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Isolation of a mesenchymal cell population from murine dermis that contains progenitors of multiple cell lineages

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Abstract

The skin contains two known subpopulations of stem cells/epidermal progenitors, a basal keratinocyte population found in the interfollicular epithelium and cells residing in the bulge region of the hair follicle. The major role of the interfollicular basal keratinocyte population may be epidermal renewal, whereas the bulge population may only be activated and recruited to form a cutaneous epithelium in case of trauma. Utilizing three-dimensional cultures of murine skin under stress conditions in which only reserve epithelial cells would be expected to survive and expand, we demonstrate that a mesenchymal population resident in neonatal murine dermis has the unique potential to develop an epidermis *in vitro*. In monolayer culture, this dermal subpopulation has long-term survival capabilities in restricted serum, and inducible capacity to evolve into multiple cell lineages, both epithelial and mesenchymal, depending on culture conditions. When grafted subcutaneously, this dermal subpopulation gave rise to fusiform structures, reminiscent of disorganized muscle, that stained positive for smooth muscle actin and desmin; on typical epidermal grafts, abundant melanocytes appeared throughout the dermis that were not associated with hair follicles. The multipotential cells can be repeatedly isolated from neonatal murine dermis by a sequence of differential centrifugation and selective culture conditions. These results suggest that progenitors capable of epidermal differentiation exist in the mesenchymal compartment of an abundant tissue source and may have a function in mesenchymal-epithelial transition upon insult; moreover these cells could be available in sufficient quantities for lineage determination or tissue engineering applications.

Keywords

mesenchymal progenitors; epidermal precursors; murine organotypic culture; keratinocytes; murine dermis

Introduction

Skin bioengineering has advanced the production of *in vitro* human skin reconstructs for trauma patients with damaged areas too large to repair by the natural wound healing process (1). However, the growth of mouse skin reconstructs *in vitro* has not been as successful to date. Three-dimensional murine skin cultures would be useful to specifically study epidermal-mesenchymal cell signaling. Through the power of mouse genetics and utilizing primary cells derived from genetically altered mouse skins, the underlying biology of a number of diseases

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would be studied. *In vitro* skin regeneration would be a useful model for the study of genetic determinants of wound healing. Human and mouse skin differ in epidermal thickness and appendage content, and it is thus possible that the niche responsible for epidermal renewal during wound healing may be different in mouse and human (2) (3) (4).

Many adult tissues contain populations that have renewal ability under circumstances such as trauma, disease or aging (1) (5). There is also ample evidence that a population of skin specific precursors capable of restoring skin integrity upon insult resides in a niche identified as the bulge region of the hair follicle (6) (7) (8). Another such population, most likely responsible for normal epidermal homeostasis is known to reside in the interfollicular basal layer of the epidermis (9) (10).

Epidermal-mesenchymal interactions are necessary to provide the context for tissue development (11), for differentiation (12), and for tissue renewal upon injury (13) (14). In skin, the mesenchyme immediately adjacent to the hair follicle epithelium has an inductive effect on follicle development. Specialized mesenchymal cells, called dermal papilla cells are embedded in the lower portion of the hair follicle and surrounded by undifferentiated epithelial cells. These fibroblast-like dermal papilla cells produce and respond to the signals that regulate hair cycles (15) (16). Hair could be re-grown when hair follicles were grafted onto nude mice with dermal papilla cells and not in their absence (17). Furthermore, the characteristics of the evolving hair were determined by the species of origin of the dermal papilla cell preparations. These findings could be expanded and explained with an *in vitro* model for murine epidermis.

In establishing such a culture system, we were unsuccessful in adapting methods reported for human epidermal cells in human skin reconstructions. However, selected epidermal subpopulations had the ability to survive, while cultured in cell-to-cell contact with a dermal component, at the air-liquid interface under culture conditions that had previously been reported (18). During the course of these studies, we identified a dermal subpopulation that could, by itself, expand in culture and express epidermal markers, while cultured in the absence of contact with a dermal component, at the air-liquid interface under epidermal growth conditions. This subpopulation had the ability to survive in minimal medium conditions over time, a property that might be expected of a progenitor population. For the purposes of these studies, a progenitor cell has the capacity to create progeny that are more differentiated than itself and the ability to survive and propagate under minimal culture conditions. Further analysis of the properties of our isolated subpopulation, indicate characteristics consistent with a multipotent mesenchymal progenitor population.

Materials and Methods

Tissue culture media and other chemicals

Minimum essential medium (S-MEM) without calcium (Gibco, Rockville, MD) was used to prepare the main two types of culture media. Standard medium was S-MEM containing 8% fetal bovine serum (Gemini BioProducts) treated with Chelex resin (Bio-Rad laboratories, Hercules, CA). High calcium medium contained 1.4 mM Ca^{2+} . This medium was typically used to plate and attach keratinocytes or hair follicle buds and to culture some of the dermal derived populations unless stated. Low calcium medium contained 0.05 mM Ca^{2+} , typically used when cultures were grown under submerged conditions to allow for basal keratinocyte proliferation. In some experiments cells were cultured in medium containing 2% serum. All other chemicals were from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. KGF (R&D systems) was used at 8.4 ng/ml in the three dimensional cultures.

Skin fractionation

Cellular subfractionation from both epidermis and dermis of neonatal BALB/c mice is summarized in Fig.1A and explained in detail in Supplementary Methods.

Epidermal fraction, thereafter abbreviated Epi was prepared as described (19). Dermal fractions were made following a previously described method of preparing fibroblasts from the skin (20). Briefly, dermises were separated from epidermis by overnight trypsin incubation and chopped coarsely in 2 ml of 0.35% collagenase (Worthington, Lakewood, NJ) per dermis. Collagenase suspension was incubated with agitation in a water bath at 37°C for 30 minutes and DNase I (Worthington; 20,000 units/ml in PBS) was added at 125 µl/ml of collagenase suspension and incubated for 10 minutes. This suspension was filtered through a coarse 100 micron Nytex filter and diluted to 100 ml per 5 dermises. Tubes were centrifuged at 800 rpm for 5 minutes, and the supernatant was placed in new tubes to be centrifuged at 1400 rpm for 5 minutes. The pellets obtained at the end of this spin were pooled and designated as Fraction D.

