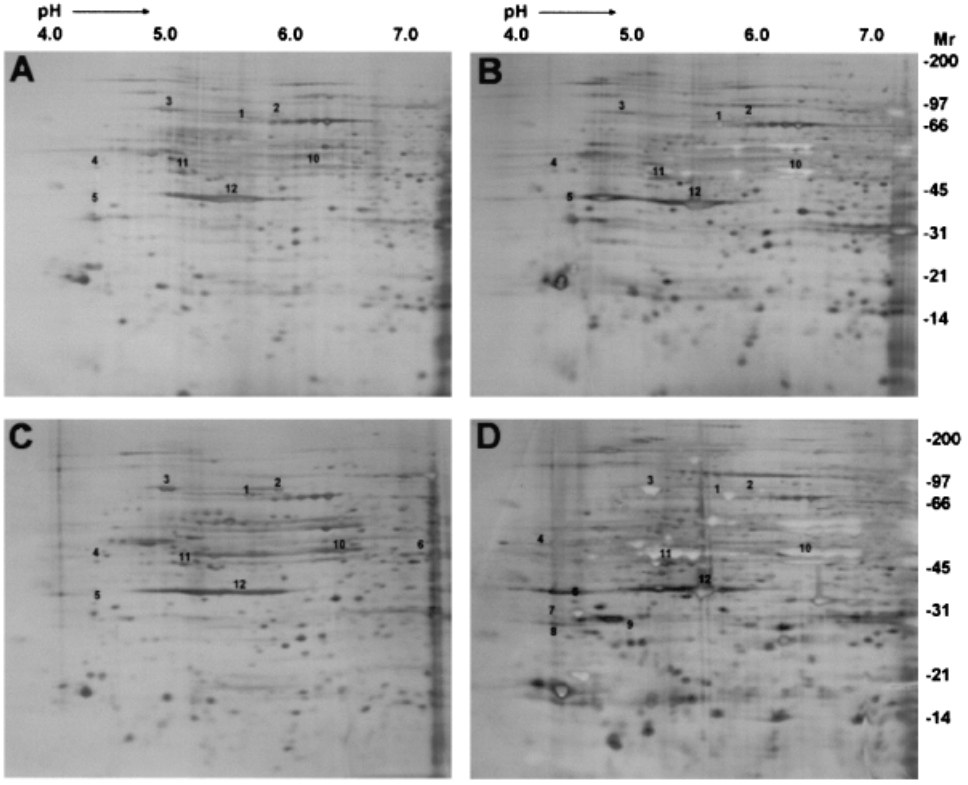

Figure 3
Two-dimensional (2D) gel electrophoresis of proteins isolated from stem cells and TA cells from 20- and 65-y-old human skin. Various ages of human epidermal cells were sorted into stem and TA cell populations, then 2D gel electrophoresis performed on the isolated proteins. Examples of 2D gels from two ages are shown here: (*A*) 20-y-old stem cells, (*B*) 20-y-old TA cells, (*C*) 65-y-old stem cells, (*D*) 65-y-old TA cells. Numbers on the gels sit just above the proteins listed in Table II, except for in D #8 is just below the protein. 1, HSC 71; 2, HSP 70; 3, glucose-regulated protein; 4, Myc-associated protein; 5, cyclin D1; 6, GAS-7; 7, CD2; 8, F-box protein 2; 9, CCR-4-NOT transcription complex; 10, keratin 14; 11, keratin 16; 12, actin.

Table II. Proteins expressed in sorted adult human epidermal stem and TA cells

	20–40 y[a]		65–75 y[a]		90 y[b]	
Proteins	**Stem**	**TA**	**Stem**	**TA**	**Stem**	**TA**
Heat shock cognet 71 (protects against apoptosis)	[c]	[d]	[c]	[d]	[c]	[d]
Stress protein 70 (protects against apoptosis)	[c]	[d]	[c]	[d]	[c]	[e]
Myc-associated protein	[d]	[c]	[d]	[c]	[d]	[c]
Cyclin D1	[d]	[c]	[d]	[c]	[d]	[c]
GAS-7 (growth arrest specific protein)	[e]	[e]	[c]	[e]	ND	ND
CD2 (antigen-binding protein)	[e]	[e]	[e]	[c]	ND	ND
F-box protein 2	[e]	[e]	[e]	[c]	ND	ND
CCR-4-NOT transcription complex	[e]	[e]	[e]	[c]	ND	ND
Glucose-regulated protein	[c]	[c]	[c]	[c]	[c]	[c]
Keratin 14	[c]	[c]	[c]	[c]	[c]	[c]
Keratin 16	[c]	[c]	[c]	[c]	[c]	[c]
Actin	[c]	[c]	[c]	[c]	[c]	[c]

[a] n = 3.
[b] n = 1.
[c] High expression.
[d] Only a little expression.
[e] Absent or too low to detect.
ND, not done or outside the area of the 2D gel that could be tested. TA, transit amplifying.

