Stem Cell Research 23 (2017) 109–118

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Constitutive transgene expression of Stem Cell Antigen-1 in the hair follicle alters the sensitivity to tumor formation and progression

Rikke Christensen [a,c], David M. Owens [b,f], Annette C. Füchtbauer [d], Anders Gunnarsson [a,c], Mette Ramsing [e], Ernst-Martin Füchtbauer [d], Uffe Birk Jensen [a,*]

[a] Department of Clinical Genetics, Aarhus University Hospital, Brendstrupgaardsvej 21C, 8200 Aarhus N, Denmark
[b] Department of Dermatology, Columbia University Medical Center, 1150 St. Nicholas Avenue, New York, NY 10032, USA
[c] Department of Biomedicine, Aarhus University, Bartholins Allé 6, 8000 Aarhus C, Denmark
[d] Department of Molecular Biology and Genetics, Aarhus University, C. F. Møllers Allé 3, 8000 Aarhus C, Denmark
[e] Department of Pathology, Aarhus University Hospital, Palle Juul- Jensens Boulevard 99, 8200 Aarhus N, Denmark
[f] Department of Pathology & Cell Biology, Columbia University Medical Center, 1150 St. Nicholas Avenue, New York, NY 10032, USA

ARTICLE INFO

Article history:
Received 22 March 2017
Accepted 6 July 2017
Available online 8 July 2017

Keywords:
Stem cell markers
Stem Cell Antigen-1

ABSTRACT

The cell surface protein Stem Cell Antigen-1 (Sca-1) marks stem or progenitor cells in several murine tissues and is normally upregulated during cancer development. Although the specific function of Sca-1 remains unknown, Sca-1 seems to play a role in proliferation, differentiation and cell migration in a number of tissues. In the skin epithelium, Sca-1 is highly expressed in the interfollicular epidermis but is absent in most compartments of the hair follicle; however, the function of Sca-1 in the skin has not been investigated. To explore the role of Sca-1 in normal and malignant skin development we generated transgenic mice that express Sca-1 in the hair follicle stem cells that are normally Sca-1 negative. Development of hair follicles and interfollicular epidermis appeared normal in Sca-1 mutant mice; however, follicular induction of Sca-1 expression in bulge region and isthmus stem cells reduced the overall yield of papillomas in a chemical carcinogenesis protocol. Despite that fewer papillomas developed in transgenic mice a higher proportion of the papillomas underwent malignant conversion. These findings suggest that overexpression of Sca-1 in the hair follicle stem cells contributes at different stages of tumour development. In early stages, overexpression of Sca-1 decreases tumour formation while at later stages overexpression of Sca-1 seems to drive tumours towards malignant progression.

1. Introduction

The epidermis is a multilayered epithelium that provides a protective cover of the external body surface. It consists of the interfollicular epidermis (IFE), the hair follicle (HF) and the sebaceous gland (SG). A complex hierarchy of distinct progenitor cell populations resides in the epidermis in order to maintain a functional epidermis during normal homeostasis (Page et al., 2013). The progenitor cells in the various compartments can be identified by the expression of subsets of different molecular markers. The bulge region stem cells of the hair follicle express CD34 and keratin 15 (Blanpain et al., 2004; Lyle et al., 1998; Trempus et al., 2003). Partly overlapping populations of the progenitor cells in the lower part of the bulge region and hair germ express Lgr5 (Jaks et al., 2008) and Gli1 (Brownell et al., 2011) respectively. The isthmus and upper isthmus contains multiple partly overlapping populations marked by the expression of Lgr6, Plet1/Mts24, and Lrig1 (Jensen et al., 2009; Nijhof et al., 2006; Snippert et al., 2010). Upon wounding, all progenitor cell populations contribute to tissue repair irrespective of the population of origin (Page et al., 2013). Common to all the stem cell populations identified so far in the epidermis is the absence of Sca-1 expression, which is interestingly as Sca-1 expression marks stem/progenitor in the hematopoietic system (Bradfute et al., 2005; Ito et al., 2003), muscle (Asakura et al., 2002; Jankowski et al., 2001), heart (cardiac stem cells) (Oh et al., 2003; Wang et al., 2014), mammary gland (Welm et al., 2002), liver (Wright et al., 2008) and prostate (Xin et al., 2005). Sca-1 belongs to the Ly6 gene family encoding highly homologous, glycosyl-phosphatidylinositol-anchored membrane proteins (van de Rijn et al., 1989). Although the precise function of Sca-1 remains unclear, Sca-1 has in several studies been shown to play a role in proliferation and differentiation (Epting et al., 2008a; Epting et al., 2004; Henderson et al., 2002; Jensen et al., 2008; Mitchell et al., 2005).

Abbreviations: Sca-1, Stem Cell Antigen-1; DMBA, 7,12-Dimethylbenz[*a*]anthracene; TPA, 2-*O*-tetradecanoylphorbol-13-acetate; EdU, 5-Ethynyl-2-deoxyuridine; TGF-β, transforming growth factor-β.

* Corresponding author.
E-mail addresses: rikkchis@rm.dk (R. Christensen), do2112@cumc.columbia.edu (D.M. Owens), acf@mbg.au.dk (A.C. Füchtbauer), gunnarssonanders@live.se (A. Gunnarsson), metramsi@rm.dk (M. Ramsing), emf@mbg.au.dk (E.-M. Füchtbauer), uffejens@rm.dk (U.B. Jensen).

http://dx.doi.org/10.1016/j.scr.2017.07.002

Sca-1 is highly expressed in the interfollicular epidermis outside the stem cell niches where most proliferating keratinocytes reside (Jensen et al., 2008; Triel et al., 2004) and expression has also been detected in other non stem cell populations such as proliferating myoblasts (Mitchell et al., 2005) and mature T cells (Spangrude et al., 1988). Several malignant tissues show elevated levels of Sca-1 expression including retinoblastomas (Seigel et al., 2007), prostate tumours (Xin et al., 2005), mammary tumours (Grange et al., 2008; Li et al., 2003; Yin et al., 2009), and chronic myeloid leukemia (Perez-Caro et al., 2009).

It has furthermore been speculated whether Sca-1 plays a role in stem cell self-renewal. Thus, some experiments with Sca-1 −/− knock-out mice investigating hematopoietic and mesenchymal progenitor cells have pointed to a role for Sca-1 in self-renewal (Bonyadi et al., 2003; Ito et al., 2003). However, other studies on hematopoietic stem cells and muscle stem cells indicate that Sca-1 does not seem to affect self-renewal (Bradfute et al., 2005; Kafadar et al., 2009). A complex phenotype has been observed in Sca-1 −/− knock-out mice including defects in the regulation of lineage commitment in hemapoietic stem cells (Bradfute et al., 2005), osteoporosis, reduced muscle size in aging KO mice (Bonyadi et al., 2003) and delayed muscle repair after injury (Epting et al., 2008b). In mammary tumor cells, Sca-1 has been shown to regulate cell migration, cell adhesion to several extracellular matrix substrates and tumor development in early lesions (Batts et al., 2011) and a study using a mammary adenocarcinoma cell line could demonstrate that Sca-1 suppresses GDF10-dependent TGF-β signaling by disrupting the heterodimerization of the TGF-β receptors (Upadhyay et al., 2011). Additionally, TGF-β acts as a negative regulator of Sca-1 expression in both myoblasts and splenic T cells (Long et al., 2011).

Since lack of Sca-1 seems to be a common factor for most stem cell populations in the hair follicle, we hypothesized that overexpression of Sca-1 in stem cells would disturb normal hair follicle generation and maintenance and possibly tumour formation. Therefore, to explore the role of Sca-1 in normal and malignant skin we generated mice that overexpress Sca-1 in the hair follicle progenitor cells that are normally Sca-1 negative. Here we show that overexpression of Sca-1 in the bulge and upper isthmus stem cells decreases the incidence of benign tumours but seems to increase the frequency of progression from benign to malignant tumour. No effect was observed on hair follicle morphogenesis.

2. Materials and methods

2.1. Vector construction

To produce transgenic mice expressing Sca-1 in the basal layer of the skin we utilized a CRE-regulated Rosa26-DEST knock-in vector system (Hohenstein et al., 2008) to create an altered Rosa26 locus, *Rosa26*TM$^{116(Ly6a)Emfu}$. The targeting vector Rosa26-DEST-Sca-1 (hereafter Rosa-Sca1) was produced by a 2-step procedure. The 0.7 kb full-length mouse Sca-1 cDNA fragment was isolated from plasmid pCMV-Sport6-Sca1 (RZPD) and blunt end ligated into the pENTR11 vector (Invitrogen, Life Technologies, Naerum, DK) to obtain the vector pENTR-Sca-1. The pENTR-Sca-1 then served as entry vector and pRosa26-DEST as destination vector in a Gateway LR clonase reaction (Invitrogen) to yield the pROSA26-DEST-Sca-1 vector. The LR clonase enzyme mix was transformed into Stbl3 *E. coli* cells and grown at 30 °C. The construct was confirmed by sequencing.

