Published in final edited form as:

Gene Expr Patterns. 2018 January ; 27: 76–84. doi:10.1016/j.gep.2017.10.003.

Characterization of a new, inducible transgenic mouse model with GFP expression in melanocytes and their precursors

Sandeep S. Joshi², Bishal Tandukar², Maira Castaneda⁵, Shunlin Jiang⁵, Ganesh Diwakar⁵, Ronna P. Hertzano⁴, and Thomas J. Hornyak^{1,2,3,5,*}

¹Research and Development Service, VA Maryland Health Care System, Baltimore, Maryland, USA

²Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland, USA

³Department of Dermatology, University of Maryland School of Medicine, Baltimore, Maryland, USA

⁴Otorhinolaryngology-Head & Neck Surgery, University of Maryland School of Medicine, Baltimore, Maryland, USA

⁵Dermatology Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland, USA

Abstract

Melanocytes are neural crest-derived cells that are responsible for mammalian hair follicle (HF) pigmentation. The *Dct-LacZ* transgenic mouse is extensively used to study melanocyte biology but lacks conditionally-inducible labelling and fluorescent labelling, enabling specific, viable isolation of melanocytes using fluorescence-activated cell sorting (FACS). Here, we have generated a Tet-off bitransgenic mouse model, *Dct-H2BGFP*, containing *Dct-tTA* and TRE-H2BGFP transgenes. Characterization of *Dct-H2BGFP* mice confirmed a pattern of *Dct-H2BGFP* expression in melanoblasts, melanocyte stem cells (McSCs), and terminally differentiated melanocytes similar to the expression pattern of previously published mouse models *Dct-LacZ* and *iDct-GFP*. GFP expression is regulated by doxycycline. GFP is shown to co-localize with melanocyte label-retaining cells (LRCs) identified through BrdU retention. The GFP-expressing cells identified *in vivo* in the bulge and the secondary hair germ of telogen HFs of *Dct-H2BGFP* mice express the melanocyte and melanocyte stem cell markers *Dct* and *Kit*. Using *Dct-H2BGFP* mice, we separated GFP-expressing cells from the telogen HF based on FACS and showed that GFP-expressing cells express high levels of *Kit* and *Dct*, and lower levels of HF epithelial keratin genes.

*Corresponding author: Thomas J. Hornyak, Research & Development Service, VA Maryland Health Care System and Departments of Dermatology and Biochemistry and Molecular Biology, University of Maryland School of Medicine, 108 N. Greene St., Room 308A, Baltimore, Maryland 21201 U.S.A., Thomas.Hornyak@va.gov.

AUTHORS CONTRIBUTIONS

S.S.J., B.T., and T.J.H. designed the study, performed the experiments and contributed to data interpretation. G.D., and S.J. generated transgenic mice and S.S.J. and M.C. characterized transgenic mice. R.P.H. characterized cochlear melanocytes. S.S.J., B.T., and T.J.H. wrote the manuscript and prepared the figures.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

We also show that GFP-expressing cells express high levels of the melanocyte differentiation genes *Tyr*, *Tyrb1*, and *Pmel17*, further substantiating their identity within the melanocyte lineage. Thus, *Dct*-H2BGFP mice are not only useful for the *in vivo* identification of melanocytic cells, but also for isolating them viably and studying their molecular and biological properties.

INTRODUCTION

Melanocyte stem cells (McSCs) are neural crest-derived cells responsible for regeneration of differentiated melanocytes during each hair follicle (HF) cycle, thereby contributing to pigmentation. During embryonic development, neural crest cells generate melanoblasts which migrate along the dorsolateral region of the neural tube. After migration, melanoblasts invade the overlying epidermis and enter the developing hair follicle, where they continue to proliferate and differentiate into early postnatal life. McSCs can be distinguished from more differentiated melanocytes of the HF matrix based upon a distinct expression profile, including the expression of *Dct* and *Pax3*, but low expression of *Sox10*, as early as postnatal day (P) 2 in the bulge/lower permanent portion (LPP) HF niche (Osawa et al., 2005). McSCs in HF telogen are identified by quiescence, by expression of *Dct*, and by their localization within the HF including the bulge/LPP region and a region previously described as the subbulge (Nishimura et al., 2002).

Mammalian HFs cycle recurrently through a growth phase (anagen), a regression phase (catagen), and a resting phase (telogen) initiated by the activation of epithelial HF stem cells (Cotsarelis, 2006; Schneider et al., 2009). HF cycle initiation requires HF epithelial SC sensitivity to a TGF- β 2 signal (Oshimori and Fuchs, 2012), with subsequent elaboration of a Wnt signal by HFSCs initiating proliferation and differentiation of McSCs (Rabbani et al., 2011). During anagen, terminally differentiated melanocytes in the hair bulb of the mature HF produce and transfer pigment to the hair matrix. When catagen begins, melanocytes and matrix cells in the hair bulb undergo apoptosis (Tobin et al., 1998), retracting the dermal papillae out of the bulb, and the HF returns to telogen. In telogen, the lower HF component consists of two regions, the bulge/LPP (Cotsarelis et al., 1990) and secondary hair germ (SHG) (Ito et al., 2004), an epithelial extension at the bottom. These compartments can be distinguished based upon region-specific markers, with the bulge compartment expressing the CD34 membrane glycoprotein (Trempe et al., 2003) and the SHG selectively expressing the intracellular adhesion protein P-cadherin/Cdh3 (Muller-Rover et al., 1999). McSCs along with other HF stem cells are found to be present in both compartments.