Until recently, this type of study has been hampered by the lack of homogenous populations to examine. Several published assays based upon cell surface markers, size, or *in vitro* adhesion have been shown to enrich for epidermal stem cells to varying degrees (Barrandon and Green, 1985; Mackenzie *et al*, 1989; Morris *et al*, 1990; Jones and Watt, 1993; Bickenbach, 1998; Li *et al*, 1998; Tani *et al*, 2000). Keratins 19 and 15 have also been proposed as epidermal stem cell markers, but these appear to mark hair follicle bulge stem cells, not interfollicular stem cells (Michel *et al*, 1996; Cotsarelis, 1999). We developed a laser cell sorting method that uses combined modifications of two other methods, one based on cell size and one based on the presence of an ABC transporter that can actively pump out the vital Hoechst 33342 dye in a verapamil-dependent manner (Barrandon and Green, 1985; Morris *et al*, 1990; Goodell *et al*, 1996, 1997; Dunnwald *et al*, 2001; Liang and Bickenbach, 2002). Although we originally designed this sorting method to yield a homogenous population of adult murine epidermal LRCs (Dunnwald *et al*, 2001), the later experiments determined that non-labeled neonatal and adult murine and human cells could be sorted while still preserving the quality and integrity of the stem and TA cells. We reported that only the stem cells could maintain an epidermis long term, and that only the stem cell population could be reprogrammed during the development of a mouse (Dunnwald *et al*, 2001; Liang and Bickenbach, 2002). How these epidermal stem cells become "reprogrammed" is not clear. It may be that they can specifically respond to the developmental environment or it may be that they have fused with the embryonic stem cell as reported for hematopoietic stem cells (Terada *et al*, 2002; Vassilopoulos *et al*, 2003; Wang *et al*, 2003). Either way, the injected epidermal stem cells responded, but the epidermal TA cells did not, thus demonstrating a fundamental difference between the two sorted cell populations.

We also found that these "reprogrammed" cells persisted into adulthood, a trait long proposed for all stem cells (Cairns, 1975; Lajtha, 1979; Potten, 1997). Long-term persistence of grafted cells has been reported for several tissues (for examples see Flowers *et al*, 1990; Bianchi *et al*, 1996; Krause *et al*, 2001). Such data suggest that stem cells exist in all tissues, and that stem cells may have a very long life. Whether stem cells age in the same sense as other cells age has not yet been determined. It could be that they age in a fashion similar to other cells, but it could also be that they are held out of the normal cellular aging processes and are used to replace dysfunctional or dead cells, thereby restoring failing tissues. Studies that support both views exist. On the one hand, the numbers and capacity of bone marrow stem cells to form bone marrow, myeloid cells, lymphoid cells, or bone marrow stroma were reported to decrease with increasing age (Quarto *et al*, 1995; Stephen *et al*, 1998; Marley *et al*, 1999; Offner *et al*, 1999). On the other hand, the numbers of murine hematopoietic stem cells were shown to fluctuate widely during aging and to correlate instead with the strain of mouse (de Haan and Van Zant, 1999). Furthermore, the ability of stem cells to respond to environmental influences may not be diminished with increasing age. Hematopoietic stem cells from old mice were shown to repopulate host mice in numbers similar to stem cells from young mice (Chen *et al*, 1999), which is similar to our findings for epidermal stem cells. Long-term *in vitro* assays may not also correlate with the age of the cell. In a reevaluation of skin fibroblast cell lines, Cristofalo *et al* (1998) found both long- and short-lived cell lines in cultures from all ages, and they concluded that the health status and biopsy conditions correlated better with long-term culture than did the age of the donor. They did, however, find higher proliferative potential in cell lines established from fetal or neonatal skin than in those established from adults. This is similar to the findings reported for human skin keratinocytes (Michel *et al*, 1997). In another study, examining growth properties of skin fibroblasts from centenarians and younger controls, a similar growth potential of fibroblasts irrespective of the age of the donor was found (Tesco *et al*, 1998). Thus, Cairns (1975) postulation that stem cells may live as long as the organism may be correct.

How stem cells survive this long is not clear, but may be due to an intrinsic, yet to be definitively identified, set of stem cell genes. One likely candidate might have been telomerase, which has been shown to be upregulated in direct relation to telomere length in fibroblasts and cell proliferation (Hiyama *et al*, 1995; Harle-Bachor and Boukamp, 1996; Masutomi *et al*, 2003), It has not been found, however, in the isolated population of stem cells of either the hematopoietic