2.2. ES cell culture

CJ7 embryonic stem cells, derived from 129S1/Sv mice (Swiatek and Gridley, 1993), were electroporated with 25 μg *Kpn*I-linearized targeting vector pROSA26-DEST-Sca-1 and screened for resistance towards G418 (350 μg/ml). DNA from individual clones was isolated and digested with *Eco*RV according to (Ramirez-Solis et al., 1993) and subsequently analyzed by Southern blot. Targeting efficiency was 11%.

To test the expression level of Sca1 from the ROAS26 promoter, the neomycin phosphotransferase expression casette was excised in five selected clones by transient expression of Cre recombinase by electroporation.

Two days after transfection, the pools of Cre transfected cells were harvested by trypsinisation and plated on gelatinized plates for 45 min to remove feeder cells. The non-attached ES cells were stained with PE-conjugated Ly-6A/E (Sca-1, BD A/S, Albertslund, DK) and subjected to flow cytometry analysis.

2.3. Transgenic mice

10–12 ES cells from one positive clone were injected into B6D2F2 blastocysts (Wertz and Füchtbauer, 1994), which were then transfered to pseudopregnant NMRI female mice that gave birth to chimeric animals. Male chimeras were mated with C57Bl/6J female mice and agouti offspring (indicating germ line transmission of the manipulated 129S1/Sv ES cells) were tested for the presence of the Rosa-Sca1 mutation by PCR using genomic tail DNA.

Heterozygous offspring from the chimeras was backcrossed ten generations with C57Bl/6j, designated *Rosa*^{Sca1}. The resulting mice were crossed with K14-Cre mice (Tg(KRT14-cre)1Amc/J, The Jackson Laboratory, Bar Harbor, Main, USA) to obtain mice heterozygous for both the K14-Cre and Rosa-Sca1 alleles, designated *Rosa*^{Sca1}*:Cre*$^{+}$. These mice were then further crossed with a heterozygous *Rosa*^{Sca1} mouse to obtain mice homozygous for the Rosa-Sca1 allele and with and without one copy of the K14-Cre construct, designated *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and designated *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ respectively. For the experiments, siblings homozygous for Rosa-Sca1 and heterozygous for K14-Cre (*Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$) were crossed with siblings homozygous for the Rosa-Sca1 and without Cre (*Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$) in order to obtain a 50/50 ratio of *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ mice.

Mice were housed under a light/dark cycle of 12 h with free access to food and water, and bred under specific pathogen-free conditions. All animal experiments were conducted in accordance with institutional guidelines and approved by the Animal Experiments Inspectorate in Denmark.

2.4. Genotyping

Presence of the Rosa-Sca1 and K14-Cre transgenes were determined in a multiplex PCR on DNA isolated from a tail biopsy by using the REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich, St. Louis, MO, USA). The identification of the Rosa-Sca1 transgene was performed by using the primers Rosa forward and Sca1 seq reverse flanking the upper cloning site in the transgenic construct and the primers IMR1084 and IMR 1085 were used in order to verify the integration of the K14-Cre transgene. Actin and IMR 42/43 primers were used as a control of the DNA preparation. The sequences of the primers are: Rosa forward: 5′-CATCAAGCTGATCCGGAACCC-3′; Sca1-seq reverse: 5′-CTGCACACA GTAGGGCCACAAG-3′; actin sense: 5′-CTGTGCTGTCCCTGTATGCC-3′; actin antisense: 5′-GTGGTGGTGAAGCTGTAGCC-3'; IMR1084: 5′-GCGGTCTGGCAGTAAAAACTATC-3′; IMR1085: 5′-GTGAAACAGCA TTGCTGTCACTT-3′; IMR42: 5′-CTAGGCCACAGAATTGAAAGATCT-3′; IMR43: 5′-GTAGGTGGAAATTCTAGCATCATCC-3′. The DNA was amplified by using the REDExtract-N-Amp Tissue PCR kit according to the manufacturer's instructions. Amplification parameters were as follows – after an initial denaturation step at 94 °C for 3 min, amplification was performed for 35 cycles at 94 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min.

2.5. Flow cytometry

Primary adult epidermal keratinocytes were isolated from 7 week old female mice as previously described (Nowak and Fuchs, 2009). Freshly isolated epidermal keratinocytes were suspended in 0.1% BSA/

PBS and surface markers stained with PE-conjugated CD49f (BD A/S), PE-Cy7-conjugated Ly-6A/E (Sca-1, BD A/S) and Alexa Fluor 647-conjugated CD34 (eBiosciences, San Diego, CA, USA) antibodies. Tumor cells were furthermore stained with APC-Cy7 conjugated CD45 (BD A/S) antibody to eliminate blood cells and V450-conjugated rabbit anti-active Caspase-3 (BD A/S) to detect apoptotic cells. Freshly isolated keratinocytes were stained with PI to eliminate dead cells. Cells were subjected to FACS analysis using a FACS Aria sorter and FACS Diva 6.1 software (BD, Franklin Lakes, NJ, USA). In all cases, cells flew under 20-psi pressure through a 100 μm nozzle. FITC, PE, PE-Cy7, Alexa Fluor 647 and APC-Cy7 signals were collected through 530/30, 585/42, 780/60, 660/20, and 780/60 band pass filters, respectively.

2.6. Fixation of cells

ZBF-fixation: Cells were suspended in 1 vol. PBS. Ten vol. ZBF-buffer (0.1 M Tris-HCl pH 7.8, 0.05% $(CH_3COO)_2Ca$, 0.5% $(CH_3COO)_2Zn$, 0.5% $ZnCl_2$) was added while vortexing. The cells were incubated at 4 °C over night. Cells were either frozen in glycerol (1:1) or washed carefully 3 times in TBS prior to use (total 30–45 min).

2.7. Label-retention analysis

To obtain label-retaining cells, EdU (0.05 mg/g mouse) was injected s.c. every 12 h on day 10 and 11 and the mice were sacrificed either 4 h after last pulse (day 11), or when mice were 21 days (first telogen phase) or 7 weeks old (second telogen phase). The isolated cells were subjected to ZBF-fixation. EdU was detected using a Click-iT assay (Molecular Probes, Life Technologies) using Alexa Fluor 488 azide and combined with surface markers as described (Christensen et al., 2011).

2.8. Clonogenicity assay

Keratinocytes were sorted by FACS directly into tissue culture dishes (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) at 10,000 cells per well in 6-well plates containing epidermal keratinocyte medium (Cnt-57, CELLnTEC, Bern, Schwitzerland) or in dishes pre-plated with mitotically-arrested 3T3 fibroblasts (strain J2) in complete serum containing keratinocyte medium (modified from (Rheinwald and Green, 1975) as described by Wu et al. (Wu et al., 1982)). The medium consists of three parts of Dulbecco's modified Eagle's medium (Invitrogen, Life Technologies) and one part Ham's F12 medium (Invitrogen, Life Technologies) supplemented with 10% fetal bovine serum (firma), 100 U/ml of pencillin and streptomycin (Gibco, Life Technologies), 2 mM of L-glutamine (Gibco, Life Technologies), 0.5 μg/ml of hydrocortisone (Calbiochem, San Diego, CA, USA), 1.8×10^{-4} M of adenine (Sigma-Aldrich), 5 μg/ml of insulin (Sigma), 1×10^{-10} M of cholera enterotoxin (ICN, Costa Mesa, CA, USA) and 10 ng/ml of EGF (Gibco, Life Technologies). Cultures were incubated at 32 °C for approximately 2 weeks, after which the 3T3 cells were removed and keratinocyte colonies were fixed and stained with 0.6% w/v methylene blue in methanol. The experiment was repeated 3 times.

2.9. Histology

Samples from tail skin, dorsal skin, and skin tumours were frozen in dry ice-cooled *n*-hexane after embedding in O.C.T. medium (Sakura, Alphen aan den Rijn, NL). Frozen sections were stained with hematoxylin and eosin. Tumor sections were graded as papillomas or SCCs.

2.10. Epidermal wholemount staining

Epidermal wholemounts were processed and stained essentially as described previously (Liakath-Ali et al., 2014). In brief, skin was detached from tails from 14-week old male Sca-1 transgenic *Rosa*$^{Sca1/Sca1}$:Cre^{+} and control littermate mice (*Rosa*$^{Sca1/Sca1}$:Cre^{-}). Pieces (0.5 cm × 0.5 cm^2) of skin were incubated in 5 mM EDTA in PBS at 37 °C for 4 h. The epidermis was gently detached from the dermis and fixed in NBF (10% Neutral buffered formalin, Cellpath Ltd., Newtown, UK) for 1 h at room temperature. Fixed epidermal sheets were washed in PBS.