The 800-rpm pellet was dissociated and centrifuged at 300 rpm for 5 minutes yielding dermal-derived hair follicles, abbreviated DHF. Supernatant from the 300-rpm pellet was twice centrifuged at 1000 rpm for 5 minutes and both pellets were pooled and designated fraction E.

Organotypic (three-dimensional) cultures

Cells were allowed to adhere overnight to the upper side of an insert membrane while suspended in 1.4 mM Ca^{2+} medium. Insert membranes used were Millipore® (Multiwell), uncoated, 12 mm diameter, abbreviated MU membrane (both 0.4 or 3.0 µm pore size used), and Collagen-Coated Transwell®-COL Inserts (Corning Inc., Acton MA), 12 mm diameter abbreviated CC membrane (0.4 or 3.0 µm pore size). These membranes were coated an equimolar mixture of collagen (Type I and III), derived from bovine placentae. The medium was changed to 0.05 mM Ca^{2+} medium and the cultures were submerged for 72 hours. Following this submerged culture incubation, the upper side of the membrane was air exposed for 72 hours to promote differentiation while a dermis in contact with the membrane was submerged in 0.25 mM Ca^{2+} medium containing 40 µg/ml ascorbic acid (21) (18). Cultures were fixed on day 6 in 3% formalin for 5 minutes, and stored in 70% ethanol before histological processing.

Dermatological punches and histological cassettes and sponges (all of them from Daigger, Vernon Hills, IL) were used. Viability and morphology of the cultures were assessed by H+E staining of random sections. In some experiments, cells from Fractions B, D and E were attached on MU membranes and submerged in low Ca^{2+} medium containing KGF at 8.4 ng/ml. After 7 days, membranes were air exposed in high Ca^{2+} medium containing 40µg/ml ascorbic acid. This exposure lasted 48 hours to promote differentiation, and was followed by one or more rounds of submerged conditions to mimic the wound-healing environment. Cells were then fixed at different time points.

Cell sorting and analysis by flow cytometry

Cells were stained with antibodies to CD49f and CD90 (FITC, Pharmingen, San Jose, CA). CD34 and CD45 (PE, Pharmingen, San Jose, CA), CD11b (PE) and CD117 (CyChrome, Caltag, Burlingame, CA) and CD105 (Chemicon, Temecula, CA), using standard procedures. Analysis was carried out on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Cells were stained for the SP phenotype (Side Population) (22) by incubating cells (1×10^6 cells per ml in DMEM with 2% FBS) with 3 µg/mL Hoechst 33342 (Molecular Probes, Eugene, OR) for 90 minutes (37°C, 5% CO_2 , 100% relative humidity). The cells were washed once in cold DMEM with 2% FBS and resuspended in the same for sorting. Cell sorting was performed on a FACSVantage SE with DiVa option (BD Biosciences). Percents were compared from the unfractionated epidermis and dermis and the individual fractions.

Cell survival and colony formation assays

Unsorted cells, SP and CD49f positive cells from fractions were placed in monolayer culture using media containing different levels of calcium and serum. After approximately four weeks, MTT analysis was performed to stain mitochondria to locate any living cells (Roche Diagnostics, Indianapolis, IN), according to manufacturer's instructions. Prior to solubilization, bright field pictures were taken of representative fields in all cultures. In order to study proliferative capacity, equal numbers of cells, enough to produce confluent monolayers, were plated in duplicate wells of 24 well plates. Epi were cultured in low calcium medium and dermal-derived fractions were cultured in high calcium medium. Cells were serially passaged until viable cells were no longer observed in the wells. In order to study whether some contaminating epidermal progenitors could affect proliferative capacity of the dermal fractions, DNase was left out from the dermal preparations, thus increasing keratinocyte cross-contamination of the fractions.

Immunoblotting and immunostaining

Freshly isolated cell pellets were lysed with 0.25 M Tris HCl buffer pH 6.8, containing 3% β -mercapto ethanol and 5% SDS; cells that migrated through a membrane and adhered to tissue culture plates were lysed in situ. Electrophoresis was run on NuPage gels (Invitrogen, Carlsbad, CA) and proteins were electrophoretically transferred onto PVDF membranes (Immobilon-P, Millipore, Bedford, MA). Antibodies for immunoblotting included anti keratins 1, 5, 10, 14 (Covance, Princeton, NJ), all at 1:2000 dilution; actin monoclonal (C4, Chemicon, Temecula, CA), rabbit polyclonal to β -tubulin and to POU3F2 (Abcam, Cambridge, MA) at 1:10000 and 1:1000 dilution respectively. Secondary antibodies were anti-rabbit or anti-mouse IgG horseradish peroxidase linked (Amersham, Piscataway, NJ). Chemiluminescent detection was done with West Pico (Pierce, Rockford IL).