Previously, a tetracycline-regulated system was described (*iDct-GFP*) which permits expression of a stable H2BGFP fusion protein from the *Dct* promoter (Zaidi et al., 2011). Characterization of this system showed expression of H2BGFP in migratory melanoblasts during embryonic development and in differentiated melanocytes during follicular morphogenesis following administration of doxycycline in post-natal life.

Characterization of HF epithelial stem cells has been advanced by the development of inducible, keratin-promoter driven transgenic system that drive expression of the stable H2BGFP protein constitutively, without the need to administer doxycycline to induce gene expression. This system utilized the Tet-Off protein (tTA) driven by a tissue-selective

promoter to effect constitutive gene expression in the absence of doxycycline (Tumbar et al., 2004). With the ability to turn off gene expression at the onset of new HF cycles, this system made it possible to identify quiescent, label-retaining cells (LRCs) because of the stable nature of the H2BGFP protein. These cells could be isolated viably and profiled, leading to advances in the understanding of transcriptional and epigenetic determinants of HF keratinocyte differentiation and developmental potential (Ezhkova et al., 2009; Greco et al., 2009; Tumbar et al., 2004).

A similar system could facilitate advances in understanding McSC function. To identify McSCs in telogen, without the need to administer doxycycline constitutively, we developed *Dct*-H2BGFP mice. These are bitransgenic *Dct*-tTA; TRE-H2BGFP mice which express *Dct*-H2BGFP constitutively in adult murine HFs. We show that *Dct*-H2BGFP mice exhibit a constitutive expression pattern of H2BGFP consistent with endogenous *Dct* expression (Steel et al., 1992) and the expression pattern of widely-used *Dct*-*LacZ* mice (Hornyak et al., 2001; Mackenzie et al., 1997; Zhao and Overbeek, 1999), including expression in retinal pigmented epithelial (RPE) cells and cochlear melanocytes. During HF telogen, expression of the *Dct*-H2BGFP reporter is present in both the bulge and subbulge/SHG region as previously described for *Dct*-expressing McSCs, where it colocalizes with expression of endogenous melanocyte and McSC markers *Dct* and *Kit* (Nishimura et al., 2002). GFP expression is suppressed during development and during HF anagen by doxycycline, demonstrating Tet-regulation of gene expression. Labeling of cells during HF anagen with bromodeoxyuridine (BrdU) reveals expression of GFP in melanocyte LRCs in telogen after an extended chase period. Gene expression analysis of FACS-isolated *Dct*-H2BGFP cells also shows high expression of *Dct* and *Kit*, as well as the melanocyte differentiation genes *Tyr*, *Tyrl*, and *Pmel17*. These findings establish bitransgenic *Dct*-H2BGFP mice as a model for the viable isolation and analysis of murine melanocytes, their precursors, and melanocyte LRCs.

RESULTS

Constitutive expression pattern of *Dct*-H2BGFP transgene in embryonic melanoblasts and adult HF melanocytes

The *Dct*-H2BGFP bitransgenic mouse was generated by crossing *Dct*-tTA and TRE-H2BGFP (Tumbar et al., 2004) mice. The *Dct*-tTA transgenic line was prepared using homologous recombination of a *Dct*-tTA-SV40p(A) construct into the *Hprt* locus of the X chromosome. The expression of tTA under the control of the *Dct* promoter was predicted to transactivate TRE-H2BGFP expression exclusively in melanocytes (Figure 1A). We evaluated transgene expression in *Dct*-H2BGFP mice. The expression of *Dct*-H2BGFP was observed in melanoblasts in the trunk, sub- and supra-optic region at E12.5 (Figures 1B, S1). *Dct*-H2BGFP expression in the eye was restricted to the retinal pigment epithelium layer (RPE) at E12.5 (Figure 1C). Truncal sections of embryos at E12.5 reveal that *Dct*-H2BGFP-expressing cells are present in the dorsolateral pattern of neural crest cell migration, consistent with migrating melanoblasts (Figure 1D).

Postnatally, we found GFP-expressing melanocytes in the stria vascularis layer of the cochlea, as suggested by co-localization of phalloidin, which labels filamentous actin, and

GFP, in P12 *Dct*-H2BGFP mice (Figure 1E). We determined *Dct*-H2BGFP transgene expression during follicular morphogenesis and found its expression in anagen, catagen and telogen HF. In anagen and catagen HFs, *Dct*-H2BGFP expression was observed in both the upper bulge/LPP location and lower bulb cells. In telogen HFs, *Dct*-H2BGFP expression was observed in bulge and SHG HF McSCs of *Dct*-H2BGFP mice (Figure 2A-2C). The constitutive expression of *Dct*-H2BGFP mice was comparable to the expression pattern of *Dct-LacZ* (Hornyak et al., 2001; Mackenzie et al., 1997; Nishimura et al., 2002) and *iDct*-GFP mice (Zaidi et al., 2011), which express the transgene in melanoblasts, melanocyte progenitors in bulge/LPP, and terminally differentiated melanocytes.