Mouse monoclonal K15 (LHK-15) antibody was directly conjugated to Alexa Fluor 568 using the Zenon mouse IgG labeling kit (Molecular Probes, Life Technologies, Eugene, OR, USA) according to manufacturer's instructions. NBF-fixed epidermal sheets were placed in 2 ml tubes and blocked in PB buffer (0.5% skimmed milk powder, 0.25% fish skin gelatin, 0.5% Triton ×100, 1 × HEPES-buffered saline) for 30 min at room temperature on a rocking table. Epidermal sheets were stained with K14 antibody directly conjugated with Alexa Fluor 488 (1:500, LL002-Alexa Fluor 488, kind gift, (Purkis et al., 1990)), directly conjugated K15 antibody and DAPI (1:500, 1 mg/ml) diluted in PB buffer for 2 h at room temperature on a rocking table. Samples were washed three times with PBS/0.2% Tween solution at room temperature for 15 min per wash, rinsed in MilliQ water and mounted on glass slides in Mowiol with 2.5% Dabco.

2.11. Tumor studies

Seven-wk.-old female *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and female control littermate mice (*Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$) received one topical application of 50 μg 7,12-dimethylbenz[*a*]anthracene (DMBA, Sigma-Aldrich) in 200 μl acetone or 200 μl acetone alone. One week later, the mice received triweekly applications of 4 μg 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Sigma-Aldrich) in 200 μl acetone or 200 μl acetone alone for 15 weeks. Papillomas larger than 1 mm and SCC were recorded once a week for up to 50 weeks after the start of the promotion. When one of the tumours on the mouse exceeded 2 cm in one direction the mouse was sacrificed and a small piece from the papillomas and SCCs were frozen and stained as described and the rest purified according to Yuspa et al. (Yuspa et al., 1986) and subjected to flow cytometry analysis.

2.12. Acute TPA treatment

Seven-week-old female Sca-1 transgenic (*Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$) and Rosa$^{Sca1/Sca1}$:Cre^{-} control littermate mice received topical applications of 4 μg 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Sigma-Aldrich) in 200 μl acetone or 200 μl acetone alone on day 1 and 3. On day 4, all mice were injected s.c. with EdU (2 mg/mouse) and the treated back skin was collected 2 h later. Cells isolated from the skin were subjected to ZBF-fixation and EdU was detected using a Click-iT assay (Molecular Probes, Life Technologies) using Alexa Fluor 647 azide and combined with the surface markers PE-conjugated CD34 (BD A/S), PE-Cy7-conjugated Ly-6A/E (Sca-1, BD A/S) and FITC-conjugated CD49f (BD A/S) antibodies as described (Christensen et al., 2011).

2.13. Acute DMBA and TPA treatment for apoptosis analysis

Seven-week-old Sca-1 transgenic (*Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$) and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ control littermate mice received one topical application of 50 μg 7,12-dimethylbenz[*a*]anthracene (DMBA, Sigma-Aldrich) in 200 μl acetone or 200 μl acetone alone. One week later, the mice received one application of 4 μg 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Sigma-Aldrich) in 200 μl acetone or 200 μl acetone alone. The day after the skin was harvested and frozen. Frozen sections were stained with rabbit anti-active Caspase-3 (BD Pharmingen) followed by staining with secondary antibody conjugated to Alexa Fluor 488, and nuclei were visualized using hoechst.

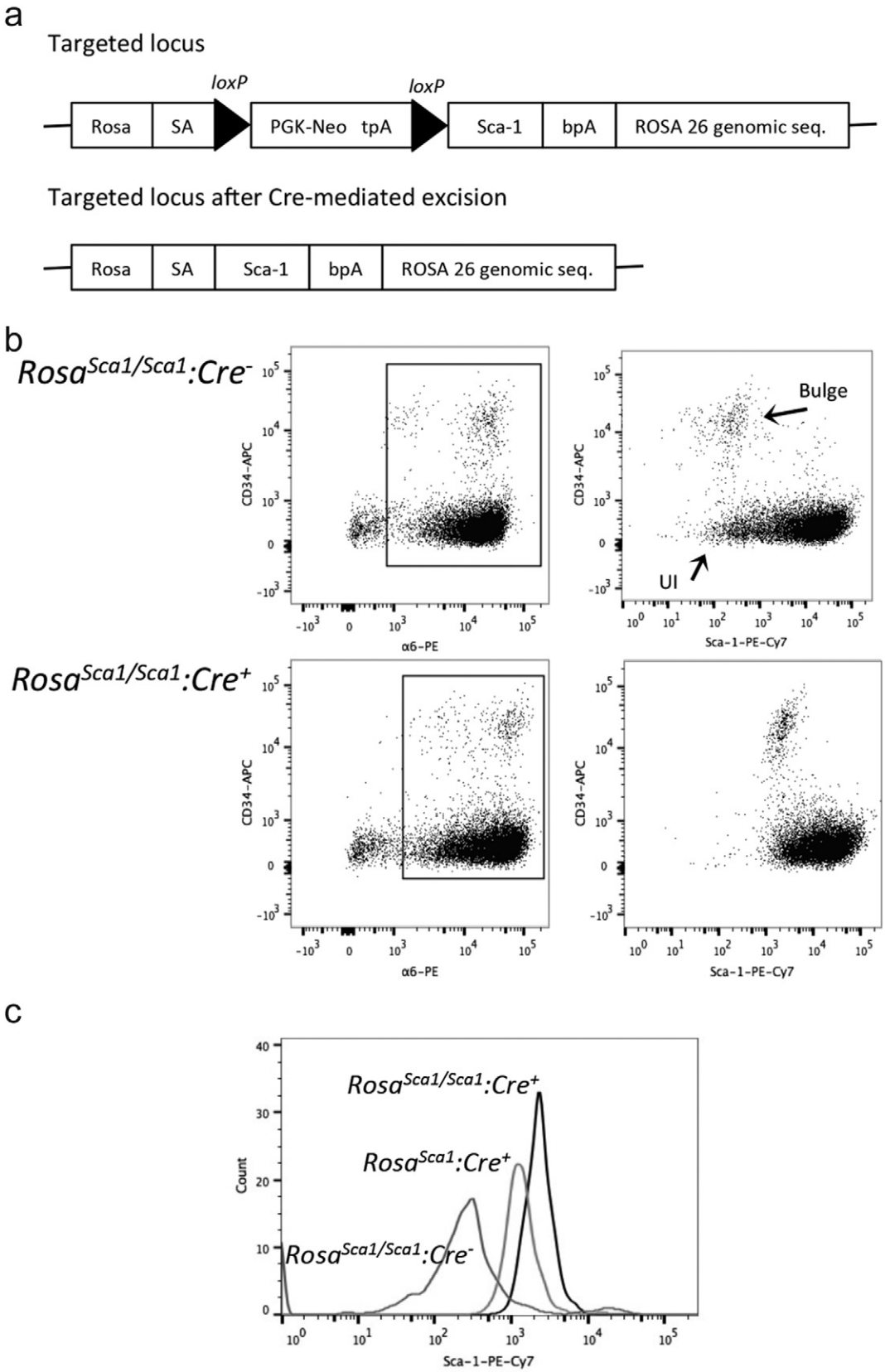

Fig. 1. Development of *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice with expression of Sca-1 in hair follicle stem cells. (a) Diagram of the targeted locus in Rosa-Sca1 transgenic mice. The construct contains a floxed neo gene separating the ROSA26 promoter from the Sca-1 gene. Upon crossbreeding to a strain expressing Cre from the K14 promoter, Sca-1 becomes expressed in Cre-active cells that have recombined out the floxed neo gene. (b) Flow cytometry analysis of control mice (upper panel) and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ transgenic mice (lower panel). Epidermal cells were harvested and stained with Sca-1, CD34 and ItgA6. UI: Upper isthmus. (c) The level of Sca-1 in the bulge stem cell population (CD34^{+}) measured by flow cytometry in control mice, *Rosa*^{Sca1}*:Cre*$^{+}$ and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice.

2.14. Statistical analysis

Data are expressed as means ± SD. Statistical analyses were performed using the unpaired Student *t*-test and chi-square test. Values of $p < 0.05$ were considered significant.

The statistical significance of differences in papilloma formation ≥ 2 mm and number of papillomas ≥ 5 mm in diameter per mouse between *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ control mice was determined with a Wilcoxon two-sample test.

3. Results

3.1. Generation of mice overexpressing Sca-1 in hair follicle progenitor cells

To analyze the role of Sca-1 in both normal and malignant skin we developed a transgenic mouse model that express Sca-1 in the hair follicle progenitor cells that are normally Sca-1 negative.

We generated a Rosa26 knock in allele (*Rosa26*$^{TM15(Ly6a)Emfu}$) from which the Sca-1 cDNA can be conditionally expressed (designated *Rosa*^{Sca1}). This allele was generated using the Rosa26-DEST knock-in vector system (Hohenstein et al., 2008) (Fig. 1a). This allows us to activate Sca-1 expression in specific tissues upon cross breeding to a mouse line expressing Cre recombinase from a tissue specific promoter.

