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Global gene expression and comparison between multiple populations in the mouse epidermis

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ABSTRACT

Several populations with stem cell characteristics have been identified in the mouse hair follicle, and some are characterized by specific subsets of surface markers.
Here we investigate to what extend a multicolor panel of the four surface markers CD34, Sca-1, Integrin-α6 and Plet-1 is sufficient for separating previously described populations. We used global transcriptome profiling to characterize these, and we also performed transcriptome analysis of selected populations after two weeks of culturing. This has not been done before in a context of multiple populations.
We identified eight populations of which two have not been described previously: A subset expressing integrin-α6 only and a subset expressing all markers but CD34. Both subsets are highly clonogenic. Transcriptome profiling also showed expression of *Hspa2* in a population negative to all markers and immunostaining identified this population as inner root sheath keratinocytes.
All cultured populations lost characteristics from the parent population and could not be separated based on the gene expression profile.
Our data shows that flow cytometry using multicolor panels can identify further subsets of cells within the epidermis and also highlights a marked discrepancy in gene expression between directly isolated cells and tissue cultured cells.

1. Introduction

The mammalian epidermis and its annex are continuously renewed throughout life. This regenerative capacity relies on stem cells or progenitor cells that are located in anatomical sites that are sometimes termed stem cell niches (Fuchs and Horsley, 2011). Several markers have been identified for labeling the various compartments of the mouse hair follicle. Cluster of differentiation 34 (CD34) was the first surface marker that allowed for the isolation of cells with stem cell characteristics by fluorescence activated cell sorting, FACS (Trempus et al., 2003). Identification of hair follicle cells outside the bulge with colony forming potential was published by Nijhof et al. using the antibody MTS24 (Nijhof et al., 2006). This was later shown to identify the glycoprotein Placenta-expressed transcript 1, Plet-1 (Trempus et al., 2003; Depreter et al., 2008). Common to both bulge cells and the colony forming Plet-1^{pos} cells were high levels of Integrin-α6 (Itgα6). However, by using a combination of antibodies against CD34, Itgα6 and Sca-1 (Stem cell antigen-1) we have previously shown the existence of a population with low levels of Itgα6 and negative to CD34 and Sca-1 that could regenerate the entire epidermis including the annexes (Jensen

Abbreviations: ANOVA, analysis of variance; *A6*, Plet-1^{neg}Sca-1^{neg}Itgα6^{high}CD34^{neg} keratinocyte population; Blimp1, B lymphocyte-induced maturation protein 1 (encoded by *Prdm1*); *Bulge*, Plet-1^{neg}Sca-1^{neg}Itgα6^{high}CD34^{pos} keratinocyte population; CD34, cluster of differentiation 34; CFE, colony forming efficiency; DAVID, database for annotation, visualization and integrated discovery; EdU, 5-ethynyl-2′-deoxyuridine; FACS, fluorescent activated cell sorting; FC, fold change of gene expression; HF, hair follicle; Hspa2, heat shock 70 kDa protein 2 (encoded by *Hspa2*); IF, immunofluorescence; IFD, infundibulum; *IFE*, interfollicular epidermis, Plet-1^{neg}Sca-1^{pos}Itgα6^{high}CD34^{neg} keratinocyte population; IPA, ingenuity pathway analysis; Itgα6, Integrin-α6 (encoded by *Itga6*); *IRK*, Plet-1^{neg}Sca-1^{neg}Itgα6^{low}CD34^{neg} Inner root sheath keratinocyte population; IRS, inner root sheath; Krt, keratin; Lgr5, leucine-rich repeat-containing G-protein coupled receptor 5 (encoded by *Lgr5*); Lgr6, leucine-rich repeat containing G protein-coupled receptor 6 (encoded by *Lgr6*); LRC, label retaining cells; Lrig1, leucine-rich repeats and immunoglobulin-like domains 1 (encoded by *Lrig1*); mRNA, messenger RNA; ORS, outer root sheath; P, probability, students *t*-test; Plet-1, placenta expressed transcript 1 (MTS24, encoded by *Plet1*); *Plet*, Plet-1^{pos}Sca-1^{neg}Itgα6^{low}CD34^{neg} keratinocyte population; *PletA6*, Plet-1^{pos}Sca-1^{neg}Itgα6^{high}CD34^{neg} keratinocyte population; *PletA6Sca*, Plet-1^{pos}Sca-1^{pos}Itgα6^{high}CD34^{neg} keratinocyte population; RNA, ribonucleic acid; RT-PCR, real-time polymerase chain reaction; Sca-1, stem cell antigen-1 (encoded by *Ly6a*); TA, transit amplifying.