Immunostaining of paraffin embedded sections was performed according to standard protocols using deparafination with Histochoice (Amresco, Solon OH) and antigen retrieval with 0.01% trypsin, or postfixing frozen sections with 4% paraformaldehyde. Immunostaining of freshly isolated cells was performed according to standard protocols, on flash-frozen cell spreads postfixed with 4% paraformaldehyde for 10 minutes. Primary antibodies were anti keratins K14-FITC, K-10-FITC, (Covance, Princeton, NJ) at 1:200 dilution, anti involucrin, kind gift of Dr. Richard Eckert at 1:500 dilution, anti K15 (BD-Pharmingen, San Jose, CA) at 1:100 dilution, anti MRP8, kind gift Dr. Joannes Roth at 1:200 dilution, α pep8/DCT, kind gift of Dr. Vincent Hearing at 1:500 dilution. Anti smooth muscle actin (1:300) dilution was from Biocare Medical. Anti-desmin was from DAKO, used at 1:500 dilution. Secondary antibodies were either Alexa-488 or Alexa-594 conjugates (Molecular Probes, Eugene, OR). For confocal images, fluorescent cells were examined with a Zeiss LSM 510 Confocal Microscope (Carl Zeiss Inc, Thornwood, NY, USA) using a 40x 1.3 NA Plan Neofluar objective. Images were collected sequentially where the FITC, Texas Red and DAPI signals were collected with a BP 505-550 filter, LP560 filter and BP 385-470 filter after excitation with 488 nm, 543 nm and 364 nm laser lines, respectively.

Multipotential differentiation assays

Differentiation assays for the osteogenic, adipogenic, myogenic and chondrogenic lineages were performed according to (23) and (24). Prior to induction of differentiation, cultures containing epidermal sub-fraction B were grown to confluence in 0.05 mM Ca^{2+} whilst cultures containing fractions D or E were grown to confluence in 1.4 mM Ca^{2+} containing medium.

Osteogenic differentiation was induced with DMEM containing 10% FBS, 1% penicillin/streptomycin, non essential aminoacids, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, 10 mM β -glycerol phosphate, and 10 nM dexamethasone for up to 3 weeks (24). Mineralization was visualized by staining

with Alizarin Red S (2% w/v Alizarin Red S adjusted to pH 4.1 using ammonium hydroxide) for 5 minutes at room temperature followed by a wash with water. Osteoblasts were shown by the formation of calcium-rich hydroxyapatite in the extracellular space which stains orange-red with Alizarin red S. Adipogenic differentiation was induced by treating the cultures with DMEM containing 10% FBS, 1% penicillin/streptomycin, non essential aminoacids, 10% rabbit serum, 20 μ M ETYA, 10 nM dexamethasone, and 25 μ g/ml insulin (24). For the subsequent feedings, the medium lacked dexamethasone and rabbit serum concentration was increased to 15%. Adipocytes were visualized by the presence of highly refractive intracellular lipid droplets in phase contrast microscopy. Chondrogenic differentiation was induced by plating 5 million cells per well of a round bottom 96 well plate. Cells were fed with 1.4 mM Ca^{2+} medium containing 50 μ g/ml ascorbic acid, 100nM dexamethasone and 1 ng/ml TGF- β (24). After 3 weeks in culture, cell clumps were harvested with a wide bore pipet tip, spread onto poly-L-lysine coated slides, flash frozen and post-fixed with 4% paraformaldehyde followed by staining with 0.1% Safranin O. Myogenic differentiation of freshly isolated fraction E cells plated at confluency was observed following infection with adenovirally expressed GFP (25). Infection was done in serum-free medium containing 2.5 μ g/ml polybrene (Sigma, St. Louis, MO) at 10-30 multiplicity of infection (MOI) for 30 min at room temperature. Fresh complete medium was added thereafter. Cells were observed and photographed daily. Movie was acquired using an LSM 510 confocal microscope.

Array analysis

Focused arrays (GEArray S series Mouse Stem Cell Array, SuperArray, Bethesda, MD) were used to establish the signature of epidermal and dermal subpopulations after one day in culture. The arrays contain 258 known genes that encode markers expressed by stem and differentiating cells as well as varied growth factors and other regulators of stem cell behaviour. Cells from epidermal sub-fraction B, from dermal fractions D, E and from a combination of D and E (termed Fb) were isolated and plated overnight in high Ca^{2+} containing medium. Total RNA was prepared with Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Arrays were hybridized according to manufacturer's protocols for the non-radioactive format. For analysis, the manufacturers recommended method was used (www.superarray.com), which involved densitometric analysis of the 30 second exposures to X-ray film and then superimposing figures with a grid pattern for quantification of raw pixels. From raw pixel data, most of row 17 (which contains no printed cDNAs) was used to subtract background. Once individual pixel values were obtained, the results were processed with Excel software. Rpl13a was selected as the housekeeping gene to normalize data, as Rpl13a was the least variable housekeeping gene among all four fractions. Then individual values were divided by the averaged value for Rpl13a in each fraction. A number of genes that were marginally upregulated in only one or two fractions were removed from the analysis by setting up the threshold at 20% expression.

Statistics

Numerical values are expressed as mean $\pm$ s.e.m. For comparison among all groups in the study, multiple comparisons of more than two groups were done by one-way analysis of variance, ANOVA. Unpaired Student *t*-test was used for comparisons between two groups; *p* values are reported.

Results

Isolation and characterization of epidermal and dermal subpopulations

In the search for murine skin subpopulations that would provide a good model for epidermal formation in vitro, we adapted sedimentation and differential adhesion protocols already used in our laboratory (26) for the isolation of cellular subpopulations from neonatal mouse skin.