By cross breeding *Rosa*^{Sca1} to a strain expressing Cre from the keratin 14 promoter we have obtained mice that express Sca-1 in cells and their progeny if the K14 promoter have been active. We produced mice that were homozygous with respect to the Rosa locus and heterozygous with respect to the K14-Cre locus (*Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$). Littermates that were homozygous in the Rosa locus but without the K14-Cre construct served as negative controls (*Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$).

To confirm that expression of Sca-1 is induced in the stem cells in these mice, primary adult epidermal keratinocytes were isolated from *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice and compared to cells isolated from *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ control mice. For comparison mice that were heterozygous with respect to the Rosa locus (*Rosa*^{Sca1}*:Cre*$^{+}$) was included. The cells were stained with the markers Sca-1, CD34 and ItgA6 and subjected to flow cytometry analysis. The level of Sca-1 is clearly up-regulated in both the bulge (CD34^{+}, Sca-1^{-},ItgA6^{high}) and upper isthmus (CD34^{-}, Sca-1^{-},ItgA6^{low}) stem cell populations in *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice compared to *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ controls (Fig. 1b). Higher levels of Sca-1 were obtained in homozygous knock-in mice compared to heterozygous knock-in mice (Fig. 1c).

3.2. Normal follicular morphogenesis in *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice

Whole mount skin preps from *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ control mice were stained with K14 and K15 antibodies and scored for epidermal abnormalities as previously described (Liakath-Ali et al., 2014). Despite up-regulated expression of Sca-1 in the stem cells, hair follicles developed and appeared normal (Fig. 2 a and b) and the skin had the same thickness as in normal mice (Fig. 2 c and d).

There were no obvious phenotypic differences in *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and control mice when followed up to an age of 15 months. The histopathology of the skin was analyzed to observe whether pathologic differences appeared with age. Sections from tail and back skin of *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ control mice were prepared at ages 6, 9, 12 and 15 months and stained with hematoxylin and eosin. No clear difference could be observed between the two groups (data not shown). Epidermal keratinocytes were isolated from the same aged mice as described above and the cells stained with the markers Sca-1, CD34 and ItgA6. Flow cytometric analysis did not show any difference in the percentage of CD34^{+} cells between *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ controls at any time points (data not shown).

3.3. Clonogenic assay and label retention assay

To determine whether Sca-1 can influence the keratinocyte stem cell clonogenic potential, freshly isolated CD34^{+} epidermal keratinocytes from *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ controls were sorted using FACS directly into tissue culture plates containing FAD medium and mitotically arrested 3T3 fibroblasts. After culturing for 2 weeks, keratinocytes were visualized using methylene blue. CD34^{+} bulge

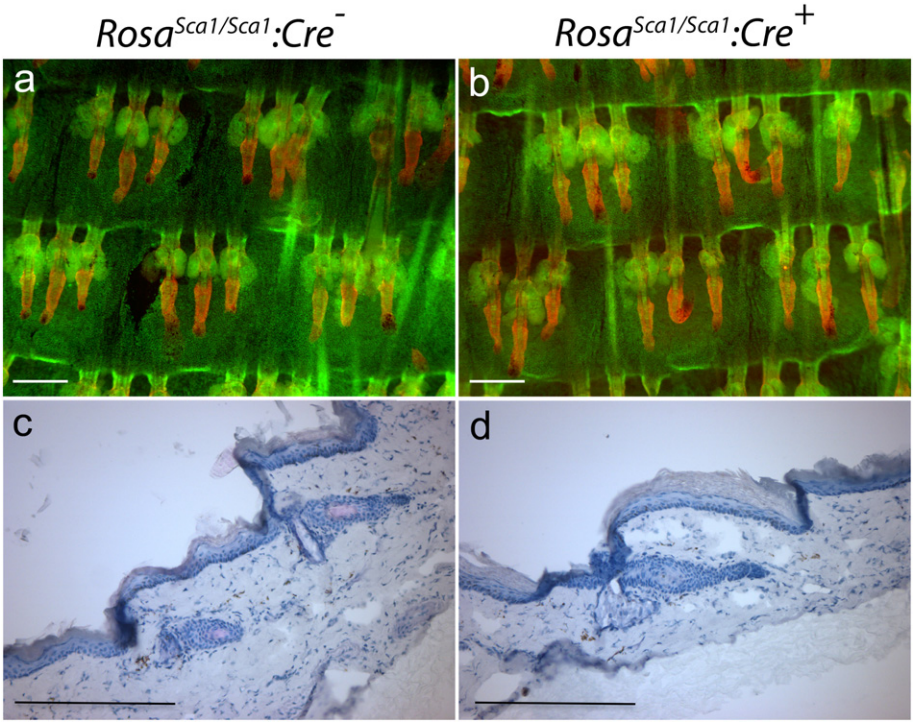

Fig. 2. Morphology of the skin in *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ transgenic mice. (a + b) Epidermal wholemount images of control and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ transgenic mice labeled for K14 (green) and K15 (red). Scale bars, 200 μm. (c + d) Histological sections stained with hematoxylin and eosin. Scale bars, 200 μm.

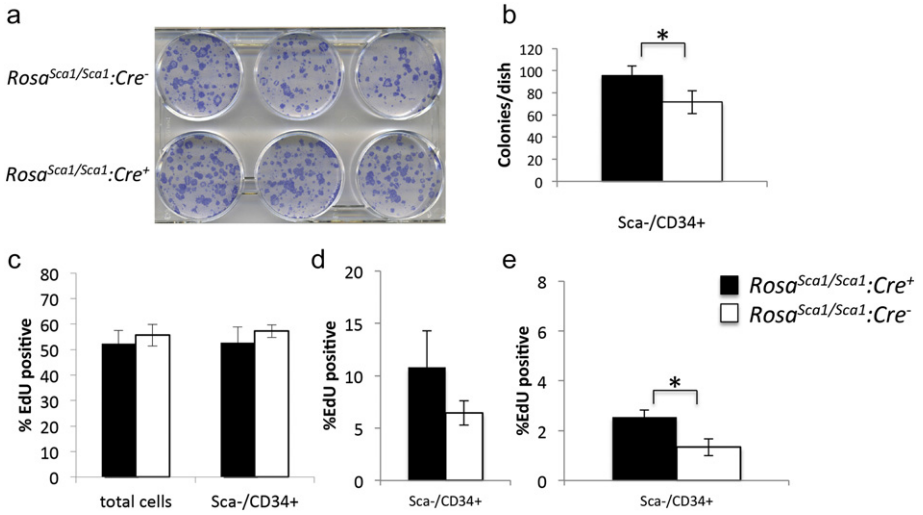

Fig. 3. Colony forming efficiency and label retention in Sca-1 induced stem cells. (a) $CD34^+$ epidermal stem cells from $Rosa^{Sca1/Sca1}:Cre^+$ and control mice were plated at equal density. Cultures were stained 2 weeks after with methylene blue and (b) the average total colony number was quantified and statistically compared using Student's *t*-test (*$P < 0.05$, $n = 3$ plates per group, error bars denote SD). Black bars: $Rosa^{Sca1/Sca1}:Cre^+$ mice, white bars: control mice.(c–e) Cells retaining label are represented as the average percentage of EdU-positive cells within the Sca-/$CD34^+$ population or within the total cell population. Mice were pulse labeled four times at day 10 and 11. Black bars: $Rosa^{Sca1/Sca1}:Cre^+$ mice, white bars: control mice. (c) $Rosa^{Sca1/Sca1}:Cre^+$ ($n = 7$), control mice ($n = 6$) sacrificed at day 11. (d) $Rosa^{Sca1/Sca1}:Cre^+$ ($n = 10$), control mice ($n = 3$) sacrificed at age 21 days. (e) $Rosa^{Sca1/Sca1}:Cre^+$ ($n = 4$), $Rosa^{Sca1/Sca1}:Cre^-$ control mice ($n = 8$) sacrificed at 7 weeks of age. *$P = 0.0001$ (Student's *t*-test). Error bars denote SD.

cells from $Rosa^{Sca1/Sca1}:Cre^+$ mice exhibited higher clonogenic capacity compared to bulge cells from $Rosa^{Sca1/Sca1}:Cre^-$ controls (Fig. 3 a and b). Similar results were obtained when FACS-sorted populations were plated in serum-free epidermal keratinocyte medium (Cnt-57) (data not shown). There were no difference in morphology between clones from $Rosa^{Sca1/Sca1}:Cre^+$ and control mice.