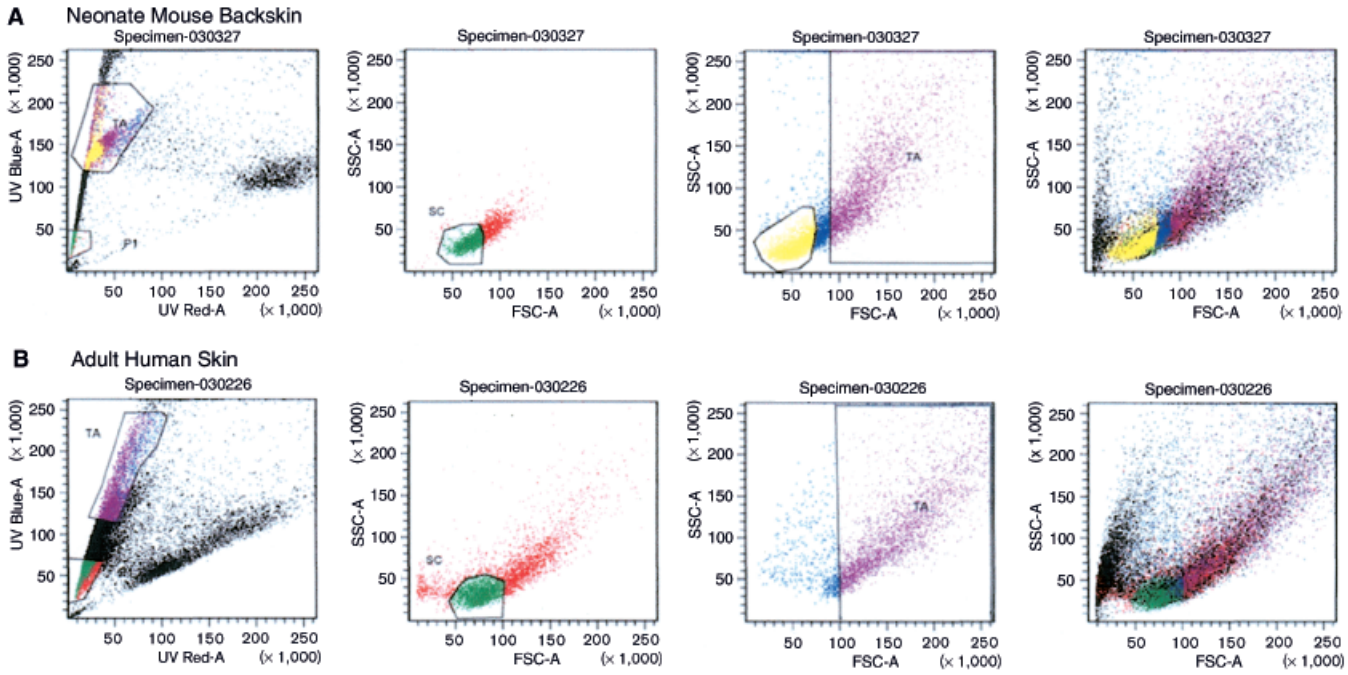

Figure 4
Scatter diagrams of stem and transit-amplifying (TA) cell sort parameters for mouse and human epidermal cells. Stem cells are first sorted according to low Hoechst red/blue fluorescence (green in *panel 1* in *A* and *B*) and then sorted by small size (green in *panel 2* in *A* and *B*). TA cells are sorted by high Hoechst red/blue fluorescence (purple in *panel 1* in *A* and *B*), then by size (purple in *panel 3* in *A* and *B*). *Panel 4* in *A* and *B* show relative sizes of all populations. Note similarity of scatter plots for neonate mouse backskin cells (*A*) as compared with adult human skin cells (*B*).

system (Hiyama *et al*, 1995; Chiu *et al*, 1996) or the skin (Ramirez *et al*, 1997; Bickenbach *et al*, 1998; Masutomi *et al*, 2003). Several other genes may also be candidates. Some genes such as CD71, β-catenin, and PA-FAB, which are upregulated in highly proliferative cells may specifically mark the TA cell population (Li *et al*, 1998; Zhu and Watt, 1999; O'Shaughnessy *et al*, 2000). Other genes, such as α6 integrin and β1 integrin may be regulated between the stem and TA cell compartments, but may also require the activation of several other genes to fully distinguish the two compartments (Li *et al*, 1998; Jensen *et al*, 1999). Genes, such as p63, which are upregulated in cells at the edge of appendage development or the edge of epithelial differentiation, such as at the limbal cornea junction, may be also involved in stem cell maintenance (Mills *et al*, 1999; Yang *et al*, 1999; Pellegrini *et al*, 2001), whereas activation of transcription factors such as c-myc, Lef-1, or Tcf-3, may drive epidermal stem cells to differentiate along a specific epithelia lineage pathway (Gandarillas and Watt, 1997; Fuchs and Segre, 2000; Merril *et al*, 2001; Waikel *et al*, 2001; Watt, 2002). Other genes, such as those in the delta–notch pathway, may signal the boundary between the stem cell and the TA cell compartments via differential regulation (Lowell *et al*, 2000). As yet, we do not have a definitive picture of how stem cells are regulated. It is also possible that a unique set of stem cell genes provides these cells with the ability to sort their DNA strands into original and duplicates. If so, stem cells could retain the original strand and thereby the integrity of the DNA for the future. This type of DNA segregation has been shown to be possible in the epithelia of the small intestine (Potten *et al*, 2002), but is only proposed to be possible in other tissues including the skin (Cairns, 1975, 2002). Our data suggest that stem cells have an intrinsic property that distinguishes them from the TA cells, and that stem cells also can be reprogrammed by extrinsic factors in the developmental environment, a finding that may be a general phenomenon evolutionarily inherited (Wei *et al*, 2000). Whether stem cells in general or epidermal stem cells specifically contain a unique set of genes remains to be determined.