Flow charts of the protocols and characterization of all the fractions are presented in Supplementary Fig. 1 and Supplementary Table 1. By immunoblot, unplated, freshly prepared fractions contained varying amounts of basal and differentiated keratins (Supplementary Table 1 and Supplementary Fig. 1). Keratin 1 or 10 and K14, were mostly detected in epidermal and hair follicle-derived fractions. In the dermal derived fractions, K1 and K10 were present in fibroblast (D and E) fractions and this could mean a small keratinocyte contamination to these fractions; however, we noticed that keratinocyte positive populations expanded from E upon culture in high or low calcium (Supplementary Table 1 and fig 4) and in minimal medium conditions (Fig 5). CD49f (integrin $\alpha 6$) and CD34 were used as markers of early keratinocyte precursors (27) (28) (29). CD117 (c-kit) was used as a potential bulge marker (30), as was K15 (31) and p63 (32), (33). All epidermal fractions had equivalent, nearly 100% expression of CD49f. Dermal fractions derived from hair follicles (26), had lower amounts of CD49F positive cells (ca 30%). The dermal fibroblast fractions D and E contained the highest amount of CD34+ (ca 45%) cells and the lowest amount of CD49f+ (ca 10%). CD117+ cells had low abundance overall and were slightly higher in dermal fractions D and E although not statistically significant. These CD117+ cells might be dermal melanocyte precursors (34). Moreover, in epidermal fractions, CD117 positive cells were CD49 intermediate bright whereas in dermal fraction E, the CD117 positive cells were distinct from CD49f and CD34, thus arguing that the CD117+ population would not come from epidermal contamination of the dermal fractions (Fig. 1C).

The Side Population (SP) (35) has been associated with mesenchymal stem cells, although skin stem cells may not share this marker (36). Side Population (SP) positive cells were much more abundant in epidermal fractions and in hair follicle buds from the dermis. Dermal fractions D and E presented the most reproducible data on SP and, within them, fraction E had a higher percentage, albeit not significantly different from D. These data suggested that it was possible to isolate early precursors but not keratinocyte stem cells by SP population analysis of total skin (Fig. 1A). In fractions D and E, CD34+ and CD49f+ represented distinct sub-populations within the SP population. There were slightly more SP cells in E than in D and also 10% more CD34/CD49f double negative cells in fraction E thus we concluded that this fraction may contain a subset of precursors that is absent from dermal fraction D.

We used immunofluorescence of freshly isolated cell spreads to investigate K15, a marker of hair follicle bulge region, and p63 (Supplementary Table 1 and Fig 6 E). Percent of K15+ cells were higher in Epi and DHF and very low in D and E ($p < 0.0001$ Epi vs E). Likewise p63 distinguished Epi and DHF from D and E (Epi vs E $p = 0.0051$, D and E were not significantly different). MRP8, an inflammation-related molecule found in endothelial cells and in keratinocytes (37), was found in low amounts in Epi, and within this fraction, it was present in fraction C. Most cells in dermal fraction E were positive and there was a very small amount detected in dermal sub-fraction G (see Supplemental Table 1). The mesenchymal marker CD105 (38), (39) was significantly higher in the hair-follicle containing populations Epi and DHF (Epi vs E $p = 0.0129$, D vs E non significant). CD90 (40) had a higher percent in the dermal fractions and slightly higher in E than D albeit not significant (CD90 Epi vs E $p = 0.0032$, D vs E, non significant). As MRP8 might be associated with immune infiltrates, we confirmed by CD11b that populations containing granulocytes, macrophages and monocytes were not significantly different amongst the fractions. The sum of these findings suggested that our protocol selectively enriches for epidermal precursors associated either with the hair follicle bulge or dermal papillae, or with as of yet uncharacterized mesenchymal progenitor cells present in the dermis.

Growth of the fractions in three dimensions

Epidermal populations containing keratinocytes and hair follicle buds grew occasionally and not reproducibly in three-dimensional cultures. Reproducibility improved when we selected hair follicle buds (epidermal sub-fraction A) and when we used membranes in contact with fresh dermis (18) (Supplementary Fig. 2). We compared the growth of epidermal cells (sub-fraction B) with dermal fractions D and E. Consistently we observed that fraction E but not D was able to form monolayers that stained H&E positive and appeared viable. Epidermal cells alone did not survive (Fig 2A) but a mixture of B and E did (Fig 2B). Taken together these observations suggested that fraction E contains functional epidermal precursors.

In order to understand whether a small population of contaminating follicles could be responsible for the proliferative capacity of dermal fraction E, we looked at the proliferative capacity of Epi, DHF, D and E in monolayer culture. When DNase was included in the dermal preparations detectable levels of Keratin 10 and 14 decreased, suggesting that the use of DNase substantially decreases cross contamination of fractions (Supplementary Fig. 3). When we plated equal number of cells from a preparation that did not include DNase, we were able to carry Epi in low calcium medium for 3 passages. Dermal-derived fractions (DHF, D and E), cultured in high calcium medium, failed to survive past passage 7. When DNase was added to the preparation, Epi failed to survive past passage 2, dermal derived hair follicles (DHF) failed to survive past passage 9 and dermal derived fractions D and E survived to passage 18; at this point, only fraction E had viable cells, which had largely senescent morphology and contained some adipocyte-looking cells in the culture. In the same experiment, we maintained cells that remained in the well after passage 1 for a month with only one medium change. In the epidermal fraction, no cells survived. In DHF cells had lifted and died by 3 weeks, however a monolayer of cells from both fractions D and E was still present after a month, similar to what we report in Fig. 3.

Survival and differentiation of monolayer cultures in reduced serum

We hypothesized that epidermal precursors in the dermis would mobilize to restore damaged epidermis and we could mimic this process in vitro. We tested whether we could functionally identify which fraction would respond to wound healing cues. Those cells, we hypothesized, would be able to survive harsh conditions through quiescence prior to responding to wound healing signals. In order to test the former, we isolated and/or sorted these fractions and placed them in monolayer culture for one month in medium containing 2% serum and either 0.05 mM or 1.4 mM Ca^{2+} . We then assayed cell survival by MTT. The non-sorted epidermal sub-fractions B and C had increased survival, likewise, side population (SP) sorted cells from hair follicle sub-fractions (B, F and G) showed the greatest ability to survive in minimal media containing 0.05 mM Ca^{2+} (Fig 3A). Epidermal fractions sorted based on the integrin marker CD49f survived only under low calcium conditions (Fig. 3B) The moderately increased survival of a CD49f-population in high calcium suggested that expansion of an epidermal-like population under high calcium and air exposure (see Figs 2 and 4) may originate in a population distinct from CD49f+. In SP-sorted fractions, a population of small, vigorously proliferating cells expanded from fraction E, and not from D or epidermal sub-fraction B, under high calcium conditions (Fig 3C). Selection by c-Kit (CD117) showed no differences between dermal fractions D and E (data not shown). Together these observations suggested that a subpopulation of SP+ dermal cells and distinct from the CD49+ population, contains epidermal precursors capable of expanding in higher calcium conditions. Therefore, the effects we had observed when fractions B and E were mixed and cultured in three dimensions (i.e. air-exposed in high calcium, Fig. 2) could be explained by the ability of selected dermal cells to form epidermis in vitro.