Transgene inducibility and identification of melanocyte LRCs

H2BGFP expression is driven by the tTA (Tet-Off) transactivator (Gossen and Bujard, 1992), which is negatively regulated by tetracycline and its derivatives. To validate regulation of H2BGFP expression *in vivo* in *Dct*-H2BGFP mice, doxycycline was administered beginning at P19, just prior to the first anagen and continued until full anagen at P30. Comparison of P30 HFs from doxycycline-treated mice with untreated controls shows efficient extinction of GFP expression from HF anagen bulb melanocytes (Figure 3A,B), as expected for a tightly Tet-regulated system. Additionally, administration of doxycycline to pregnant dams suppressed the expression of GFP through P8 (Supplementary Figure 2). We then determined whether the system could facilitate detecting, in the telogen HF, quiescent melanocyte LRCs, as defined by cells retaining a BrdU label administered during anagen followed by an extensive chase period (Nishimura et al., 2002). H2BGFP alone was sufficient to reveal quiescent keratinocyte LRCs in a previous study (Tumbar et al., 2004). The result of this experiment (Figure 3C,D) showed co-localization of a retained BrdU label with H2BGFP expression in P56 telogen HFs, indicating the potential for *Dct*-H2BGFP to reveal quiescent McSCs. The percentage of GFP-expressing cells that have retained BrdU in the bulge and SHG regions was quantified in P56 HFs. Quantification showed that 57(+2)% and 19(+10)% of GFP cells co-expressed a BrdU label, respectively, in the SHG and bulge regions of the HFs (Figure 3E), a significant difference ($p < 0.021$). This result shows not only that a significant fraction of GFP-expressing melanocytes are BrdU LRCs, but also suggests that there could be differences between the bulge- and SHG-resident HF melanocytes in telogen that reflect functional differences between them.

Characterization of H2BGFP-expressing McSCs in telogen HF of *Dct*-H2BGFP mice

In telogen HFs, the quiescent McSCs along with other HF stem cells are found in the bulge and SHG compartments. Here, we showed that *Dct*-H2BGFP cells identified in bulge and SHG compartment of second telogen HF expressed the McSC markers Kit (Figure 4A) and Dct (Figure 4B). In addition, similar to cells from the Tet-On *iDct*-GFP mice (manuscript in preparation and (Zaidi et al., 2011)), *Dct*-H2BGFP cells were present in both the CD34-expressing bulge region (Hsu et al., 2011; Trempus et al., 2007) of the HF and the CD34-negative, secondary hair germ (SHG) region (Oshimori and Fuchs, 2012) at the base of the telogen HF (Figures 4C). To scrutinize the expression pattern for evidence of ectopic GFP expression observed previously with a randomly inserted *Dct*-tTA transgene (manuscript in preparation) and with a non-inducible, *Dct*-H2BGFP transgenic mouse generated with random insertion (Okamoto et al., 2014; Ueno et al., 2014), we quantified the *Dct*-H2BGFP

expressing cells within and outside the HF using collagen IV, a basement membrane marker (Sriwiranont et al., 2011). We found that >90% *Dct*-H2BGFP cells were coming from within the HFs, with a few on the border and only rare cells outside the follicle (Figures S3 and Table S1). Our data supports the absence of ectopic expression with inducible *Dct*-H2BGFP mice.

Isolation and marker analysis of GFP-expressing McSCs of *Dct*-H2BGFP mice

The constitutive GFP-expression in HF McSCs of *Dct*-H2BGFP mice suggested a strategy to separate these from dermal cells to evaluate their molecular properties. Single cell suspensions prepared from shaven, dorsal skin of approximately 8-week-old (P56) mice, an age when all HFs are synchronously in the telogen (Schneider et al., 2009), were prepared for fluorescence-activated cell sorting (FACS). The percentage yield of live cells comprising these populations differed slightly between experiments, in general 0.8 – 1% of the dermal cell suspension was comprised of *Dct*-H2BGFP cells (Figure 5A and Figures S4), comparable to previous findings with *iDct*-GFP mice. To further evaluate the specificity of these cell populations, RNA was isolated, and relative gene expression for specific marker genes determined. These results confirmed that *Dct*-H2BGFP cells expressed endogenous McSC markers *Dct* and *Kit* at significantly higher levels than the basal keratinocyte gene *Krt14*.

The ability to separate subsets of HF McSCs, defined by *Dct*-H2BGFP expression during telogen, prompted us to evaluate the relative expression of the melanogenic genes *Tyr*, *Typr1*, and *Pmel17* within these subsets. Quantitative RT-PCR results (Figure 5B) showed that relative expression of all three melanogenic genes measured was significantly higher in the *Dct*-H2BGFP expressing cells present in the telogen HF compared to dermal cells, confirming that the *Dct*-H2BGFP cells isolated are melanocytic in origin and represent HF McSCs populations from telogen HFs.

DISCUSSION

Here we generated and characterized *Dct*-H2BGFP mice which constitutively express GFP exclusively in melanocytes. *Dct*-H2BGFP mice are Tet-off, bitransgenic mice comprising both *Dct*-tTA and TRE-H2BGFP transgenes. Characterization of *Dct*-H2BGFP mice confirmed a pattern of *Dct*-H2BGFP expression in melanoblasts, McSCs, and terminally differentiated melanocytes similar to the expression pattern of previously published mouse models *Dct-LacZ* (Hornyak et al., 2001; Mackenzie et al., 1997; Nishimura et al., 2002) and *iDct*-GFP (Zaidi et al., 2011). *Dct*-H2BGFP mice not only are useful for the *in vivo* identification of melanocytic cells, but also may be useful for isolating them viably and studying their molecular and biological properties.