Conclusion

In a general way, we propose that a stem cell is first defined in the developing blastocyst, before (or as) the embryoblast cells are imprinted or committed. This may be a direct response to its environment. As the organism develops, we hypothesize that these stem cells are distributed throughout all tissues where they reside in stem cell niches, and retain an ability to respond as an uncommitted stem cell if needed.

This ability gives them an intrinsic "stemness" that keeps them fundamentally different from the rest of the proliferative cells, thereby allowing the stem cells to respond to environmental signals in a "stem cell-like manner." In aging, it seems likely that both the stem cell itself and its environmental niche in the tissue may be important. Cairns (1975, 2002) suggested that stem cells that accumulate too much damage may be specifically eliminated. This may be what happens in the telomerase knockout mice that show premature aging and eventual death, but not until the fifth or sixth generation (Blasco *et al*, 1997; Liu *et al*, 2004). This type of reasoning may also be true for the stem cell's niche, in that damage to the surrounding cells and/or changes

in the macromolecules that make up the niche may result in loss of proper environmental input, which could lead to a "functional death" of that stem cell. Aging could be the irreversible accumulation of such defects, which results in continual depletion of stem cells until the tissue ultimately fails. This model is still consistent with the idea that stem cells may be intrinsically different from TA cells, and that old stem cells may retain their function as long as their niche is maintained.

Materials and Methods

Epidermal stem cell and TA cell isolation Epidermal stem and TA cells were prepared from neonatal mouse backskin, adult mouse footpad skin, and adult human skin in a similar manner. Wild-type C57BL/6 mice and transgenic C57BL/6 mice carrying the EGFP transgene (TgN(ACTbEGFP)1Osb) were originally obtained from Jackson Labs (Bar Harbor, Maine) and breeding colonies of both were established and maintained in The University of Iowa's Animal Care Facility under the direct supervision of a certified veterinarian. Mice were anesthetized then euthanized as recommended by the Panel on Euthanasia of the American Veterinary Medical Association and with full approval of The University of Iowa ACURF. Adult human skin was obtained as discarded specimens from the Surgical Pathology Department in The University of Iowa Hospitals and Clinics with full approval of The University of Iowa IRB. Tissues were used without regard to sex, race, or ethnic background, but age was recorded. Skin tissues were dissected and cut into strips, then incubated in dispase II overnight at 4°C. The epidermis was mechanically removed from the dermis, and the basal cells were dissociated by incubating the epidermis in 0.25% trypsin at 37°C for 30 min. The dissociated basal cells were centrifuged and resuspended at 5×10^6 cells per mL in SMEM (Life Technologies Inc., Rockville, Maryland) with 0.05 mM Ca^{2+}, 1 mM Hepes, 1% penicillin–streptomycin–actinomycin D, and 5 μg per mL Hoechst 33342. Cells were incubated at 37°C for 90 min, centrifuged, resuspended in 1 μg per mL propidium iodide in SMEM, and kept on ice until the flow cytometric procedure. A modification of previously reported methods for sorting stem and TA cells (Dunnwald *et al*, 2001; Liang and Bickenbach, 2002) was performed on a Becton Dickinson FACS DiVa (Bectin-Dickinson, Franklin Lakes, New Jersey). Forward and orthogonal scatter signals were generated using 100 mW Argon Ion Laser at 488 nm. Hoechst 33342 and propidium iodide was excited with 100 mW of a Krypton Laser (351–364 nm). Hoechst fluorescence was measured through a 440/60 nm bandpass filter whereas propidium iodide was measured through a 670/14 nm bandpass filter. Both mouse and human epidermal stem cells were sorted using gates placed around the population exhibiting low Hoechst and propidium iodide fluorescence and low forward and orthogonal scatter; TA cells were sorted with gates placed around the population exhibiting medium to high Hoechst fluorescence and medium to high orthogonal scatter (in Fig 4, stem cells, green; TA cells, purple). It is important to note that the stem cell population could not be sorted on Hoechst dye exclusion alone because not all low Hoechst dye cells showed high proliferative potential or could maintain an epidermis (see red cells in Fig 4). Also, stem cells could not be sorted using only small size because many TA cells were also small in size, but they did not show high proliferation and could not maintain an epidermis (see blue and yellow cells in Fig 4) (Dunnwald *et al*, 2001).

Blastocyst injection To test whether aged epidermal stem cells could function in a manner similar to neonatal epidermal stem cells during development, we injected GFP^+ epidermal stem or TA cells from the footpads of 21-mo-old-transgenic EGFP mice into 3.5-d blastocysts from C57BL/6 non-transgenic mice. We compared the offspring derived from blastocysts injected with aged stem or TA cells to offspring derived from blastocysts injected with neonatal stem or TA cells. We could easily distinguish cell lineages derived from the GFP^+ cells from those formed from the inner cell mass of the C57BL/6 non-transgenic blastocyst. The injection of epidermal cells was performed by personnel in The University of Iowa Gene Targeting Core and the procedure was similar to injection of ES cells. Each C57BL/6 blastocyst was injected with 10 GFP^+ epidermal stem or TA cells. Approximately 10 blastocysts were transferred to the uterus of each ICR pseudopregnant recipient mother. The procedure for adult footpad cells was performed three times with approximately three mothers each receiving stem cell-injected blastocysts or TA cell-injected blastocysts. Around 50% of all mothers became pregnant; all pregnant mothers gave birth to approximately five pups each. There appeared to be no specific detrimental effect in the resultant pups from either stem cell-injected or TA cell-injected blastocysts. For comparison, ten blastocysts injected with either ten GFP^+ neonatal stem or ten neonatal GFP^+ TA cells were transferred each to one ICR pseudopregnant recipient mother. All resultant offspring were allowed to grow normally for 2–4 mo.