Survival and differentiation of dermal fraction E in three dimensional cultures

Based on the results in monolayer cultures, we reasoned that dermal subpopulations survived more readily than others because they contained early progenitor cells. Upon wounding, healing would arise from those populations and provide rapidly amplifying cells for epidermal formation. Accordingly, we simulated conditions that favor epidermal cell expansion. In three-dimensional cultures, these conditions consisted of low Ca^{2+} containing medium with KGF while submerged and high Ca^{2+} and ascorbic acid-containing medium (21) when air exposed. Fraction E was plated in 1.4 mM Ca^{2+} to favor attachment to the membrane and 24 h later the medium was changed to 0.05 mM Ca^{2+} medium containing KGF. Cultures were grown submerged for a week and air-exposed in medium containing 1.4 mM Ca^{2+} for 2 days, 6 days and 9 days (Fig 4A, B, C). Fraction E was grown submerged and air-exposed as in panel A, then it was submerged again for 4 days and air-exposed for additional 7 days (Fig 4G). The early cultures (Fig 4D), stained positive for CD34 (data not shown).

We compared the expansion of K10/K14 staining cells in the cultures. Fig 4 E and H demonstrate K14 staining of the membranes shown in panels D and G. Fig 4 F and I, shows stronger staining for K10 of the panel G sample suggesting that a second phase of submerge culture followed by air-exposure directs differentiation of keratinocytes in vitro. The co-localization of a later keratinocyte marker involucrin with K14 further supported this differentiation (Fig 4J). This experiment was subsequently repeated with epidermal fraction B but the cells were nonviable. Immunoblots of dermal subpopulations that migrated through 0.3 μ membranes, adhered to the plastic underneath and were cultured in two individual wells with 0.05 mM or 1.4 mM Ca^{2+} , suggested that fraction E more than D, contained cells able to migrate through membrane pores and expand keratin positive cells under both calcium conditions, although the differences were not statistically significant (Fig 4K).

Differentiation assays

To test whether fraction E had multilineage potential, we conducted the following assays (24). Fractions B, or total epidermal preparation, and D were used as comparison controls. Fraction E was positive for osteogenesis, chondrogenesis and adipogenesis (Fig 5A and B). Under starvation and 0.05 mM Ca^{2+} conditions, fraction E and Epi but not D, expanded a K5 positive population while fraction D more than E, expanded a melanocyte positive population (as marked by the early melanocyte marker Tyrp8/Dct) (Fig 5E). DCT, an early melanoblast marker, has been proposed to be a marker of the bulge (16). In addition to these findings both fractions D and E, but most prominently fraction E, gave rise in culture to cells with contractile behaviour (Fig 5D and supplementary movie). We observed this phenotype on confluent monolayers and on air-exposed three-dimensional cultures as well (data not shown).

Stem cell and differentiation markers were differentially expressed in skin subpopulations

Epidermal cells (sub-fraction B), and dermal fractions D and E were plated in complete high calcium medium. Total RNAs were prepared from the overnight-adhered cells and hybridization to mouse stem cell arrays was carried out. These arrays confirmed functional observations of differences between epidermal and dermal fractions and within the dermal sub-fractions D and E. Differentiation and stem cell markers were differentially expressed in those populations (Fig 6A). As examples, in red are circled genes that were up-regulated in fraction E whereas blue circles mark genes that were below detection limits in fraction E, while being up-regulated in the others. Fig 6C shows many genes were exclusively below the limits of detection in E while up-regulated in the other fractions suggesting that negative selection might enrich for stem cells in fraction E (24,41). Specifically genes involved in cellular adhesion such as integrin $\beta 4$ and ICAM 5 were below detection in E whilst highest in D. Fig 6D represents the genes that were detected in E as well as in other sub-fractions and their percent distribution.

Changes in the expression of K14, 10 and of the transcription factor Pou3f2 were confirmed at the protein level by immunofluorescence and immunoblotting (fig 6F).

In vivo engraftment and differentiation

Cells from Epi, DHF, D and E fractions were engrafted into skin of immunodeficient mice in two different ways. They were either subcutaneously injected or grafted under a silicon dome covering the graft bed, as typical epidermal grafts.

Isolated subpopulations were injected at defined subcutaneous sites on the back of immunodeficient mice. Mice were sacrificed at one and two week post-injection. In the one-week histology sections, clusters of cells in the injected sites of fractions Epi and DHF could not be found. Fraction D formed a fibrotic mass that had abundant vascularity, consistent with previous reports (42). In the fraction E group, one week after subcutaneous injection, some pigmented aggregates were observed within host muscle (Fig 7A, Fontana Masson stain). In addition both fraction D and E, but most prominently fraction E, displayed fusiform structures that stained positive for desmin and smooth muscle actin in the periphery and positive for smooth muscle actin toward the center (Fig 7A, 1 week). These structures were not as prominent in the two-week histology sections. These staining patterns suggested early differentiation of the mesenchymal cells into the myogenic lineage. In the two-week post injection set, we could find the injected Epi and DHF cells and those formed abundant cysts, which were larger in DHF (Fig. 7A, 2 weeks) whilst fraction D cells could not be found. Fraction E showed some few and small cysts, numerous melanocytes surrounding those and interspersed in the dermal tissue as well as some non-melanized, fusiform structures (Fig 7A, arrowhead).