Doxycycline administration to *Dct*-H2BGFP mice inhibits constitutive H2BGFP expression in melanocytic cells. This system is similar to the K5-tetVP16/TRE-H2BGFP Tet-off mouse model system used to visualize, identify and isolate quiescent keratinocyte HF stem cells (Tumbar et al., 2004). In this paper, we have shown that melanocyte LRCs in telogen, identified by the classical method of extended BrdU administration during anagen (Cotsarelis et al., 1990; Nishimura et al., 2002), also express *Dct*-H2BGFP, demonstrating

that *Dct*-H2BGFP can be used to identify melanocyte LRCs. Additional work will be required to determine whether LRCs can be identified after doxycycline-dependent suppression of *Dct*-H2BGFP expression during anagen (Tumbar et al., 2004) and whether there is overlap between any population of *Dct*-H2BGFP LRCs and the population of McSCs defined by BrdU retention. In addition, *Dct*-H2BGFP mice may complement existing mouse models for probing mechanisms underlying initiation and progression of melanoma. *iDct*-GFP mice, a Tet-On bitransgenic model, were utilized to identify interferon- γ as a key regulator in ultraviolet radiation-induced melanomagenesis (Zaidi et al., 2011). In contrast to *iDct*-GFP mice, *Dct*-H2BGFP mice do not require administration of doxycycline for expression of *Dct*-H2BGFP in melanocytic cells, advantageous for certain studies. Ectopic expression of GFP was reported in a previous mouse model in which GFP expression was driven directly by the *Dct* promoter (Okamoto et al., 2014). The absence of ectopic transgene expression with *Dct*-H2BGFP mice may be due to the combination of precise integration and copy number of the *Dct*-tTA transgene construct, homologously recombined at the neutral murine X chromosome *Hprt* locus (Palais et al., 2009; Touw et al., 2007), and control of TRE-H2BGFP expression level resulting from its activation by the tTA tetracycline-regulated transactivator and not by randomly-inserted *Dct* promoters directly (Figure S3, Table S1).

Another *Dct*-driven transgenic model was used to identify McSCs in the early post-natal HF mice for characterization by co-localization with putative McSC markers and for single-cell gene expression profiling. In this model, *Dct^{tm1(Cre)Bee}* mice were used to recombine the *CAG-CAT-EGFP* transgene (Kawamoto et al., 2000) to result in durable expression of EGFP in cells expressing or which had expressed *Dct* (Osawa et al., 2005). The analysis was performed in early post-natal life (P6) soon after entry of McSCs into the LPP niche and during the anagen phase associated with follicular morphogenesis. This approach has the potential to drive expression of a GFP reporter not only in cells actively transcribing *Dct*, but also in cells that previously had expressed *Dct* at a sufficiently high level to activate the *Dct-Cre* transgene to trigger a recombination event, but no longer express *Dct*. Our model does not necessarily have this capability. Although the TRE-H2BGFP reporter used in our system is a stable protein which has been used to identify and isolate quiescent, label-retaining cells (LRCs) weeks after active transcription is suppressed (Osawa et al., 2005), it is not likely that this property extends indefinitely. However, the *Dct-Cre; CAG-CAT-EGFP* transgene only demonstrated a recombination efficiency of approximately 50%; hence, it is likely that the model we have described captures most or all of the melanocyte and McSC population, in contrast to the recombination model (Osawa et al., 2005). Although clearly useful for the localization and characterization of P6 McSCs, the recombination ultimately may be most suitable for lineage tracing to analyze the cellular fate of McSCs and differentiated melanocytic progeny. Our model may reveal melanocytes and McSCs most robustly, while providing certain capabilities to identify quiescent McSCs after *Dct* transgene expression has been extinguished by a tetracycline derivative.

In that regard, our results also exhibit an intriguing difference with those reported using the lineage-tracing model. Our expression analysis revealed significant levels of expression of the melanocyte differentiation genes *Tyr* and *Pmel17 (Si)* in P56 telogen-phase McSCs, whereas the expression of these genes in single cells isolated from the HF lower permanent

portion during anagen at P6 was not detectable (Osawa et al., 2005). Although it is possible that this apparent difference in expression level is solely attributable to differences in HF stage and mouse age, it is more likely, in our opinion, that these results reflect differences between cells isolated exclusively from the LPP, commonly referred to as the bulge, and the entirety of the telogen follicle including the McSCs we have identified in the SHG region. Our results in Figure 3E suggest that there is a functional difference between the bulge/LPP-resident and SHG-resident McSCs in telogen, and our gene expression results suggest the possibility of a previously-unrecognized heterogeneity within the quiescent, telogen McSC population.

EXPERIMENTAL PROCEDURES

Generation of *Dct*-H2BGFP bitransgenic mouse model

Dct-H2BGFP mice were generated by intercrossing *Dct*-tTA and TRE-H2BGFP mice (gift of E. Fuchs) to the homozygous state. *Dct*-tTA mice were generated commercially by genOway (Lyon, France) by homologous recombination of a SpeI-PvuI fragment from the vector *Dct*-tTA-SV40p(A) into the *Hprt* locus on the X chromosome of 129O1a (E14) ES cells. Following blastocyst injection, 3 highly chimeric males were selected from 2 different ES cell clones. 2 males from a single ES clone yielded agouti pups at 50% frequency when crossed with C57BL6 females to indicate germline transmission. Southern blotting confirmed the presence of the recombined allele in the female F1 progeny. Female progeny were crossed with C57BL6 males to generate F2 *Dct*-tTA progeny, which were subsequently crossed with TRE-H2BGFP transgenic mice to generate bitransgenic *Dct*-H2BGFP progeny. *Dct*-H2BGFP mice were then intercrossed to generate double homozygous females or males hemizygous for the *Dct*-tTA gene and homozygous for TRE-H2BGFP.