Next, the levels of label-retaining cells (LRC) in the hair follicle bulge stem cell pool were compared between $Rosa^{Sca1/Sca1}:Cre^+$ and control littermates. Ten-day old mice were pulse labeled with 5-ethynyl-2-deoxyuridine (EdU). Skins were examined for EdU^+ LRCs at day 11, day 21 and day 49. $Rosa^{Sca1/Sca1}:Cre^+$ and control mice exhibited similar levels of initial EdU uptake (Fig. 3c, total cells). The level of EdU^+ cells in the $CD34^+$ population was the same at day 11 in $Rosa^{Sca1/Sca1}:Cre^+$ mice and control littermates (Fig. 3c). In the first telogen phase, there were not statistically significant more LRC cells in the $CD34^+$ population in $Rosa^{Sca1/Sca1}:Cre^+$ transgenic mice compared to control littermates (Fig. 3d $p = 0.060$). However, in the second telogen phase, the level of LRC cells in the $CD34^+$ population was significantly higher ($p = 0.0001$) in the transgenic mice compared to control littermates (Fig. 3e).

3.4. Tumor formation

Since the clonogenic assay and the label retention assay may suggest that Sca-1 expression alters the number or behavior of progenitor cells in the bulge we asked if this alteration could have an impact on the ability to form tumours and on the progression from benign to malignant tumour. To investigate this we applied a two-step chemical carcinogenesis protocol on the $Rosa^{Sca1/Sca1}:Cre^+$ mice and $Rosa^{Sca1/Sca1}:Cre^-$ control littermates. The mice were treated once with DMBA and subsequentially 3 times per week with TPA for 15 weeks. DMBA induces H-ras mutations and repeated TPA treatments cause tumour promotion (Owens and Watt, 2001). Tumour formation and development was followed and compared in the mice for up to one year. Papillomas began to appear 6 weeks after start of TPA treatment in both types of mice. After 12 weeks of promotion, transgenic $Rosa^{Sca1/Sca1}:Cre^+$ mice showed a statistically significant reduction in the number of papillomas as compared to $Rosa^{Sca1/Sca1}:Cre^-$ controls (Fig. 4a) ($Rosa^{Sca1/Sca1}:Cre^-$: 9.2 papillomas ≥2 mm/mouse; $Rosa^{Sca1/Sca1}:Cre^+$: 3.8 papillomas ≥ 2 mm/mouse; $P = 0.01$). The number of large papillomas (≥5 mm in diameter) per mouse was also decreased in the transgenic $Rosa^{Sca1/Sca1}:Cre^+$ group (Fig. 4b), however this difference was not statistically significant (week 34: $Rosa^{Sca1/Sca1}:Cre^-$: 2.6 papillomas ≥ 5 mm/mouse; $Rosa^{Sca1/Sca1}:Cre^+$: 0.9 papillomas ≥ 5 mm/mouse; $P = 0.37$). The number of papillomas ≥ 2 mm started to drop after 13 weeks of TPA treatment in both groups of mice, however, the difference in papilloma load between the mice remained until week 44. The proportion of mice that developed papillomas was the same for the two groups of mice (95% of $Rosa^{Sca1/Sca1}:Cre^+$ vs. 100% control). No papillomas were observed in control or $Rosa^{Sca1/Sca1}:Cre^+$ mice initiated with DMBA and promoted with acetone vehicle. Very few papillomas developed in mice initiated with acetone vehicle and promoted with TPA and the papillomas appeared much later (Fig. 4a).

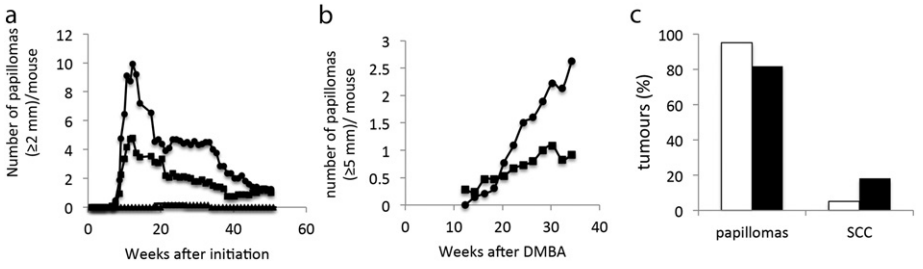

Fig. 4. Responsiveness of $Rosa^{Sca1/Sca1}:Cre^+$ mice to two-stage skin carcinogenesis. $Rosa^{Sca1/Sca1}:Cre^+$ (■, $n = 19$) and control mice (●, $n = 17$) were treated with one topical application of DMBA. One week later, the mice received triweekly applications of TPA for 15 weeks. $Rosa^{Sca1/Sca1}:Cre^+$ (—, $n = 7$) and control mice (▲, $n = 6$) were treated with TPA only for 15 weeks. (a) Average number of papillomas per mouse exceeding 2 mm. (b) Average number of papillomas per mouse exceeding 5 mm. (c) The percentage of tumours above 5 mm in $Rosa^{Sca1/Sca1}:Cre^+$ and control mice graded as either papilloma or SCC. Black bars: $Rosa^{Sca1/Sca1}:Cre^+$ mice (SCC, $n = 2$; papillomas, $n = 9$), white bars: control mice (SCC, $n = 1$; papillomas, $n = 19$).

The histopathology was examined for tumours above 5 mm and the tumours were classified according to their degree of dysplasia. The histopathology of the papillomas of *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice was not phenotypically distinct from those of control mice. The papillomas tended to be well-differentiated with a low grade of dysplasia in both groups of mice. Only few of the tumours developed into SCCs. In *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice, two tumours developed into SCCs. One tumour was well differentiated and the other was poorly differentiated. In control mice, only one of the tumours developed into a well-differentiated SCC. The proportion of papillomas above 5 mm developing into SCCs was higher in *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice (18%) than in control mice (5%) (Fig. 4c). Although the difference was not statistical significant (χ^2 test, P = 0.23) due to the small number of tumours, the data indicate a tendency to a higher malignant conversion frequency in the *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice.

Flow cytometry analysis of tumours from the different mice using the markers CD34, Sca-1 and ItgA6 showed that there were less Sca-1 negative cells in tumours from *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice (Supplementary Fig. 1 c and d) than control mice (Supplementary Fig. 1 a and b). Furthermore, in both *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ controls, SCCs and papillomas with a high grade of dysplasia (Supplementary Fig. 1 b and d) contained fewer cells with low Sca-1 expression than papillomas with a low grade of dysplasia (Supplementary Fig. 1 a and c) indicating that Sca-1 is upregulated during tumor progression.

Caspase-3 is a protease that is activated during the early stages of apoptosis. Staining of the tumours with antibody against active Caspase-3 showed no difference in the number of Caspase-3 positive cells in tumours from *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ control mice (Supplementary Fig. 2).

3.5. Response to acute DMBA and TPA exposure

The effect of acute treatment with DMBA and TPA on *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and control mice was analyzed by staining tissue sections from mice treated with one topical application of DMBA and TPA 1 week apart with anti-active Caspase-3. Treatment with DMBA and TPA did not induce apoptotic cells in the epidermis or hair follicles in neither *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ nor *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ control mice (data not shown). An increase in apoptotic cells was only observed in the dermis (data not shown).

To investigate whether tumor promotion with TPA resulted in altered proliferation rates in the skin in *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{-}$ control mice, we treated mice with TPA twice 48 h apart. The mice were pulse labeled with EdU the day after and 2 h later the skin was harvested. The numbers of EdU positive cells were similar in control and *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice in the bulge (CD34^{+}, Sca1^{-}, ItgA6^{high}; Supplementary Fig. 3a), upper isthmus stem cells (CD34^{-}, Sca1^{-}, ItgA6^{low}; Supplementary Fig. 3b) and interfollicular epidermis (Sca-1^{+}, ItgA6^{high}; Supplementary Fig. 3c) both before and after exposure to TPA. All populations responded to TPA with an increase in cell proliferation with the highest increase observed in the interfollicular epidermis.

4. Discussion

Sca-1, encoded by the gene *Ly6a*, has been known for >30 years and is being used extensively for isolating hematopoietic stem cells in the mouse. The role of Sca-1 is still shrouded in uncertainty although a great number of publications have tried to address this issue (for review see (Holmes and Stanford, 2007)). We have previously shown that Sca-1 is useful when studying mouse hair follicle stem cells (Gunnarsson et al., 2016; Jensen et al., 2008) as it allows you to separate cells below the infundibulum of the hair follicles from cells of the infundibulum and interfollicular epidermis which express high levels of Sca-1.