For the epidermal grafts, five days post-grafting the silicon domes were removed to facilitate wound healing. At that time animals grafted with fraction E had a significantly bloodier wound, which nonetheless healed faster as shown in Fig. 7 B. All four subpopulations had graft take as evidenced by the presence of dermal melanocytes not associated with hair follicles, consistent with previous reports (43). A very dark healed wound was appreciated in DHF, which contained numerous hair follicles and cysts as observed in the histology (Fig. 7B). Animals grafted with fraction E displayed a dark ring around the edges of the wound site (Fig. 7B, arrow). On histology, it could be observed that there were pigmented nests deep within the host dermis, where a few contaminating hair follicles accumulated. The environment of the wound edge has been reported to be more favorable to hair follicle development (44), (43). In the graft central areas, away from wound edges, very few melanocytes were observed in the case of Epi and D fractions; in DHF they were concentrated in areas surrounding pre-existing follicles and cysts whereas in fraction E, melanocytes could be found in the center of the graft. Melanocytes appeared vacuolated in Epi and had lighter color than those in the E fraction (Fig. 7B).

Discussion

Murine skin bioengineering lags behind the progress made in the human skin bioengineering field (45) (46). A complete epidermis may be reformed following the growth of mouse keratinocytes on nonviable, de-epidermized dermis (47) (10), and some studies have shown that mixing human and murine skin components may reconstruct an epithelial layer (48). Collectively, these studies suggest that there may be different requirements for the *in vitro* formation of mouse and human skin, such as growth factors and cellular selection. *In vivo*, an epidermis can be formed by grafting epidermal cells with a dermal component onto nude mice (49). This dermal component was made up of a combination of fractions D and E, described in this paper. Weinberg et al. were able to demonstrate hair growth when hair follicles were grafted with dermal papilla and not in their absence (17). Our work aimed at expanding these findings *in vitro*. We could not consistently replicate the *in vitro* formation of an epidermal

layer when we used neonatal mouse epidermal cells cultured on artificial inserts with gamma irradiated dermal cells cultured on the underside (50). Therefore, we focused on isolating different cellular subpopulations from neonatal mouse skin taking advantage of properties such as differences in buoyant density or differential attachment to extracellular matrix components (51) which can selectively enrich for hair follicle derived cells (52) (17). Our isolation scheme, selected 7 populations (3 derived from the epidermis: A, B and C) and 4 derived from the dermis (D, E, F, G), characterized as shown in Supplementary Table 1. Again, we had very limited success with sub-populations obtained from the epidermal compartment on organotypic cultures. Among the dermal fractions, F grew well under submerged conditions in low calcium but failed to grow in 3D cultures. Co-cultures of epidermal cells and dermal fractions, designed to enrich in mesenchymal cells, grew successfully in three-dimensional cultures; moreover we observed that de-epidermized dermis controls quite often reformed an epithelium even after gamma irradiation and thus the dermis may contain radio-resistant progenitors. We reasoned that our isolation procedure could shed cells from the epithelial fractions into the mesenchymal component of the dermis. Those precursors would be derived from the bulge or the dermal papilla sections of the hair follicle and would, in conjunction with fibroblasts, favor epidermal formation *in vitro*. We tried to assess the effects of contamination of epidermal cells in the dermal preparations. Increasing keratinocyte contamination was found when DNase was left out of our isolation protocol (the use of DNase has been a current practice in our laboratory as described in (53), which did not specify why it was added). To our knowledge, other papers published looking at dermal cells such as (54), describing DEEP-1 cells, fail to use DNase and we demonstrate that the use of DNase in preparations of dermal cells significantly reduces hair follicle contamination.

When plated on membranes, mesenchymal cell preparations formed multiple layers and migrated through pores to the underside. Furthermore, in submerged conditions, keratinocyte monolayers formed that stratified at the air-medium interphase and expressed keratin 14 and other markers as seen in Fig. 4.

The mesenchymal cells expressed cell surface markers that distinguished them from keratinocytes. They expressed low levels of CD49f, were rich in CD34, had detectable CD117, and had higher CD90 expression. These cells distinctly expressed MRP8 (S100A8) and this was not due to differential content in cells that express the type 3 complement receptor, CD11b. Changes in expression of S100 A8 have been reported in wound fibroblasts (55). A distinguishing characteristic of the mesenchymal population was the ability to survive and propagate under minimal culture conditions. This may have parallels *in vivo* as reserve or hematopoietic stem cells are reported to survive for long periods in a poorly oxygenated niche (56). Thus, the long-term survival of a mesenchymal precursor population may require that the cells have enhanced survival capabilities under stressful conditions.

The mesenchymal cells (from fraction E) could differentiate into different lineages thus forming osteocytes, chondrocytes, myocytes and adipocytes. We repeatedly observed that when dermal cells were placed in three-dimensional cultures, under submerged conditions, only isolated cells survived on the membranes, and those cells were evenly spaced. We hypothesized that those cells would differentiate into epidermis if we gave them an opportunity to expand with appropriate growth factors and tested this by simulating the skin wound healing environment. Our simple model of skin wound healing consisted of growth of Fraction E alone, under alternating submerged-air exposed-submerged+KGF-air exposed conditions. Early cultures were positive for the stem cell marker CD34 and for the basal keratinocyte marker K14 whereas cultures that had undergone more than one round of submerged-air-exposed conditions, acquired the early differentiation keratinocyte markers K10 and involucrin.