The following primer sets can be used to genotype the mice using PCR:

Dct-tTA (amplicon size: 322 bp):

Forward 5' - GCTTGCAGGATCATAATCAGCCATACC -3'

Reverse 5' - GCCGACTTCGAGTTTGAGCAGATGT -3'

TRE-H2BGFP (amplicon size: 314 bp):

Forward 5' -GCCACAAGTTCAGCGTGTCC-3'

Reverse 5' -GATGCCCTTCAGCTCGATGC-3'

For both primer sets, standard PCR conditions with 60°C annealing temperature can be used.

Immunofluorescence microscopy

Dorsal skin obtained from transgenic mice at the indicated ages was embedded in OCT compound and cryosections cut with a thickness of 10µm to observe H2BGFP expression. For immunofluorescence, cryosections were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and blocked with blocking buffer (10% FBS, 1% BSA and 0.1% Triton-X) for 1h at room temperature. Primary antibodies for c-Kit (rat monoclonal antibody, Cedarlane), *Dct* (α-PEP8, rabbit polyclonal antibody, a gift from Dr. Vincent

Hearing, NIH), CD34 (rat monoclonal antibody, clone Ram34, BD Biosciences), or Col IV (rabbit polyclonal antibody, Abcam) at 1:200 dilution were added and incubated at 4°C overnight. To detect the primary antibody, isotype-matched Cy3-conjugated secondary antibodies (Jackson Immunogenetics) or Alexa 647-conjugated secondary antibodies (Invitrogen) were added at a 1:1000 dilution and incubated for 1h at room temperature. Coverslips were mounted using mounting solution with DAPI (Vectashield, Vector Laboratories). Fluorescence was detected using an Olympus upright fluorescence microscope, and Slidebook imaging software. To determine the co-localization of H2BGFP expression with cochlear stria vascularis, the sections of cochlea were stained with 1:800 Alexa Fluor™ 546 labelled phalloidin antibody for an hour at room temperature (Thermo Fisher Scientific).

Doxycycline-dependent suppression of H2BGFP expression in *Dct*-H2BGFP mice

To suppress H2BGFP expression during first anagen, P19 *Dct*-H2BGFP mice (n=3) were provided 2 g/l doxycycline (doxycycline hyclate, Sigma) dissolved in drinking water for 11 days. The doxycycline containing water was changed every 24-48 hours. Control groups received regular water only. The mice were euthanized at P30. Dorsal skins were removed from the mice and embedded in Oct compound (Tissue-Tek) to prepare 10µm cryosections. The tissue was fixed with 4% paraformaldehyde for 10 minutes followed by a 1 hour incubation in blocking solution containing 10% FBS, 1% BSA and 0.1% Triton-X. The sections were probed with Dct (1:200, α-PEP8, rabbit polyclonal antibody, a gift from Dr. Vincent Hearing, NIH) followed by Cy3 conjugated secondary antibody (1:1000, Jackson laboratories). DMI8 Leica fluorescence microscope was used to take the immunofluorescence images.

To suppress H2BGFP expression during embryogenesis and follicular morphogenesis, *Dct*-H2BGFP mice were arranged in breeding trios and immediately given doxycycline (doxycycline hyclate, Sigma) at the dose of 2 g/L dissolved in drinking water. The doxycycline containing water was changed every 24-48 hours. Doxycycline was continued until the dams gave birth to pups and the pups were at P8. Control group received regular water only. The *Dct*-H2BGFP pups were euthanized at P8. Dorsal skins were removed from the mice and embedded, fixed, and mounted as described above. Fluorescence microscopy was performed with the DMI8 Leica fluorescence microscope.

Administration of BrdU to reveal label retaining cells (LRCs)

To generate BrdU-LRCs for co-localizing with *Dct*-H2BGFP-expressing McSCs, 3 *Dct*-H2BGFP transgenic mice were injected intraperitoneally with a 10mM solution of BrdU (Molecular Probes), with final volume calculated with drug to body weight ratio of 50µg/g, twice a day from P21 to P30. One *Dct*-H2BGFP mouse was treated similarly with vehicle only. The mice were euthanized at P56 followed by preparation of cryosections from dorsal skins (Figure 3C).

Isolation of HF McSCs from subepidermal skin and fluorescence activated cell sorting (FACS)

Dorsal skin samples were obtained from transgenic mice at the indicated ages immediately following euthanasia and fat was removed from the dermis using fine forceps. Defatted skin was incubated in 0.5% of trypsin (USB) dissolved in PBS at 37°C for 30min. Epidermis was peeled away from the dermis following incubation, and the remaining dermis including HFs was cut into small pieces. The dermal fragments were incubated in digestion medium containing 0.2mg/mL Liberase Thermolysin low (Roche) in a 37°C water bath for 45 to 60min. The digested dermal mixture was added into PBS containing 0.05% DNase (Sigma) and 5% FBS. Single cells were extracted from the dermis by repeated plunging with a 60mL syringe followed by filtration through 40µm nylon mesh (BD Falcon). The dermal cell suspension is prepared in 5% FBS/PBS prior to incubation with respective antibodies and FACS. This procedure was done under aseptic conditions.