Histology Tissues were harvested at 60 d and at 4 mo after birth. Tissues were cryopreserved in 30% sucrose, embedded in OCT compound (Electron Microscopy Sciences, Washington, Pennsylvania), frozen in liquid nitrogen, and blocks stored at −80°C. Sections were made at 8–10 μm on a cryostat, and the slides stored at −80°C. Each slide containing four serial sections was fixed for 15 min–1 h in 4% paraformaldehyde at room temperature, then sections were examined for GFP expression and adjacent sections stained with hematoxylin and eosin.

RNA isolation RNA was isolated from the sorted stem and TA cells by using the Trizol LS method (Invitrogen, Carlsbad, California). The cells directly from the flow cytometer were centrifuged at $200 \times g$, all but 30 μL of the supernatant was removed and ∼ 60 μL of DEPC water was added to the pellet to a total aqueous volume of 100 μL. Then 300 μL of Trizol LS was added and allowed to sit at room temperature for 5 min, then 200 μL chloroform was added for every 750 μL of trizol, the samples mixed and incubated at room temperature for 15 min and then centrifuged for 15 min at $12{,}000 \times g$ at 4°C. The aqueous phase was removed from the tube, and placed in a new RNAse free tube. The organic phase and the interphase were saved for DNA and protein isolation. 0.5 μL of a 20 g per liter glycogen solution was added to the aqueous phase, vortexed briefly, then 0.5 mL of 2-propanol was added for every 0.75 mL of trizol, mixed well, and incubated at room temperature for 10 min. The sample was then centrifuged for 15 min at $12{,}000 \times g$ at 4°C, the supernatant removed and 1 mL of 75% ethanol added to the pellet for each 0.75 mL of trizol, and vortexed well. The sample was centrifuged at $7500 \times g$ for 5 min at 4°C, the supernatant removed, the sample air-dried for 5 min at room temperature, then resuspended in ∼ 25 μL of RNAse free water per 1 million cells (to ∼ 1 μg RNA per liter). Aliquots were stored at −80°C.

Real-time RT-PCR Total RNA, isolated from 3.75×10^5 cells, was first treated with DNase I at room temperature for 5 min to remove any residual genomic DNA, then the RNA was reverse transcribed using the TaqMan Reverse Transcription (RT) Kit (Applied Biosystems, Foster City, California) according to the manufacturer's recommendation for a final volume of 30 μL. Real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems), with 1–3 μL of the RT product in a final volume of 25 μL for PCR. Real-time PCR was performed with the first cycle at 50°C for 2 min, 95°C for 10 min, then at 95°C for 15 s; at 60°C for 1 min for each of 40 cycles. Fluorescent emissions were detected on the ABBI PRISM 7700 Sequence Detection System (Applied Biosystems). Expression levels of the target genes (x) were shown as ΔCt values standardized with GAPDH as an endogenous reference (r), using the following equation: $\Delta Ct = Ct(x) - Ct(r)$. Relative expression levels were compared between the stem and the TA

cells, such that the levels of expressions of the target genes in the TA cells relative to the level of expression of the target gene in the stem cells were determined by the following formula: $2^{-((Ct,x,TA)-Ct,r,TA)-(Ct,x,SC-Ct,r,SC))}$.

The sequences of primers used were as follows: (ABCG2, sense, 5′-GCA CCT CAA CCT GCC CAT T-3′, antisense, 5′-AGG GTG CCC ATC ACA ACG-3′) (p63, sense, 5′-CAA GTA TCG GAC AGC GCA AAG-3′, antisense, 5′-TCT TGA TGG AAG TCA TCT GGA TTC-3′) (integrin α6, sense, 5′-GAT GTC ACC GCT GCT GCT C-3′, antisense, 5′-GGG AAA CAC CGT CAC TCG AA-3′) (integrin β1, sense, 5′-GCA ACA ATG AAG CTA TCG TGC A-3′, antisense, 5′-CAC GCC TGC TAC AAT TGG G-3′) (CD71, sense, 5′-TTG TCA GAG AAG TTG AAC TCC ATA GAG-3′, antisense, 5′-TTT TCA ATA TAA TAG GCA AGA CTT TCA TCT-3′) (c-myc, sense, 5′-CGA GCT GTT TGA AGG CTG GAT-3′, antisense, 5′-GGC ATC GTC GTG GCT GTC-3′) (GAPDH, sense, 5′-AAA ATG GTG AAG GTC GGT GTG-3′, antisense, 5′-GCC ACT GCA AAT GGC AGC-3′).