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et al., 2008). Furthermore, it was shown that these cells were actively cycling as compared to bulge cells.

Sca-1 has for a number of years been used as a positive marker to isolate stem cells or progenitor cells from a number of tissues (Spangrude et al., 1988; Klimmeck et al., 2014; Holmes and Stanford, 2007). Inclusion of antibodies against Sca-1 in the antibody panels is now more or less standard when FACS isolating mouse epidermal cells as it separates the lower hair follicle and the upper hair follicle (isthmus) from the infundibulum and the interfollicular epidermis which are Sca-1^{pos} (Jensen et al., 2008). The basal keratinocytes located within the junctional zone and isthmus are positive for the Myc target gene Lrig1 and constitutes a highly proliferative epidermal population (Jensen et al., 2009). In contrast to the Sca-1^{pos} cells, they also show high colony forming efficiency *in vitro*. In the murine dorsal skin hair follicle, Lrig1 and Plet-1 seem to be co-expressed (Page et al., 2013) and it has been suggested that Plet-1 regulates cellular motility by preventing tight attachment to the underlying matrix (Raymond et al., 2010).

Other markers located within the upper isthmus and junctional zone have also been identified. B lymphocyte-induced maturation protein 1, Blimp1 was initially thought to mark sebocyte progenitor cells (O.C.D. et al., 2006), but this has recently been re-evaluated. Blimp1 is now believed to be expressed in terminally differentiated cells located within the upper hair follicle, sebaceous glands and granular layer of interfollicular epidermis where it co-express with the differentiation markers involucrin and Krt31 (Kretzschmar et al., 2014). In addition, *SCD1* gene expression has also been coupled to the sebaceous glands (Zheng et al., 1999). Gli1-expressing cells can be found within the isthmus and also the distal portion of the bulge, where they span from Krt15^{pos}CD34^{pos} lower bulge to Krt15^{pos}CD34^{neg} hair germ cells (Brownell et al., 2011). The Lgr6^{pos} cells seem dispersed and are located at the lower isthmus, sebaceous glands and interfollicular epidermis and based on the global transcription profile, the Lgr6^{pos} keratinocytes are biochemically distinct from the highly cycling and clonogenic Lgr5^{pos} population of the lower bulge (Snippert et al., 2010).

Several pairwise gene expression profiles have been made between hair follicle stem cells expressing a marker of interest and a negative control (Nijhof et al., 2006; Page et al., 2013; Snippert et al., 2010; Morris et al., 2004; Blanpain et al., 2004; Jaks et al., 2008; Nath et al., 2011). However, the negative controls are often the remaining cells or CD34^{pos} cells alone. These pairwise comparison studies give us a better understanding on what separates the different stem cell populations on a gene expression level and in the long term their functionality. Still, the use of different negative controls or selected genes on RT-PCR makes it difficult to compare their results and draw conclusions on how they relate to each other. This study emphasizes the usefulness of multicolor flow cytometry as we can identify multiple distinct populations without the need for transgenic animals. The populations are tested for clonogenic capacity and we show for the first time a more comprehensive comparison of gene expression profiles of different epidermal stem cell populations.