We have not conclusively determined the origin of these mesenchymal cells. It is possible that cells from the hair follicle bulge area get dislodged and mixed in with the dermal populations in our isolation protocol. It has been reported that putative human keratinocyte stem cells have the lowest amounts of desmoglein (57) and therefore could become loose and remain in the dermal subpopulations separated from the dermal hair follicle cells. Thus these precursors could either come from the bulge (10) or from the dermal papilla, which may contain early transient amplifying cells (16). However the significant lower levels of p63 and K15 between E, D and DHF argues against bulge contamination of our cultures.

Stem cells and early precursors express lineage specific genes at low levels before lineage commitment (41). Analysis of gene array data showed that among the few genes detected in fraction E were the intermediate filament keratins 14 and 17. K14 is a basal keratin that precedes K1 and the later stages of epidermal squamous differentiation; K14 was relatively more abundant in fraction E than in D, thus confirming our immunoblotting results. K17 is an early epidermal progenitor marker (58) (59) and a marker for bulge (16) (60). Injury to the skin results in an induction of K17 concomitant with activation of keratinocytes for reepithelialization. K17 was not significantly different in fraction E. Only Acta2 was more prominent in fraction E than in the others. Acta2 encodes the alpha form of smooth muscle actin. The contractile isoform alpha-2 actin is also named smooth muscle and aorta isoform and is present in musculoskeletal connective tissue cells and has been previously demonstrated in early progenitors (61) (62). The expression of smooth muscle actin suggests that the population might contain myofibroblast precursors (63). The bone morphogenic proteins BMP3 and BMP6, signals required for stem cells, the stem cell marker CD34, GJB1, a member of the gap junction connexin family, and nerve growth factor, were about equally expressed in all the fractions analyzed and thus were not specific to fraction E. However Cnp1, a marker for myelin, was expressed at much lower levels in E and B than in D and Fb, suggesting that oligodendrocytes or their precursors could be present in the D preparation and absent in fractions E and B. Cnp positive cells were detected in bulge cells after differentiation in culture (64). Some genes whose expression was lower in the E fraction were Fzd1 and 8, which were more prominent in D and B, consistent with recently published patterns of frizzled genes in skin and hair follicles (65). Many stem cells and differentiation genes from the array were exclusively below the limits of detection in E while upregulated in the other fractions.

When injected subcutaneously, fraction E gave rise to structures that stained positive for smooth muscle actin and desmin. In silicon chamber grafts, which are conducive to epidermal differentiation, melanocytes not associated with hair follicles, were abundant in the dermis. Smooth muscle actin-positive cells and melanoblasts are both neural crest derivatives and one or the other may predominate depending on the microenvironment (66). Collectively, our grafting and subcutaneous injection results suggest that dermal fraction E has the ability to differentiate into muscle-like structures, when subcutaneously grafted or into melanocytes when grafted in an environment in which the canonical β -catenin pathway predominates.

In summary, we isolated a mesenchymal population devoid of hematopoietic cell markers and capable of forming adipocytes, chondrocytes, osteoclasts, functional smooth muscle cells and keratinocytes *in vitro*. Moreover, this population was able to generate an epidermal layer in a 3D model of murine skin. By comparison, this population lacks the expression of markers shared by epidermal and other dermal subpopulations such as ICAM 5 and β -4 integrin and the expression of a selected set of genes is strikingly different from that of other epidermal and dermal subfractions. We were able to demonstrate the relative absence from dermal fractions D and E of epithelial stem cell markers (K15, p63); CD90, a mesenchymal progenitor marker, distinguished D and E from Epi and DHF, whereas Cd105 did not. MRP8 (S100A8) is a distinguishing marker to this fraction where as CD11b did not distinguish it from the other fractions studied. This isolation procedure will be useful for the study of genetically altered

mouse strains as models for various human skin conditions. Further analysis of these cells may provide new insight into how mesenchymal-to epithelial transitions play a role in tissue regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HiCa	medium containing 1.4 mM Ca ²⁺
LoCa	medium containing 0.05 mM Ca ²⁺
MU	Millipore uncoated membrane
CC	Costar coated membrane (refer to Materials and Methods)

Fig 1.

A. Simplified schematic of fraction preparation (for more detailed schematic, see Supplementary Fig.1). B. Flow cytometry analysis of SP in epidermal sub-fraction B and dermal fractions D and E. C. Contour plots of CD34 and CD49f positive populations contained within the CD117+ population of epidermal (sub-fraction A) and dermal fraction E. Percent CD117+ cells were 2.7 and 6.2 respectively. E. MRP8 staining of Epi, DHF, D and E showing percent positive cells counted from 5 independent fields, DHF and D were negative. Top right panel shows MRP8 stained neonatal skin, arrow points at area shown in inset. For description of epidermal sub-fractions A and B, refer to supplemental Materials and Methods.

Figure 2. Dermal fraction E has potential for differentiation into epidermis in a three dimensional culture

A. Epidermal sub-fraction B was cultured under submerged conditions in 0.05 mM Ca^{2+} and then was air-exposed on a fresh dermis on a MU insert (0.4 μm) in 1.4 mM Ca^{2+} for 3 days.

B. A mixture of fraction B and E was cultured as in panel A. For description of epidermal sub-fraction B, refer to supplemental Materials and Methods.