Protein isolation To isolate the protein from sorted cells, ethanol was added to the interphase and organic phases of the Trizol LS described above, then mixed well, incubated for 2 min, centrifuged, and the supernatant removed. (The pellet can be saved for DNA isolation.) To the supernatant, 2-propanol was added, incubated, centrifuged, and the resulting pellet washed with guanidine hydrochloride in 95% ethanol. Then 100% ethanol was added to the pellet and vortexed, then centrifuged and vacuum dried. The protein was dissolved in rehydration buffer (see below) and stored at −80°C. The amount of protein was determined by a Bradford assay.

2D gel electrophoresis Twenty micrograms of protein from stem or TA cells was measured, lyophilized, and dissolved in 125 μL of rehydration buffer (9.8 M urea, 4% CHAPS, 100 mM DTT, 0.2% ampholine, 0.001% bromophenol blue). Isoelectric focusing was carried out in Bio-Rad PROTEAN IEF System (Bio-Rad, Hercules, California) using their protocol. Each sample was loaded into a 7 cm strip holder tray with an IPG strip (70 × 3 × 0.5 mm, pH 3.0–10.0, or pH 4.0–7.0 linear). The IPG strips were rehydrated in active mode at 50 V for 12 h at 20°C and then subjected to 250 V for 15 min in step S1, and a 4000 V for 2 h in step S2, and in step 3 the final voltage reached 10,000 V. The run was carried out until it attained a total 20,000 V h at 50 μA current per strip. The IPG strips were then equilibrated 2 × 10 min with equilibration buffer (50 mM Tris-HCl, pH 7.5, 6 M urea, 30% glycerol, 2% SDS), first with 0.5% dithiothreitol (DTT), then with the DTT replaced by 4.5% iodoacetamide, then equilibrated again. For the second dimension, the IPG strips were placed onto 12% SDS polyacrylamide vertical slab gels (1.0-mm thick), and sealed with 0.2% agarose. SDS-PAGE was carried out in the Bio-Rad Mini-Protean 3 Electrophoresis Cell at a current of 35 mA per gel until the dye front reached the base of the gel. The proteins were visualized by sliver staining.

Protein identification Differentially expressed protein spots were excised, destained, washed with ultra-pure water 3 × 10 min, dehydrated with 100% methanol, washed with 100 mM ammonium bicarbonate containing 30% acetonitrile for 10 min, and the gel pieces dried in a SpeedVac (Savan Instruments, Farmingdale, New York) for 30 min after washing with ultra-pure water. The dry gel pieces were resuspended in 50 mM ammonium bicarbonate, with 5–10 ng per μL sequence grade trypsin, then incubated overnight at 37°C. As controls, spots were cut from blank regions of the gel and processed similarly. The next day the peptides from the gel pieces were extracted by two washes of 50% acetonitrile containing 0.1% trifluoroacetic acid at room temperature, then the samples were concentrated to 4–5 μL using a SpeedVac. For the MALDI-TOF/MS, fast evaporation matrix surface were prepared according to Jensen *et al* (1997). A saturated solution of α-cyano-4-hydroxy-trans-cinnamic acid was prepared in acetonitrile:TFA (0.1%) 50:50. Two microliters of protein sample and 3 μL of α-cyano-4-hydroxy-trans-cinnamic acid saturated solution were then mixed, dried, and loaded onto the MALDI-TOF probe. After inserting the samples into the mass spectrometer, MALDI was performed in The University of Iowa Protein Core Facility. Peptide values obtained after MALDI-TOF/MS were used to identify the proteins via PeptIdent, a tool that allowed identification of proteins using their pI, Mr (molecular weight), and peptide mass fingerprinting. Experimentally measured, user-specified peptide masses were compared with the theoretical peptides calculated for all proteins in the Swiss-Prot/TrEMBL database (http://us.expasy.org/tools/peptident.html). The following search parameters were used (database: Swiss-Prot/TrEMBL, taxonomy category: *Homo sapiens* (human) protein mass range: 0–3000 kDa, protein pI range: 0.0–14.0, search for: single protein only, digest chemistry: trypsin, max missed cut: 1, modifications: none, charge state: monoisotopic, tolerance 0.5 Da. Identified proteins were compared with the keratinocyte 2D gels published on the Danish Centre for Human Genome Research web site maintained by Professor J. E. Celis and Dr M. Østergaard; (http://proteomics.cancer.dk/).

We thank the other members of the Bickenbach lab for helpful discussions, Justin Fishbaugh and Gene Hess in the UI Flow Cytometry Core for their excellent assistance in sorting the epidermal stem cells, the staff of the UI Gene Targeting Core for their technical expertise in injecting the epidermal cells into the blastocysts, and Dr Baoli Yang, Director, for his excellent advice. This project was supported by a Focused Giving Award from Johnson and Johnson (JRB), and by grants from the National Institute for Craniofacial and Dental Research (RO1 DE13001, JRB), National Institute for Arthritis, Musculoskeletal, and Skin Diseases (R21 AR48398, JRB & LL), and National Institute of Aging (RO1 AG020913, JRB & SC) at The National Institutes of Health.