Colony forming efficiency is often used to access the growth potential of epidermal sub populations and can be considered an indirect measure of stemness (Barrandon and Green, 1987; Jensen et al., 2008; Jones and Watt, 1993). One of the striking features of testing FACS sorted keratinocytes for colony forming efficiency is that Sca-1 positive cells normally perform inefficiently. However, bulge region keratinocytes from *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice that express Sca-1 showed a slightly increased clonogenic capacity in colony forming assays. This was somewhat surprising and may suggest a permissive role of Sca-1 action in terms of growth. Thus, bulge stem cells expressing Sca-1 may have a higher growth potential when cultured as compared to normal bulge stem cells and Sca-1 is normally expressed at high levels in proliferating cells in the skin. This may reflect that Sca-1 plays a role in controlling proliferation and that Sca-1 does not affect stemness directly. In other tissues like the hematopoietic system, muscle, mammary tissue, and liver, stem cells express Sca-1 in contrast to the hair follicle (Bradfute et al., 2005; Epting et al., 2008a; Epting et al., 2004; Ito et al., 2003; Mitchell et al., 2005; Welm et al., 2002; Wright et al., 2008). Therefore, expression of Sca-1 per se is not incompatible with a stem cell phenotype. We also know that colony forming efficiency does not always reflect potency as Sca-1 negative and CD34 negative cells that express high levels of alpha6 integrin do well in the colony forming assay but do not form hair in a hair reconstitution assay (Jensen et al., 2008). It could be speculated that while there was no impact on hair follicle homeostasis, there may be impact of Sca-1 induction on regeneration following wounding or grafting since bulge stem cells expressing Sca-1 were more clonogenic.

We also tested the *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice in label retaining experiments. Since label retaining cells (LRCs) are traditionally thought to reflect the number of stem cells, we measured the number of LRCs and found an increased number of label retaining cells (LRCs) in *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice as compared to control littermates.

From previous studies we know that cells above the bulge that are Sca-1 and CD34 negative also possess stem cell properties and that these stem cells are not label retaining cells, but relatively active in cell cycle (Jensen et al., 2008). This suggests that LRCs primarily reflects a certain pattern of proliferation that is characteristic for bulge stem cells and not for stem cells in the upper part of the hair follicle. Our data may suggest that overexpression of Sca-1 in the bulge stem cells leads to an accumulation of LRCs due to either inhibition of cell proliferation after the morphogenesis, or increased number of keratinocyte stem cells. The fact that equal amount of cells were labeled at day 11 may suggest that the number of stem cells has been affected. It has recently been shown that overexpression of Stat3 in bulge region keratinocyte stem cells resulted in a significant decrease in the number of LRCs in the hair follicles. Furthermore it was shown that Sca-1 is a direct transcriptional target of Stat3 in keratinocytes and it was furthermore hypothesized that the effect could be explained by an increase in Sca-1 expression in bulge region keratinocytes (Rao et al., 2015). However, in our study we have observed an increased number of LRCs in bulge region keratinocytes when directly overexpressing Sca-1 in bulge region keratinocytes. This discrepancy indicates that additional factors, regulated by Stat3, affect cell maturation.

Tumorigenesis is believed to primarily occur in stem cells. The theoretical argument is that tumorigenesis requires a certain number of genetic alterations and that stem cells are the only cells that are present in tissues over long periods of time necessary for the minimal number of alterations to occur. A two-step carcinogenesis model in mice supports this (Morris, 2004). It has been suggested that keratinocyte stem cells and in particular those in the bulge region of hair follicles could be potential targets for skin carcinogenesis (Kangsamaksin et al., 2007; Trempus et al., 2007).

To determine whether overexpression of Sca-1 in progenitor cells residing in the bulge and upper isthmus could function as an initiating event, *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice and control littermates were treated with TPA only. This treatment resulted in very few papillomas that quickly regressed and there was no significant difference between *Rosa*$^{Sca1/Sca1}$*:Cre*$^{+}$ mice and control mice. Thus, overexpression of Sca-1

in stem cells was insufficient as an initiating event. We next wanted to address whether overexpression of Sca-1 in stem cells had an effect on the promotion phase of carcinogenesis or on the malignant conversion rate. Therefore, transgenic and normal littermates were treated with a two-step carcinogenesis protocol. The DMBA/TPA protocol produces a large amount of papillomas. However, many of these regress rapidly when TPA treatment is terminated. Papillomas appeared at the same time point in transgenic mice as in normal mice. However, overexpression of Sca-1 reduced the overall number of skin tumours. In multistage chemical carcinogenesis of the mouse epidermis, malignant conversion of a benign papilloma to a squamous cell carcinoma (SCC) is a rare event (Pai et al., 1983). In our study, only few papillomas developed into SCCs. However, a larger proportion of the papillomas in the $Rosa^{Sca1/Sca1}$:Cre^{+} mice developed into SCCs as compared to control littermates which seems to indicate a higher malignant conversion frequency in the transgenic mice although the difference was not significant. A markedly undifferentiated SCC was observed in $Rosa^{Sca1/Sca1}$:Cre^{+} mice whereas the SCC derived from control mice was well differentiated further indicating increased malignancy of SCCs in the transgenic mice. A decrease in papilloma yield and a concomitant increase in the degree of malignant progression have previously been observed in the p53 null mouse model (Cui et al., 1994; Kemp et al., 1993). The authors speculated that many initiated cells die due to accumulation of DNA damage and progression through cell cycle before having repaired the damage, with consequent cell death. Furthermore, they argued that the increased benign to malignant conversion in developing tumour cells could be due to synergism between the presence of mutant H-ras, which is known to increase genetic instability (Stenman et al., 1987) and affect cell differentiation (Bar-Sagi and Feramisco, 1985; Yuspa et al., 1983), and the lack of p53, resulting in a greatly increased rate of accumulation of genetic alterations. Glick et al. previously observed that loss of TGF-β in epidermis was associated with suppression of low risk papillomas but not high risk papillomas (Glick et al., 1993). This is parallel to our observation that overexpression of Sca-1 in bulge stem cells suppressed the overall number of papillomas but not the number of papillomas that develop into SCCs. There could be several explanations for the reduction in the overall number of tumours in our model and a reduced number of stem cells would be a simple explanation. However, this is contradictory to the colony forming and label retaining experiments. Another explanation is that Sca-1 hyper expression induces an altered state of stemness in the hair follicle and that this state is only revealed under certain extreme conditions such as tissue culturing or carcinogenesis. We have recently shown that the hair follicle contains a more complex composition of subpopulations by using multi color flow cytometry (Gunnarsson et al., 2016). Here we could show that two novel populations performed well in a colony forming assay and that one of these to our surprise expressed Sca-1. From our earlier studies we also know that Sca-1 positive cells are unable to form hair in the hair reconstitution assays. As Sca-1 has been shown to play a role in proliferation and differentiation (Epting et al., 2008a; Epting et al., 2004; Henderson et al., 2002; Jensen et al., 2008; Mitchell et al., 2005) it could also be speculated whether altered proliferation in cells overexpressing Sca-1 in combination with increased genetic instability from the presence of mutant H-ras could lead to an increased rate of accumulation of genetic alterations with consequent cell death (apoptosis) at early stages and malignant progression in later stages of tumour development, similar to what was observed in the p53 null mouse (Cui et al., 1994; Kemp et al., 1993).

Measurement of apoptosis in the skin after acute treatment of DMBA and TPA show that the skin is relatively resistant to apoptosis. This has also been described previously (Raj et al., 2006). However, increased cell death in initiated cells could also happen weeks after initiation when sufficient genetic alterations have accumulated and would thereby be missed in the acute DMBA/TPA assay. Acute TPA treatment indeed resulted in increased cell proliferation in all investigated populations, however no difference could be observed between Sca-1 induced and control mice. Overall the findings suggest that overexpression of Sca-1 in the progenitor or stem cells leads to reduced formation of tumours in the hair follicle stem cells. However, in developing tumours overexpression of Sca-1 may drive the cells towards malignant conversion. In a previous study it was shown that knock down of Sca-1 in a mammary tumour cell line resulted in increased tumour outgrowth potential and accelerated tumour development (Batts et al., 2011). The data suggested that knock-down of Sca-1 primarily influenced tumour initiation and showed little effect in established tumours. Other studies have, however, observed opposite effects of Sca-1 repression. By using a mammary adenocarcinoma cell line induced by medroxyprogesterone (MP) and DMBA, Upadhyay et al. showed that repression of Sca-1 reduced tumorigenecity (Upadhyay et al., 2011). It was shown that Sca-1 served as a tumor-initiating factor by suppressing TGF-β signaling. In addition, delayed tumor development has been observed in MP/DMBA induced mammary tumours in Sca-1 knock out mice compared to WT mice (Yuan et al., 2012). Here it was shown that Sca-1 served as a negative regulator of the tumor suppressor effects of PPARγ.

When we analyzed tumor cells generated in $Rosa^{Sca1/Sca1}$:Cre^{+} mice by flow cytometry we confirmed that Sca-1 was present. Data also showed that tumours from wild type mice gradually upregulated Sca-1 during tumorigenesis although more cells were Sca-1 negative in these control mice. This is as somewhat expected as overexpression using the K14 promoter leads to upregulated expression of Sca-1 in all keratinocytes in the transgenic mice. The data show that some cells upregulate Sca-1 during progression of the tumour which is in line with the literature that has shown that Sca-1 is upregulated in cancers (Grange et al., 2008; Li et al., 2003; Perez-Caro et al., 2009; Seigel et al., 2007; Xin et al., 2005; Yin et al., 2009). The observation that overexpression of Sca-1 in Sca-1 negative stem cell populations leads to an overall reduction in the number of tumours indicates that at least some of the tumours arise from Sca-1 negative stem cells as we would only expect an effect on cells that do not normally express Sca-1. This has also been suggested by others (Kangsamaksin et al., 2007; Kim et al., 2009; Trempus et al., 2007).