A. Survival of total or SP sorted skin subpopulations in 2% serum containing medium with 0.05 mM Ca²⁺, measured by MTT

B. Survival of CD49f sorted cells in minimal medium with different Ca²⁺, MTT assay

C. Survival of SP sorted cells in minimal medium containing 1.4 mM Ca²⁺, MTT assay

Fig 3. Survival and expansion of isolated fractions in monolayer culture

A. Isolated/and or SP sorted fractions were plated in 0.05 mM Ca²⁺/2% serum containing SMEM medium for a month in, at least, triplicate wells. The MTT assay was carried out after this time. Cell survival calculated from abs 490-650 (left panel) or abs 490-650/cell input (right panel). In a separate experiment, equal numbers of SP cells were plated and the results followed the same pattern. Asterisks denote significant difference in survival of individual fractions compared to fraction E. Non sorted fractions, $p=0.0018$ B vs E, $p=0.0244$ C vs E. Sorted fractions $p=0.0036$ B vs E, $p<0.0001$ for both F and G vs E. B. Fractions B, D and E cells sorted based on CD49f were placed in 24 well plates at 10,000 cells/well and cultured with 2% serum and either 0.05 mM or 1.4 mM Ca²⁺ for a month. The MTT assay was carried out after

this time. In CD49f positive fractions $p=0.00642$ B vs E. Right set of panels show MTT stained cells. C. SP sorted dermal subpopulations were plated in duplicate wells of a 24 well plate at 10,000 cells per well in minimal medium containing 1.4 mM Ca^{2+} . In SP sorted fractions $p<0.0001$ D vs E. Right panel show MTT stained cells. These experiments were repeated at least twice; one representative set of results is shown. For description of epidermal and dermal sub-fractions, refer to supplemental Materials and Methods.

Fig 4. Dermal fraction E does not require underlying dermis to survive in 3D cultures under epidermal conditions

Fraction E was plated in a 3 μ m MU insert in high calcium containing medium to favor attachment. Next day medium was changed to low calcium containing KGF and cultured under submerged conditions for a week, followed by air exposure as detailed below. A. Air exposure for 2 days in medium containing 0.24 mM Ca²⁺. B. Air-exposure for 6 days. C. Air exposure for 9 days. D. Higher magnification of the 2 day culture. E. K14-FITC stain of 2 day culture. F. Double stain K14-FITC and K10 with a texas Red anti-rabbit secondary; some co-localization is observed as yellow signal (arrowhead). G. Fraction E was cultured submerged and air-exposed as in panel A then it was submerged again for 4 days and air-exposed for

additional 7 days. H. K14-FITC stain of sample in panel G. I. Double stain of sample in panel G with K14-FITC and K10 with a texas Red anti-rabbit secondary; yellow shows increased areas of co-localization of the texas Red and FITC signals as compared to panel F (arrowhead). J. Double stain of sample in panel G with K14-FITC and involucrin with a texas Red anti-rabbit secondary. K. Fractions D and E were on MU inserts (3 μ pore size). Cells that migrated to the plastic well below were cultured in submerged conditions for 8 days with either 0.05 mM (Lo) of 1.4 mM (Hi) Ca^{2+} medium. Fraction B was cultured in 0.05 mM Ca^{2+} for three days as a control. Lysates for one set of wells out of two replicates are shown. B-actin is shown as a loading control. Quantitation of averages for both replicates is shown.

Fig 5. Differentiation assays

A. Osteogenesis differentiation, Alizarin Red stain. B. Adipogenic differentiation, Oil Red O stain. Fraction B did not survive these conditions. C. Chondrogenesis differentiation, Safranin O stain. D. Myogenic differentiation of adenovirally infected (GFP) confluent cultures, grown in complete MEM medium for 3 weeks; some cells within fraction E had myocyte morphology and were contractile (see Supplementary video). E. Staining of cells cultured in MEM medium containing 0.05 mM Ca²⁺ and 2% FBS for a month. K5 denotes an active proliferating population expressing the basal keratinocyte marker K5. Tyrp2 denotes the melanoblast/early melanocyte marker dopachrometautomerase.

Fig 6. Additional characterization of the fractions

A. One min exposure of membrane arrays (for easier identification of genes). Some examples are highlighted, red indicates genes with a positive value in fraction E, blue indicates genes with positive values in more than one fraction and not in E. Gene intensity values were calculated from a 30 second exposed film, in which house keeping genes, shown in the bottom row, were not saturating. B. Not all of the housekeeping genes in the array were evenly present in all fractions thus Rpl13a was chosen for normalization. C. Bar graph showing percent contribution of each fraction to the total expression levels; most of the genes that were negatively regulated belong to fraction E. D. Bar graph showing percent contribution of each fraction to the total expression levels in upregulated genes; only genes in which fraction E had a positive value are shown. E. Immunostaining of freshly isolated flash frozen fractions with the markers p63 and K15, quantitation of positive stained cells from five random fields is shown below, asterisks denote significant differences between Epi and E (p63, $p=0.0051$; K15, $p<0.0001$). F. Immunoblots of proteins that were detected in fraction E in the array (K14, Fig 6B) or below detection in E, (Pou3f2, Fig 6C). Left panel shows quantitation of their relative levels by NIH image.

Fig 7. Engraftment and differentiation of fraction E in vivo

Isolated subpopulations were injected at defined subcutaneous sites on the back of immunodeficient mice. Mice were sacrificed at one and two week post-injection. A. In the fraction E group, pigmented aggregates are seen within host muscle (arrow and upper right inset). Arrowhead and lower left inset shows positive Fontana Masson fusiform structure which stains positive for desmin and smooth muscle actin in the periphery and positive for smooth muscle actin toward the center (stained sections are from the area in the lower left inset). Clusters of cells in the injected sites of fractions Epi and DHF could not be found in the animals. At two week, injected Epi and DHF cells formed abundant cysts, which were larger in DHF. Fraction E had some few and small cysts, numerous melanocytes surrounding those and interspersed in the dermal tissue as well as some non-melanized, fusiform structures (arrowhead). Fraction D cells could not be found in the animals.

B. Week three post-grafting with silicon chambers. Arrows point at graft area in all four panels. A very dark healed wound was appreciated in DHF, which contained numerous hair follicles and cysts as observed in the Fontana Masson panel below. Animals grafted with fraction E displayed a dark ring around the edges of the wound site; pigmented nests were deep within the host dermis, as seen in Fontana Masson panel below. In the graft central areas, away from wound edges, very few melanocytes are observed in Epi and many more in fraction E.