DOI: 10.1111/j.1087-0024.2004.09309.x

Manuscript received October 1, 2003; revised March 4, 2004; accepted for publication March 12, 2004

Address correspondence to: Jackie R. Bickenbach, Department of Anatomy and Cell Biology, 1-457 BSB, Carver College of Medicine, The University of Iowa, 51 Newton Road, Iowa City, Iowa 52242, USA. Email: jackie-bickenbach@uiowa.edu

References

Barrandon Y, Green H: Cell size as a determinant of the clone-forming ability of human keratinocytes. Proc Natl Acad Sci USA 82:5390–5394, 1985

Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA: Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci USA 93:705–708, 1996

Bickenbach JR: Identification of label-retaining cells in oral mucosa and skin. J Dent Res 122C:1611–1620, 1981

Bickenbach JR: Selection and growth of epidermal stem cells. In: Savage LM (ed). Bioengineering of Skin Substitutes. Southborough, MA: IBC Library Series, 1998; p 75–92

Bickenbach JR, Mackenzie IC: Identification and localization of label-retaining cells in hamster epithelia. J Invest Dermatol 82:618–622, 1984

Bickenbach JR, McCutcheon J, Mackenzie IC: Rate of loss of tritiated thymidine label in basal cells in mouse epithelial tissue. Cell Tissue Kinet 19: 325–333, 1986

Bickenbach JR, Vormwald-Dogan V, Bachor C, Bleuel K, Schnapp G, Boukamp P: Telomerase is not an epidermal stem cell marker and is downregulated by calcium. J Invest Dermatol 111:1045–1052, 1998

Blasco M, Lee H, Hande M, Samper E, Lansdorp P, DePinho R, Greider C: Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 91:25–34, 1997

Cairns J: Mutation selection and the natural history of cancer. Nature 255: 197–200, 1975

Cairns J: Somatic stem cells and the kinetics of mutagenesis and carcinogenesis. Proc Natl Acad Sci USA 99:10567–10570, 2002

Chen J, Astle CM, Harrison DE: Development and aging of primitive hematopoietic stem cells in BALB/cBy mice. Exp Hematol 27:928–935, 1999

Chiu C-P, Dragowska W, Kim NW, *et al*: Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. Stem Cells 14:239–248, 1996

Cotsarelis G: Epithelial stem cells in the skin: Definition, markers, functions, and localization. Viewpoint #1. Exp Dermatol 8:80–88, 1999

Cotsarelis G, Sun T-T, Lavker RM: Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61:1329–1337, 1990

Cristofalo VJ, Allen RG, Pignolo RJ, Martin BG, Beck JC: Relationship between donor age and the replicative lifespan of human cells in culture: A reevaluation. Proc Natl Acad Sci USA 95:10614–10619, 1998

de Haan G, Van Zant G: Dynamic changes in mouse hematopoietic stem cell numbers during aging. Blood 93:3294–3301, 1999

Dunnwald M, Chinnathambi S, Alexandrunas D, Bickenbach J: Mouse epidermal stem cells proceed through the cell cycle. J Cell Physiol 195:194–201, 2003

Dunnwald M, Tomanek-Chalkley A, Alexandrunas D, Fishbaugh J, Bickenbach JR: Isolating a pure population of epidermal stem cells for gene use in tissue engineering. Exp Dermatol 10:45–54, 2001

Flowers MED, Stockschlaeder MAR, Schuening FG, Niederwieser D, Hackman R, Miller AD, Storb R: Long-term transplantation of canine keratinocytes made resistant to G418 through retrovirus-mediated gene transfer. Proc Natl Acad Sci USA 87:2349–2353, 1990

Fuchs E, Segre JA: Stem cells: A new lease on life. Cell 100:143–155, 2000

Gandarillas A, Watt FM: c-Myc promotes differentiation of human epidermal stem cells. Genes Dev 11:2869–2882, 1997

Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC: Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. J Exp Med 183:1797–1806, 1996

Goodell MA, Rosenzweig M, Kim H, *et al*: Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 3:1337–1345, 1997

Harle-Bachor C, Boukamp P: Telomerase activity in the regenerative basal layer of the epidermis in human skin and in immortal and carcinoma-derived skin keratinocytes. Proc Natl Acad Sci USA 93:6476–6481, 1996

Hiyama K, Hirai Y, Kyoizumi S, *et al*: Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. J Immunol 155:3711–3715, 1995

Jensen ON, Podtelejnikov AV, Mann M: Identification of the components of simple protein mixtures by high-accuracy peptide mass mapping and database searching. Anal Chem 69:4741–4750, 1997

Jensen UB, Lowell S, Watt FM: The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: A new view based on whole-mount labeling and lineage analysis. Development 126:2409–2418, 1999

Jones PH, Watt FM: Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 73:713–724, 1993

Krause DS, Theise ND, Collector MI, *et al*: Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105:369–377, 2001

Lajtha G: Stem cell concepts. Differentiation 14:23–34, 1979

Lavker RM, Sun T-T: Heterogeneity in epidermal basal keratinocytes: Morphological and functional correlations. Science 215:1239–1241, 1982

Lavker RM, Sun T-T: Epidermal stem cells: Properties, markers, and location. Proc Natl Acad Sci USA 97, 2000

Li A, Simmons PJ, Kaur P: Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. Proc Natl Acad Sci USA 95:3902–3907, 1998