The separation of the hair follicle live (GFP+) melanocyte precursors and (GFP-) dermal cells from *Dct*-H2BGFP bitransgenic mice was performed using flow sorting based on expression of GFP and eliminating 7-AAD+ cells. The 7-AAD dye was added to dermal cell suspension in 5% FBS/PBS to discriminate between live and dead cells, and cell sorting was performed using BD FACS Aria1 (Becton-Dickenson) on a 100µm nozzle at 20 psi. low sheath pressure. Sorted cells were counted and used for quantitative realtime PCR (qRT-PCR) by extracting RNA from respective cell populations.

RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from sorted cells using RNeasy Micro Kit (Qiagen) as per manufacturer's protocol. Following transfer to a spin column, incubation with a DNase I, and washing, the spin column membrane was eluted with 14µl RNase free water. The quantity and quality of isolated RNA was determined with an Agilent 2100 Bioanalyzer using RNA PicoChips (Agilent). To synthesize first-strand cDNA from total RNA we used the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Total RNA was incubated with random hexamer primer along with dNTP at 65°C for 5min and added to a mixture of reverse transcriptase containing reaction buffer incubated first at 25°C for 10min, then at 50°C for 50min, and the reaction terminated by heating at 85°C for 5 min. qRT-PCR analysis to determine differential gene expression between the sorted cell populations was performed using SsoFast EvaGreen Supermix (Bio-Rad Laboratories) and running them on CFX96 real-time system-C1000 Thermal Cycler (Bio-Rad Laboratories).

Statistical analysis

The statistical analysis for the qRT-PCR of differential gene expression among sorted cells (Figure 5B) was performed using Student T-Test.

List of Primers

The primer sequences for mouse *Dct*, *Tyr*, *Tyrp1*, *Pmel17* (Lanning, et al, 2005), mouse β -Actin, *Krt14* and *Kit* were designed as described below.

Dct

Forward: 5' – TTCGCAAAGGCTATGCGC – 3'

Reverse: 5' GTTACTACCCAGGTCAGGCCAG – 3'

Tyr

Forward : 5' – CGGCCAACGATCCCATT – 3'

Reverse: 5' – TGCCTTCGCAGCCATTG – 3'

Tyrl

Forward: 5' – GTGTTCCCTAGCTCAGTTCTCTGG – 3'

Reverse: 5' – TCCTCTGACTGATACCTT – 3'

Pmel17

Forward: 5' – TCCAGGAATCAGGACTGGCTTGGT – 3'

Reverse: 5' – GTGAAGGTTGAACTGGCGTG - 3'

β-Actin

Forward: 5' – GGCTGTATTCCCCTCCATCG – 3'

Reverse: 5' – CCAGTTGGTAACAATGCCATGT – 3'

Cytokeratin 14 (Krt14)

Forward: 5' – ATCGAGGACCTGAAGAGCAA – 3'

Reverse: 5' – GGCTCTCAATCTGCATCTCC – 3'

cKit

Forward: 5' - GCCAGGAGACGCTGACTATC - 3'

Reverse: 5' - TGGGGTAGGCCTCGTATTCA - 3'

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Elaine Fuchs for permission to use the TRE-H2BGFP mouse strain in this study. Flow cytometry analyses and cell sorting were performed at the University of Maryland Marlene and Stuart Greenebaum Cancer Center Flow Cytometry Shared Service. This research was supported in part by the Intramural Research Program, Center for Cancer Research, NCI, NIH: NIH/NIAMS R01 grant 1R01AR064810; Merit Review Award I01 BX002582, United States (U.S.) Department of Veterans Affairs, Biomedical Laboratory Research and Development Service; Dean's Office funds, University of Maryland School of Medicine; and the Baltimore Research and Education Foundation, VA Maryland Health Care System. The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

References

Cotsarelis G. Epithelial stem cells: a folliculocentric view. *The Journal of investigative dermatology*. 2006; 126:1459–1468. [PubMed: 16778814]

- Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell*. 1990; 61:1329–1337. [PubMed: 2364430]
- Ezhkova E, Pasolli HA, Parker JS, Stokes N, Su IH, Hannon G, Tarakhovsky A, Fuchs E. Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. *Cell*. 2009; 136:1122–1135. [PubMed: 19303854]
- Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences of the United States of America*. 1992; 89:5547–5551. [PubMed: 1319065]
- Greco V, Chen T, Rendl M, Schober M, Pasolli HA, Stokes N, Dela Cruz-Racelis J, Fuchs E. A two-step mechanism for stem cell activation during hair regeneration. *Cell Stem Cell*. 2009; 4:155–169. [PubMed: 19200804]
- Hornyak TJ, Hayes DH, Chiu L-Y, Ziff EB. Transcription factors in melanocyte development: distinct roles for Pax-3 and Mitf. *Mech Dev*. 2001; 101:47–59. [PubMed: 11231058]
- Hsu YC, Pasolli HA, Fuchs E. Dynamics between stem cells, niche, and progeny in the hair follicle. *Cell*. 2011; 144:92–105. [PubMed: 21215372]
- Ito M, Kizawa K, Hamada K, Cotsarelis G. Hair follicle stem cells in the lower bulge form the secondary germ, a biochemically distinct but functionally equivalent progenitor cell population, at the termination of catagen. *Differentiation*. 2004; 72:548–557. [PubMed: 15617565]
- Kawamoto S, Niwa H, Tashiro F, Sano S, Kondoh G, Takeda J, Tabayashi K, Miyazaki J-i. A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. *FEBS Letters*. 2000; 470:263–268. [PubMed: 10745079]
- Lanning JL, Wallace JS, Zhang D, Diwakar G, Jiao Z, Hornyak TJ. Altered melanocyte differentiation and retinal pigmented epithelium transdifferentiation induced by Mash1 expression in pigment cell precursors. *J Invest Dermatol*. 2005; 125:805–817. [PubMed: 16185282]
- Mackenzie MA, Jordan SA, Budd PS, Jackson IJ. Activation of the receptor tyrosine kinase Kit is required for the proliferation of melanoblasts in the mouse embryo. *Dev Biol*. 1997; 192:99–107. [PubMed: 9405100]
- Muller-Rover S, Tokura Y, Welker P, Furukawa F, Wakita H, Takigawa M, Paus R. E- and P-cadherin expression during murine hair follicle morphogenesis and cycling. *Exp Dermatol*. 1999; 8:237–246. [PubMed: 10439220]
- Nishimura EK, Jordan SA, Oshima H, Yoshida H, Osawa M, Moriyama M, Jackson IJ, Barrandon Y, Miyachi Y, Nishikawa S. Dominant role of the niche in melanocyte stem-cell fate determination. *Nature*. 2002; 416:854–860. [PubMed: 11976685]
- Okamoto N, Aoto T, Uhara H, Yamazaki S, Akutsu H, Umezawa A, Nakauchi H, Miyachi Y, Saidai T, Nishimura EK. A melanocyte-melanoma precursor niche in sweat glands of volar skin. *Pigment Cell Melanoma Res*. 2014; 27:1039–1050. [PubMed: 25065272]
- Osawa M, Egawa G, Mak SS, Moriyama M, Freter R, Yonetani S, Beermann F, Nishikawa S. Molecular characterization of melanocyte stem cells in their niche. *Development (Cambridge, England)*. 2005; 132:5589–5599.
- Oshimori N, Fuchs E. Paracrine TGF-beta signaling counterbalances BMP-mediated repression in hair follicle stem cell activation. *Cell Stem Cell*. 2012; 10:63–75. [PubMed: 22226356]
- Palais G, Nguyen Dinh Cat A, Friedman H, Panek-Huet N, Millet A, Tronche F, Gellen B, Mercadier J-J, Peterson A, Jaisser F. Targeted transgenesis at the HPRT locus: an efficient strategy to achieve tightly controlled in vivo conditional expression with the tet system. *Physiological Genomics*. 2009; 37:140–146. [PubMed: 19141541]
- Rabbani P, Takeo M, Chou W, Myung P, Bosenberg M, Chin L, Taketo MM, Ito M. Coordinated activation of Wnt in epithelial and melanocyte stem cells initiates pigmented hair regeneration. *Cell*. 2011; 145:941–955. [PubMed: 21663796]
- Schneider MR, Schmidt-Ullrich R, Paus R. The hair follicle as a dynamic miniorgan. *Current biology : CB*. 2009; 19:R132–142. [PubMed: 19211055]
- Sriwiriyanont P, Hachiya A, Pickens WL, Moriwaki S, Kitahara T, Visscher MO, Kitzmiller WJ, Bello A, Takema Y, Kobinger GP. Effects of IGF-binding protein 5 in dysregulating the shape of human hair. *The Journal of investigative dermatology*. 2011; 131:320–328. [PubMed: 20944648]

- Steel KP, Davidson DR, Jackson IJ. TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor. *Development* (Cambridge, England). 1992; 115:1111–1119.
- Tobin DJ, Hagen E, Botchkarev VA, Paus R. Do hair bulb melanocytes undergo apoptosis during hair follicle regression (catagen)? *The Journal of investigative dermatology*. 1998; 111:941–947. [PubMed: 9856800]
- Touw K, Hoggatt AM, Simon G, Herring BP. *Hprt*-targeted transgenes provide new insights into smooth muscle-restricted promoter activity. *American Journal of Physiology - Cell Physiology*. 2007; 292:C1024–C1032. [PubMed: 17079332]
- Trempe CS, Morris RJ, Bortner CD, Cotsarelis G, Faircloth RS, Reece JM, Tennant RW. Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. *The Journal of investigative dermatology*. 2003; 120:501–511. [PubMed: 12648211]
- Trempe CS, Morris RJ, Ehinger M, Elmore A, Bortner CD, Ito M, Cotsarelis G, Nijhof JG, Peckham J, Flagler N, et al. CD34 expression by hair follicle stem cells is required for skin tumor development in mice. *Cancer research*. 2007; 67:4173–4181. [PubMed: 17483328]
- Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E. Defining the epithelial stem cell niche in skin. *Science* (New York, NY). 2004; 303:359–363.
- Ueno M, Aoto T, Mohri Y, Yokozeki H, Nishimura EK. Coupling of the radiosensitivity of melanocyte stem cells to their dormancy during the hair cycle. *Pigment Cell Melanoma Res*. 2014; 27:540–551. [PubMed: 24730534]
- Zaidi MR, Hornyak TJ, Merlino G. A genetically engineered mouse model with inducible GFP expression in melanocytes. *Pigment Cell Melanoma Res*. 2011; 24:393–394. [PubMed: 21392368]
- Zhao S, Overbeek PA. Tyrosinase-related protein 2 promoter targets transgene expression to ocular and neural crest-derived tissues. *Dev Biol*. 1999; 216:154–163. [PubMed: 10588869]