The many studies describing various roles of Sca-1 highlight that Sca-1 likely regulates multiple cellular processes and may interact with different ligands in different cells and tissues. Furthermore, the discrepancies in the expression pattern between tissues may indicate that Sca-1 expression as such is irrelevant to the stem cell function, but pivotal to cellular fate decision and that Sca-1 may play different roles in different tissues. The diverse roles of Sca-1 in different cell types may explain the discrepancy observed in various studies investigating the effect of overexpressing and repressing Sca-1.

5. Conclusions

Our study seeks to address the role of Sca-1 in the mouse epidermis. By overexpressing Sca-1 in compartments that normally do not express Sca-1, we find that this affects the behavior of keratinocytes in several assays. We find that Sca-1 overexpression leads to increased growth in vitro and we detect an increased number of label retaining cells in the hair follicle.

Tumorigenesis experiments showed a significant reduction in the overall number of tumours generated in transgenic mice overexpressing Sca-1 but an increased tendency of malignant progression from benign to malignant tumour.

Our data support previous studies suggesting that Sca-1 plays a role in controlling proliferation. Even though Sca-1 is not normally expressed in hair follicle stem cells, overexpression of Sca-1 did not disturb normal hair follicle generation and maintenance.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2017.07.002.

Conflict of interest

The authors state no conflict of interest.

Acknowledgements

This work was supported by The Danish Cancer Society (R.C. Grant number DP07024); Kgl. Hofbundtmester Aage Bang Fund (Grant number 55-07/08); Karen Elise Jensen Fund; Karen Kriegers Fund (Grant number 06203050); Lundbech Foundation (Grant number R19-A2286) and Danish Ministry of Higher Education and Science through Danish Center for Transgenic Mice. We are grateful to Anette Thomsen and Christian Knudsen for excellent technical assistance and Lisbeth Ahm Hansen for blastocyst injection.

References

Asakura, A., Seale, P., Girgis-Gabardo, A., Rudnicki, M.A., 2002. Myogenic specification of side population cells in skeletal muscle. J. Cell Biol. 159, 123–134.

Barrandon, Y., Green, H., 1987. Three clonal types of keratinocyte with different capacities for multiplication. Proc. Natl. Acad. Sci. U. S. A. 84, 2302–2306.

Bar-Sagi, D., Feramisco, J.R., 1985. Microinjection of the ras oncogene protein into PC12 cells induces morphological differentiation. Cell 42, 841–848.

Batts, T.D., Machado, H.L., Zhang, Y., Creighton, C.J., Li, Y., Rosen, J.M., 2011. Stem cell antigen-1 (sca-1) regulates mammary tumor development and cell migration. PLoS One 6, e27841.

Blanpain, C., Lowry, W.E., Geoghegan, A., Polak, L., Fuchs, E., 2004. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 118, 635–648.

Bonyadi, M., Waldman, S.D., Liu, D., Aubin, J.E., Grynpas, M.D., Stanford, W.L., 2003. Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. Proc. Natl. Acad. Sci. U. S. A. 100, 5840–5845.

Bradfute, S.B., Graubert, T.A., Goodell, M.A., 2005. Roles of Sca-1 in hematopoietic stem/progenitor cell function. Exp. Hematol. 33, 836–843.

Brownell, I., Guevara, E., Bai, C.B., Loomis, C.A., Joyner, A.L., 2011. Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. Cell Stem Cell 8, 552–565.

Christensen, R., Owens, D.M., Thomsen, A., Pedersen, S., Jensen, U.B., 2011. Zinc fixation for flow cytometry analysis of intracellular and surface epitopes, DNA content, and cell proliferation. Curr. Protoc. Cytom. 40 Chapter 7, Unit 7.

Cui, W., Kemp, C.J., Duffie, E., Balmain, A., Akhurst, R.J., 1994. Lack of transforming growth factor-beta 1 expression in benign skin tumors of p53null mice is prognostic for a high risk of malignant conversion. Cancer Res. 54, 5831–5836.

Epting, C.L., Lopez, J.E., Shen, X., Liu, L., Bristow, J., Bernstein, H.S., 2004. Stem cell antigen-1 is necessary for cell-cycle withdrawal and myoblast differentiation in C2C12 cells. J. Cell Sci. 117, 6185–6195.

Epting, C.L., King, F.W., Pedersen, A., Zaman, J., Ritner, C., Bernstein, H.S., 2008a. Stem cell antigen-1 localizes to lipid microdomains and associates with insulin degrading enzyme in skeletal myoblasts. J. Cell. Physiol. 217, 250–260.

Epting, C.L., Lopez, J.E., Pedersen, A., Brown, C., Spitz, P., Ursell, P.C., Bernstein, H.S., 2008b. Stem cell antigen-1 regulates the tempo of muscle repair through effects on proliferation of alpha7 integrin-expressing myoblasts. Exp. Cell Res. 314, 1125–1135.

Glick, A.B., Kulkarni, A.B., Tennenbaum, T., Hennings, H., Flanders, K.C., O'Reilly, M., Sporn, M.B., Karlsson, S., Yuspa, S.H., 1993. Loss of expression of transforming growth factor beta in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion. Proc. Natl. Acad. Sci. U. S. A. 90, 6076–6080.

Grange, C., Lanzardo, S., Cavallo, F., Camussi, G., Bussolati, B., 2008. Sca-1 identifies the tumor-initiating cells in mammary tumors of BALB-neuT transgenic mice. Neoplasia 10, 1433–1443.

Gunnarsson, A.P., Christensen, R., Li, J., Jensen, U.B., 2016. Global gene expression and comparison between multiple populations in the mouse epidermis. Stem Cell Res. 17, 191–202.

Henderson, S.C., Kamdar, M.M., Bamezai, A., 2002. Ly-6A.2 expression regulates antigen-specific CD4+ T cell proliferation and cytokine production. J. Immunol. 168, 118–126.

Hohenstein, P., Slight, J., Ozdemir, D.D., Burn, S.F., Berry, R., Hastie, N.D., 2008. High-efficiency Rosa26 knock-in vector construction for Cre-regulated overexpression and RNAi. PathoGenetics 1, 3.

Holmes, C., Stanford, W.L., 2007. Concise review: stem cell antigen-1: expression, function, and enigma. Stem Cells 25, 1339–1347.

Ito, C.Y., Li, C.Y., Bernstein, A., Dick, J.E., Stanford, W.L., 2003. Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. Blood 101, 517–523.

Jaks, V., Barker, N., Kasper, M., van Es, J.H., Snippert, H.J., Clevers, H., Toftgard, R., 2008. Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat. Genet. 40, 1291–1299.

Jankowski, R.J., Haluszczak, C., Trucco, M., Huard, J., 2001. Flow cytometric characterization of myogenic cell populations obtained via the preplate technique: potential for rapid isolation of muscle-derived stem cells. Hum. Gene Ther. 12, 619–628.

Jensen, U.B., Yan, X., Triel, C., Woo, S.H., Christensen, R., Owens, D.M., 2008. A distinct population of clonogenic and multipotent murine follicular keratinocytes residing in the upper isthmus. J. Cell Sci. 121, 609–617.

Jensen, K.B., Collins, C.A., Nascimento, E., Tan, D.W., Frye, M., Itami, S., Watt, F.M., 2009. Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis. Cell Stem Cell 4, 427–439.

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

Kafadar, K.A., Yi, L., Ahmad, Y., So, L., Rossi, F., Pavlath, G.K., 2009. Sca-1 expression is required for efficient remodeling of the extracellular matrix during skeletal muscle regeneration. Dev. Biol. 326, 47–59.

Kangsamaksin, T., Park, H.J., Trempus, C.S., Morris, R.J., 2007. A perspective on murine keratinocyte stem cells as targets of chemically induced skin cancer. Mol. Carcinog. 46, 579–584.

Kemp, C.J., Donehower, L.A., Bradley, A., Balmain, A., 1993. Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors. Cell 74, 813–822.

Kim, D.J., Kataoka, K., Rao, D., Kiguchi, K., Cotsarelis, G., Digiovanni, J., 2009. Targeted disruption of stat3 reveals a major role for follicular stem cells in skin tumor initiation. Cancer Res. 69, 7587–7594.

Li, Y., Welm, B., Podsypanina, K., Huang, S., Chamorro, M., Zhang, X., Rowlands, T., Egeblad, M., Cowin, P., Werb, Z., Tan, L.K., Rosen, J.M., Varmus, H.E., 2003. Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proc. Natl. Acad. Sci. U. S. A. 100, 15853–15858.