Liang L, Bickenbach JR: Somatic epidermal stem cells can produce multiple cell lineages during development. Stem Cells 20:3–10, 2002

Liu L, DiGirolamo C, Navarro P, Blasco M, Keefe D: Telomerase deficiency impairs differentiation of mesenchymal stem cells. Exp Cell Res 294:1–8, 2004

Lowell S, Jones P, Le Roux I, Dunne J, Watt FM: Stimulation of human epdiermal differentiation by Delta–Notch signalling at boundaries of stem-cell clusters. Curr Biol 10:491–500, 2000

Mackenzie IC, Mackenzie SL, Rittman GA: Isolation of subpopulations of murine epidermal cells using monoclonal antibodies against differentiation-related cell surface molecules. Differentiation 41:127–138, 1989

Marley SB, Lewis JL, Davidson RJ, Roberts IA, Dokal I, Goldman JM, Gordon MY: Evidence for a continuous decline in haemopoietic cell function from birth: Application to evaluating bone marrow failure in children. Br J Haematol 106:162–166, 1999

Masutomi K, Yu E, Khurts S, *et al*: Telomerase maintains telomere structure in normal human cells. Cell 114:241–253, 2003

Merril BJ, Gat U, DasGupta R, Fuchs E: Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. Genes Dev 15:1688–1705, 2001

Michel M, L'Heureux N, Auger FA, Germain L: From newborn to adult: Phenotypic and functional properties of skin equivalent and human skin as a function of donor age. J Cell Physiol 171:179–189, 1997

Michel M, Torok N, Godbout M-J, Lussier M, Gaudreau P, Royal A, Germain L: Keratin 19 as a biochemical marker of skin stem cells *in vivo* and *in vitro*: Keratin 19 expressing cells are differentially localized in function of anatomic sites, and their number varies with donor age and culture stage. J Cell Sci 109:1017–1028, 1996

Mills A, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A: p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398: 708–713, 1999

Morris RJ, Fischer SM, Klein-Szanto AJP, Slaga TJ: Subpopulations of primary adult murine epidermal basal cells sedimented on density gradients. Cell Tissue Kinet 23:587–602, 1990

Morris RJ, Potten CS: Highly persistent label-retaining cells in the hair follicles of mice and their fate following induction of anagen. J Invest Dermatol 112:470–475, 1999

Offner F, Kerre T, De Smedt M, Plum J: Bone marrow CD34+ cells generate fewer T cells *in vitro* with increasing age and following chemotherapy. Br J Hematol 104:801–808, 1999

O'Shaughnessy RFL, Seery JP, Celis JE, Frischauf A-M, Watt FM: PA-FABP, a novel marker of human epidermal trnsist amplifying cells revealed by 2D protein gel electrophoresis and cDNA array hybridization. FEBS Lett 486:149–154, 2000

Pellegrini G, Dellambra E, Golisano O, *et al*: p63 identifies keratinocyte stem cells. Proc Natl Acad Sci USA 98:3156–3161, 2001

Potten CS: Stem Cells. London: Academic Press, 1997

Potten CS, Hendry HH: Clonogenic cells and stem cells in the epidermis. Int J Radiat Biol 24:537–540, 1973

Potten CS, Owen G, Booth D: Intestinal stem cells protect their genome by selective segregation of template DNA strands. J Cell Sci 115:2381–2388, 2002

Quarto R, Thomas D, Liang CT: Bone progenitor cell deficits and the age-associated decline in bone repair capacity. Calif Tiss Int 56:123–129, 1995

Ramirez RD, Wright WE, Shay JW, Taylor RS: Telomerase activity concentrates in the mitotically active segment of human hair follicles. J Invest Dermatol 108:113–117, 1997

Stephen RP, Reilly CR, Witte PL: Impaired ability of bone marrow stromal cells to support B-lymphopoiesis with age. Blood 91:75–88, 1998

Tani H, Morris RJ, Kaur P: Enrichment for murine keratinocyte stem cells based on cell surface phenotype. Proc Natl Acad Sci USA 97:10960–10965, 2000

Terada N, Hamazaki T, Oka M, *et al*: Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:482–485, 2002

Tesco G, Vergelli M, Grassilli E, *et al*: Growth properties and growth factor responsiveness in skin fibroblasts from centenarians. Biochem Biophys Res Comm 244:912–916, 1998

Vassilopoulos G, Wang P, Russell D: Transplanted bone marrow regenerates liver by cell fusion. Nature 422:901–904, 2003

Waikel RL, Kawachi Y, Waikel PA, Wang X-J, Roop DR: Deregulated expression of c-Myc depletes epidermal stem cells. Nat Genet 28:165–168, 2001

Wang XJ, Willenbring H, Akkari Y, *et al*: Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 422:897–901, 2003

Watt F: The stem cell compartment in human interfollicular epidermis. J Dermatol Sci 28:173–180, 2002

Wei G, Schubiger G, Harder F, Muller AM: Stem cell plasticity in mammals and transdetermination in Drosophil: Common themes? Stem cells. 18:409–414, 2000

Yang A, Schweitzer R, Sun D, *et al*: p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398:714–718, 1999

Zhu AJ, Watt FM: β-catenin signaling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. Development 126:2285–2298, 1999