Figure 1. Expression pattern of *Dct*-H2BGFP reporter during embryonic development
 (A) Generation of *Dct*-H2BGFP mice. *Dct*-tTA transgenic mice were crossed with TRE-H2BGFP mice to obtain *Dct*-H2BGFP bitransgenic mice, with potential for doxycycline-regulated GFP expression. (B) Expression of *Dct*-H2BGFP in embryonic melanoblasts in the trunk (arrow) and sub- and supra-optic region (arrowhead) at E12.5. (C) Cross-section of eye show H2BGFP-expressing embryonic melanoblasts in retinal pigmented epithelium (rpe) layer and uveal (uv) region and restricted from neural retina (nr) at E12.5. (D) Transverse section of E12.5 embryonic trunk lateral to midline (ml) and neural tube (nt), and deep to surface ectoderm (ec) shows the H2BGFP-expressing embryonic melanoblasts present in the dorsolateral pathway of neural crest cell migration (two representative images). Dorsal root ganglion (drg) is shown in the second representative image. (E) Postnatal H2BGFP expression in cochlear melanocytes (arrow) of stria vascularis shows co-localization with phalloidin.

Figure 2. Expression pattern of *Dct-H2BGFP* reporter during distinct HF stages

(A) Expression pattern during anagen (P8). Expression is observed both in the bulge (arrow) as well as in the lower HF bulb region (arrowheads). (B) Expression pattern during catagen (P17). Expression (arrows) is detected within the retracting HF. (C) Expression pattern during telogen (P21). Expression observed both in the bulge (arrow) and in the subbulge/SHG region (arrowhead).

Figure 3. Ablation of GFP expression in HF melanocytes by doxycycline treatment in *Dct-H2BGFP* mice

(A) Immunofluorescence images in the top row depict Dct-expressing melanocytes in HF bulb of P30 mice with nuclear H2BGFP expression without doxycycline treatment (Dox-). (B) In contrast, immunofluorescence images in the bottom row show no nuclear H2BGFP expression in Dct-expressing melanocytes from HF bulb of P30 mouse after P19 to P30 doxycycline treatment (Dox+). (D) Schematic for BrdU chase experiment: Three *Dct-H2BGFP* mice were intraperitoneally injected with BrdU solution every 12 hours from P21 to P30 during HF anagen. One control mouse was similarly treated with vehicle only. The mice were euthanized at P56, during telogen HF, followed by preparation of cryosections from the dorsal skins and were screened for the co-localization of BrdU-LRCs with *Dct-H2BGFP*-expressing McSCs. (C) GFP-expressing cells and anti-BrdU immunofluorescence staining in P56 dorsal skin HF. Arrow depicts co-localization of SHG (top panel) and bulge (middle panel) GFP-expressing cell with BrdU stain in BrdU injected *Dct-H2BGFP* mouse telogen HF. Bottom panel shows lack of BrdU stain in vehicle injected *Dct-H2BGFP* mouse. (E) Quantification of co-localization of BrdU-LRCs with SHG and bulge *Dct-H2BGFP*-

expressing McSCs relative to total *Dct*-H2BEGFP-expressing cells of telogen HF. Data is an average of three independent experiments. (* P Value < 0.05)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Identification of H2BGFP-expressing McSCs in telogen HF by co-localization with McSC markers

(A) Co-localization of Kit and H2BGFP in bulge and SHG (arrows). Inset boxes show higher magnification of individual H2BGFP-expressing cells co-localizing with Kit expression in both bulge (left inset boxes) and subbulge/SHG (right inset boxes) McSCs. (B) Co-localization of Dct and H2BGFP in bulge and SHG (arrows). Inset boxes show higher magnification of individual H2BGFP-expressing cells co-localizing with Dct expression in both bulge (left inset boxes) and subbulge/SHG (right inset boxes) McSCs. (C) GFP-expressing cells and anti-CD34 immunofluorescence in P56 dorsal skin HF. Arrow depicts co-localization of bulge H2BGFP-expression McSC and CD34 expression while arrowhead shows SHG H2BGFP-expressing McSC in region lacking CD34 expression. Col IV staining defines the HF border.

Figure 5. Separation of HF GFP-expressing melanocyte precursor cells

(A) The GFP-expressing melanocyte precursors identified in P56 telogen HF of *Dct*-H2BGFP mice were separated using fluorescence activated sorting (FACS). The separation of live (GFP+) melanocyte precursor cells and (GFP-) dermal cells was performed using FACS based on expression of GFP and eliminating 7-AAD+ dead cells. Here we are showing a representative image of the FACS using *Dct*-H2BGFP and control C57/BL6 mice. (B) Quantitative RT-PCR analysis for the expression of *Dct*, *Kit*, *Gfp*, *Krt14*, *Tyr*, *Tyrp1* and *Pmel17* genes among the GFP+(melanocyte precursors), and GFP-(dermal cells) sorted cell populations. Here, β -*Actin* was used as reference gene. (* $P < 0.01$ by ANOVA).