Liakath-Ali, K., Vancollie, V.E., Heath, E., Smedley, D.P., Estabel, J., Sunter, D., Ditommaso, T., White, J.K., Ramirez-Solis, R., Smyth, I., Steel, K.P., Watt, F.M., 2014. Novel skin phenotypes revealed by a genome-wide mouse reverse genetic screen. Nat. Commun. 5, 3540.

Long, K.K., Montano, M., Pavlath, G.K., 2011. Sca-1 is negatively regulated by TGF-beta1 in myogenic cells. FASEB J. 25, 1156–1165.

Lyle, S., Christofidou-Solomidou, M., Liu, Y., Elder, D.E., Albelda, S., Cotsarelis, G., 1998. The C8/144B monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. J. Cell Sci. 111 (Pt 21), 3179–3188.

Mitchell, P.O., Mills, T., O'Connor, R.S., Kline, E.R., Graubert, T., Dzierzak, E., Pavlath, G.K., 2005. Sca-1 negatively regulates proliferation and differentiation of muscle cells. Dev. Biol. 283, 240–252.

Morris, R.J., 2004. A perspective on keratinocyte stem cells as targets for skin carcinogenesis. Differentiation 72, 381–386.

Nijhof, J.G., Braun, K.M., Giangreco, A., van Pelt, C., Kawamoto, H., Boyd, R.L., Willemze, R., Mullenders, L.H., Watt, F.M., de Gruijl, F.R., van Ewijk, W., 2006. The cell-surface marker MTS24 identifies a novel population of follicular keratinocytes with characteristics of progenitor cells. Development 133, 3027–3037.

Nowak, J.A., Fuchs, E., 2009. Isolation and culture of epithelial stem cells. Methods Mol. Biol. 482, 215–232.

Oh, H., Bradfute, S.B., Gallardo, T.D., Nakamura, T., Gaussin, V., Mishina, Y., Pocius, J., Michael, L.H., Behringer, R.R., Garry, D.J., Entman, M.L., Schneider, M.D., 2003. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc. Natl. Acad. Sci. U. S. A. 100, 12313–12318.

Owens, D.M., Watt, F.M., 2001. Influence of beta1 integrins on epidermal squamous cell carcinoma formation in a transgenic mouse model: alpha3beta1, but not alpha2beta1, suppresses malignant conversion. Cancer Res. 61, 5248–5254.

Page, M.E., Lombard, P., Ng, F., Gottgens, B., Jensen, K.B., 2013. The epidermis comprises autonomous compartments maintained by distinct stem cell populations. Cell Stem Cell 13, 471–482.

Pai, S.B., Steele, V.E., Nettesheim, P., 1983. Neoplastic transformation of primary tracheal epithelial cell cultures. Carcinogenesis 4, 369–374.

Perez-Caro, M., Cobaleda, C., Gonzalez-Herrero, I., Vicente-Duenas, C., Bermejo-Rodriguez, C., Sanchez-Beato, M., Orfao, A., Pintado, B., Flores, T., Sanchez-Martin, M., Jimenez, R., Piris, M.A., Sanchez-Garcia, I., 2009. Cancer induction by restriction of oncogene expression to the stem cell compartment. EMBO J. 28, 8–20.

Purkis, P.E., Steel, J.B., Mackenzie, I.C., Nathrath, W.B., Leigh, I.M., Lane, E.B., 1990. Antibody markers of basal cells in complex epithelia. J. Cell Sci. 97 (Pt 1), 39–50.

Raj, D., Brash, D.E., Grossman, D., 2006. Keratinocyte apoptosis in epidermal development and disease. J. Invest. Dermatol. 126, 243–257.

Ramirez-Solis, R., Davis, A.C., Bradley, A., 1993. Gene targeting in embryonic stem cells. Methods Enzymol. 225, 855–878.

Rao, D., Macias, E., Carbajal, S., Kiguchi, K., Digiovanni, J., 2015. Constitutive Stat3 activation alters behavior of hair follicle stem and progenitor cell populations. Mol. Carcinog. 54, 121–133.

Rheinwald, J.G., Green, H., 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6, 331–343.

van de Rijn, M., Heimfeld, S., Spangrude, G.J., Weissman, I.L., 1989. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc. Natl. Acad. Sci. U. S. A. 86, 4634–4638.

Seigel, G.M., Hackam, A.S., Ganguly, A., Mandell, L.M., Gonzalez-Fernandez, F., 2007. Human embryonic and neuronal stem cell markers in retinoblastoma. Mol. Vis. 13, 823–832.

Snippert, H.J., Haegebarth, A., Kasper, M., Jaks, V., van Es, J.H., Barker, N., van de Wetering, M., van den Born, M., Begthel, H., Vries, R.G., Stange, D.E., Toftgard, R., Clevers, H., 2010. Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. Science 327, 1385–1389.

Spangrude, G.J., Aihara, Y., Weissman, I.L., Klein, J., 1988. The stem cell antigens Sca-1 and Sca-2 subdivide thymic and peripheral T lymphocytes into unique subsets. J. Immunol. 141, 3697–3707.

Stenman, G., Delorme, E.O., Lau, C.C., Sager, R., 1987. Transfection with plasmid pSV2gptEJ induces chromosome rearrangements in CHEF cells. Proc. Natl. Acad. Sci. U. S. A. 84, 184–188.

Swiatek, P.J., Gridley, T., 1993. Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20. Genes Dev. 7, 2071–2084.

Trempus, C.S., Morris, R.J., Bortner, C.D., Cotsarelis, G., Faircloth, R.S., Reece, J.M., Tennant, R.W., 2003. Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. J. Invest. Dermatol. 120, 501–511.

Trempus, C.S., Morris, R.J., Ehinger, M., Elmore, A., Bortner, C.D., Ito, M., Cotsarelis, G., Nijhof, J.G., Peckham, J., Flagler, N., Kissling, G., Humble, M.M., King, L.C., Adams, L.D., Desai, D., Amin, S., Tennant, R.W., 2007. CD34 expression by hair follicle stem cells is required for skin tumor development in mice. Cancer Res. 67, 4173–4181.

Triel, C., Vestergaard, M.E., Bolund, L., Jensen, T.G., Jensen, U.B., 2004. Side population cells in human and mouse epidermis lack stem cell characteristics. Exp. Cell Res. 295, 79–90.

Upadhyay, G., Yin, Y., Yuan, H., Li, X., Derynck, R., Glazer, R.I., 2011. Stem cell antigen-1 enhances tumorigenicity by disruption of growth differentiation factor-10 (GDF10)-dependent TGF-beta signaling. Proc. Natl. Acad. Sci. U. S. A. 108, 7820–7825.

Wang, H., Chen, H., Feng, B., Wang, X., He, X., Hu, R., Yin, M., Wang, W., Fu, W., Xu, Z., 2014. Isolation and characterization of a Sca-1 +/CD31- progenitor cell lineage derived from mouse heart tissue. BMC Biotechnol. 14, 75.

Welm, B.E., Tepera, S.B., Venezia, T., Graubert, T.A., Rosen, J.M., Goodell, M.A., 2002. Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev. Biol. 245, 42–56.

Wertz, K., Füchtbauer, E.-M., 1994. B6D2F1 - an improved mouse hybrid strain for the production of ES cell germ line chimeras. Transgene 1, 277–280.

Wright, N., Samuelson, L., Walkup, M.H., Chandrasekaran, P., Gerber, D.A., 2008. Enrichment of a bipotent hepatic progenitor cell from naive adult liver tissue. Biochem. Biophys. Res. Commun. 366, 367–372.

Wu, Y.J., Parker, L.M., Binder, N.E., Beckett, M.A., Sinard, J.H., Griffiths, C.T., Rheinwald, J.G., 1982. The mesothelial keratins: a new family of cytoskeletal proteins identified in cultured mesothelial cells and nonkeratinizing epithelia. Cell 31, 693–703.

Xin, L., Lawson, D.A., Witte, O.N., 2005. The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. Proc. Natl. Acad. Sci. U. S. A. 102, 6942–6947.

Yin, Y., Yuan, H., Zeng, X., Kopelovich, L., Glazer, R.I., 2009. Inhibition of peroxisome proliferator-activated receptor gamma increases estrogen receptor-dependent tumor specification. Cancer Res. 69, 687–694.

Yuan, H., Upadhyay, G., Yin, Y., Kopelovich, L., Glazer, R.I., 2012. Stem cell antigen-1 deficiency enhances the chemopreventive effect of peroxisome proliferator-activated receptorgamma activation. Cancer Prev. Res. (Phila.) 5, 51–60.

Yuspa, S.H., Vass, W., Scolnick, E., 1983. Altered growth and differentiation of cultured mouse epidermal cells infected with oncogenic retrovirus: contrasting effects of viruses and chemicals. Cancer Res. 43, 6021–6030.

Yuspa, S.H., Morgan, D., Lichti, U., Spangler, E.F., Michael, D., Kilkenny, A., Hennings, H., 1986. Cultivation and characterization of cells derived from mouse skin papillomas induced by an initiation-promotion protocol. Carcinogenesis 7, 949–958.