2. Materials and methods

2.1. Media and reagents

Following reagents were used: PBS without Ca^{2+} and Mg^{2+} (Lonza, Basel, Switzerland). PBS-BSA: PBS supplemented with 0.1% Bovine Serum Albumin (Sigma-Aldrich, Seelze, Germany). D10: DMEM (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (Biosera), penicillin-streptomycin (Sigma-Aldrich, Seelze, Germany) and D-Glutamine (Sigma-Aldrich, Seelze, Germany). Trypsin-EDTA: 0.25% trypsin (Fisher Scientific, Waltham, USA) supplemented with 5 mM EDTA. TBS: Ultra filtrated water with 0.9% NaCl, 20 mM Tris-HCl, pH 7.4. FAD medium: 67.5% Dulbecco's modified Eagle's medium with 4.5 g/L glucose without Ca^{2+} (Fisher Scientific, Waltham, USA), 22.5% Ham's F12 medium (Fisher Scientific, Waltham, USA) and Ca^{2+} chelated 10% fetal calf serum (Biosera, Boussens, France) supplemented with 5 μL/mL insulin (Sigma-Aldrich, Seelze, Germany), 10^{-10} M cholera toxin (Sigma-Aldrich, Seelze, Germany), 1.8×10^{-4} M adenine (Sigma- Aldrich, Seelze, Germany), 10 ng/mL EGF (Invitrogen, CA, USA), 0.4 μg/mL hydrocortisone (Sigma-Aldrich, Seelze, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, Seelze, Germany) with Ca^{2+} added to a final concentration of 0.7 mM.

2.2. Isolation of epidermal keratinocytes

To access the dorsal hair follicle cells in the second telogen phase, C57Bl6/J female mice at 7–9 weeks of age were purchased (Janvier Labs, Saint Berthevin, France) and kept in the animal facility at Aarhus University in accordance with the guidelines of Danish Veterinary and Food Administration. Mice were sacrificed by cervical dislocation and dorsal skin was shaved using a razor machine before its removal using sterile scissors. The skin was rinsed in 5% betadine for 30 s, 70% EtOH for 2 min, and washed in PBS. Fat and connective tissues were gently scraped away from the dermal side using sterile scalpels and forceps. The skin were kept floating on Trypsin-EDTA for 16 h at 4 °C after which hair follicle keratinocytes were isolated by gently scraping the epidermal side in D10 to inhibit trypsin activity. Cells were filtered through 40 μm cell strainer (Fisher Scientific, Waltham, USA), centrifuged at 4 °C for 200 ×*g* for 10 min, re-suspended in PBS-BSA and kept on ice until further processing.

2.3. Flow cytometry and immunofluorescence staining

Freshly isolated keratinocytes were stained in PBS-BSA on ice for 30 min. The following cell surface antibodies were used for immunofluorescence staining: Brilliant Violet 421-conjugated CD34 (clone RAM34; BD Biosciences, New Jersey, USA); PE-conjugated Itgα6 (CD49f, clone GoH3; BD Biosciences, New Jersey, USA); PE-Cy/7-conjugated Sca-1 (Ly-6A/E, clone D4; BD Biosciences, New Jersey, USA); APC-conjugated Plet-1 (clone 33A10; Mubio, Susteren, Netherlands) using Lighting-link labeling kit (Innova Biosciences, Braham, UK). The intracellular antibodies Keratin 79 (clone Y-17; Santa Cruz Biotechnology, Dallas, USA) and Hspa2 (EPR4596; Abcam, Cambridge, UK) were used together with secondary antibody Alexa Fluor 488 (Fisher Scientific, Waltham, USA). Cells were analyzed and sorted through a FACSAria III cell sorter equipped with four lasers; 405 nm violet, 488 nm blue, 561 nm yellow-green and 633 nm red. A 100 μm nozzle and a pressure of 20 psi were used. Propidium iodide (Bio-Rad, Hercules, USA) was used to exclude dead cells. For subsequent immunofluorescence staining, cells were prepared by cytospin at 200 ×*g* for 5 min, fixated for 30 s in methanol, washed, stained and visualized using fluorescence microscopy.

2.4. Clonogenicity assay

For clonal growth assays, 2500 stained keratinocytes were sorted into 6-well plates pre-seeded with mitotically inactivated J2-3T3 mouse embryonic fibroblasts. Cells were grown in complete FAD medium, which were freshly replaced every second day. After 14 days of culture, feeder cells were removed with 0.15 mM EDTA and the plates were fixated with 2% neutral buffered formaldehyde (Cellpath Ltd., Newtown, UK) for 10 min, stained with 2% Rhodamine for 30 min and washed with PBS. The number of colonies and colony sizes were measured for each population. The assay was conducted as three independent experiments ($n = 3$).

2.5. Whole-mount preparation

Mouse tail skin was isolated by performing a longitudinal section and subsequently peel off the skin from the bone and connective tissue.