# Conditional Immortalization Establishes a Repertoire of Mouse Melanocyte Progenitors with Distinct Melanogenic Differentiation Potential

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## TO THE EDITOR

Epidermal melanocytes (MCs) are specialized melanin-producing cells (Slominski *et al.*, 2004; Yamaguchi *et al.*, 2007; Thomas and Erickson, 2008; Park *et al.*, 2009) that synthesize melanin within the melanosome (Slominski *et al.*, 2004) and protect individuals from harmful UV rays (Slominski *et al.*, 2004; Yamaguchi *et al.*, 2007; Thomas and Erickson, 2008; Park *et al.*, 2009). Defects in or a lack of MCs can lead to melanoma, pigment disorders, and auditory defects. MC proliferation and differentiation in skin is tightly linked to hair regeneration cycles. MCs in vertebrates are derived from neural crest (Thomas and Erickson, 2008). In hair follicles, melanoblasts are segregated into hair matrix MCs (for hair pigmentation) and melanocyte stem cells (MCSCs). The discovery of MCSCs and induced pluripotent (iPS) cells provides important resources to elucidate mechanisms underlying melanogenesis and pathogenesis of MC-related disorders (Lin and Chuong, 2011; Nishikawa-Torikai *et al.*, 2011; Nishimura, 2011; Ohta *et al.*, 2011; Yang *et al.*, 2011). Although MCSCs and iPS cells are important MC precursors for mechanistic studies, their isolation and expansion are technically challenging.

Here we investigate whether MCs can be immortalized without compromising melanogenic potential. By using primary MCs isolated from newborn mouse skin, we engineered >100 clones by introducing SV40 T antigen (SV40T), which is flanked with FRT sites (Supplementary Figure S1A online; Westerman and Leboulch, 1996). We found that although primary MCs grew slower after passage 5 immortalized MC (iMC) cells acquired high proliferative activity (Supplementary Figure S1B online); however, wide variations in proliferation were observed among clones. For instance, iMC23 grew faster than iMC65 (Figure 1a).

We analyzed melanogenic markers in iMC clones. More than half of the analyzed clones expressed progenitor marker c-kit, whereas MC progenitor markers Pax3, Sox10, and MITF-m were readily detected in most clones (Supplementary Figure S2A online). Dopachrome tautomerase and tyrosinase-related protein 1 (TRP-1) were highly expressed in most iMC clones, whereas tyrosinase was highly expressed in about half of the clones (Supplementary Figure S2B and C online). It is conceivable that early melanogenic iMCs should express c-kit and early melagonic markers but not late markers (e.g., tyrosinase). Three iMC clones were chosen for further characterization: iMC23 (melanoblast progenitor-like) (Figure 1aA), iMC65 (late-stage melanocyte-like) (Figure 1aB), and iMC37 (intermediately differentiated melanoblast-like). These results indicate that SV40T-mediated immortalization can create a repertoire of iMCs with varied melanogenic potential.

We also tested whether immortalization phenotypes were reversible by flippase recombination enzyme (FLP). By using adenovirus FLP (AdFLP) that co-expresses green fluorescent protein (GFP; He *et al.*, 1998; Luo *et al.*, 2007), we found that iMCs were effectively transduced by AdFLP or AdGFP, and SV40T expression was reduced in FLP-transduced iMCs (Figure 1bA and bB). iMC23 infected with AdFLP grew slower (Figure 1bC and bD), suggesting that the proliferative activity of iMCs may be reversed by removing SV40T. By using reporter pTyr-Gluc-expressing Gaussia luciferase (GLuc) driven by a 2.0-kb mouse tyrosinase promoter, we found that GLuc activity was increased by dexamethasone and was potentiated by removing SV40T with FLP (Supplementary Figure S3A and B online).

We further analyzed spontaneous differentiation of iMCs by assessing endogenous melanin and tyrosinase activity. Cell pellets from iMCs exhibited varied amounts of melanin. iMC65 and iMC61 exhibited the highest level of melanin and iMC23 produced the lowest level, whereas a majority of iMC clones produced low-to-modest levels of melanin (Figure 1cA). Accordingly, quantitative analysis revealed that iMC65 and iMC61 exhibited high tyrosinase activity, and seven clones including iMC23 had low tyrosinase activity, whereas iMC37 exhibited modest-to-high levels of tyrosinase activity (Figure 1cB), consistent with the qualitative results shown in Figure 1cA.

We characterized the early and late markers c-kit and HMB45, respectively, in iMC23, iMC37, and iMC65. HMB45 is a widely used mAb detecting melanocytic tumors (Gown *et al.*, 1986). iMC23 expressed a high level of c-kit, whereas iMC37 and iMC65 were strongly stained with HMB45 (Figure 1dA). Fontana–Masson staining revealed the highest level of melanin in iMC65 and a modest level in iMC37, whereas a negligent level of melanin was observed in iMC32 (Figure 1dB).

*Abbreviations: FLP, flippase recombination enzyme; GFP, green fluorescent protein; GLuc, Gaussia luciferase; iMC, immortalized melanocyte; iPS, induced pluripotent; MCSC, melanocyte stem cell; TRP-1, tyrosinase-related protein 1*

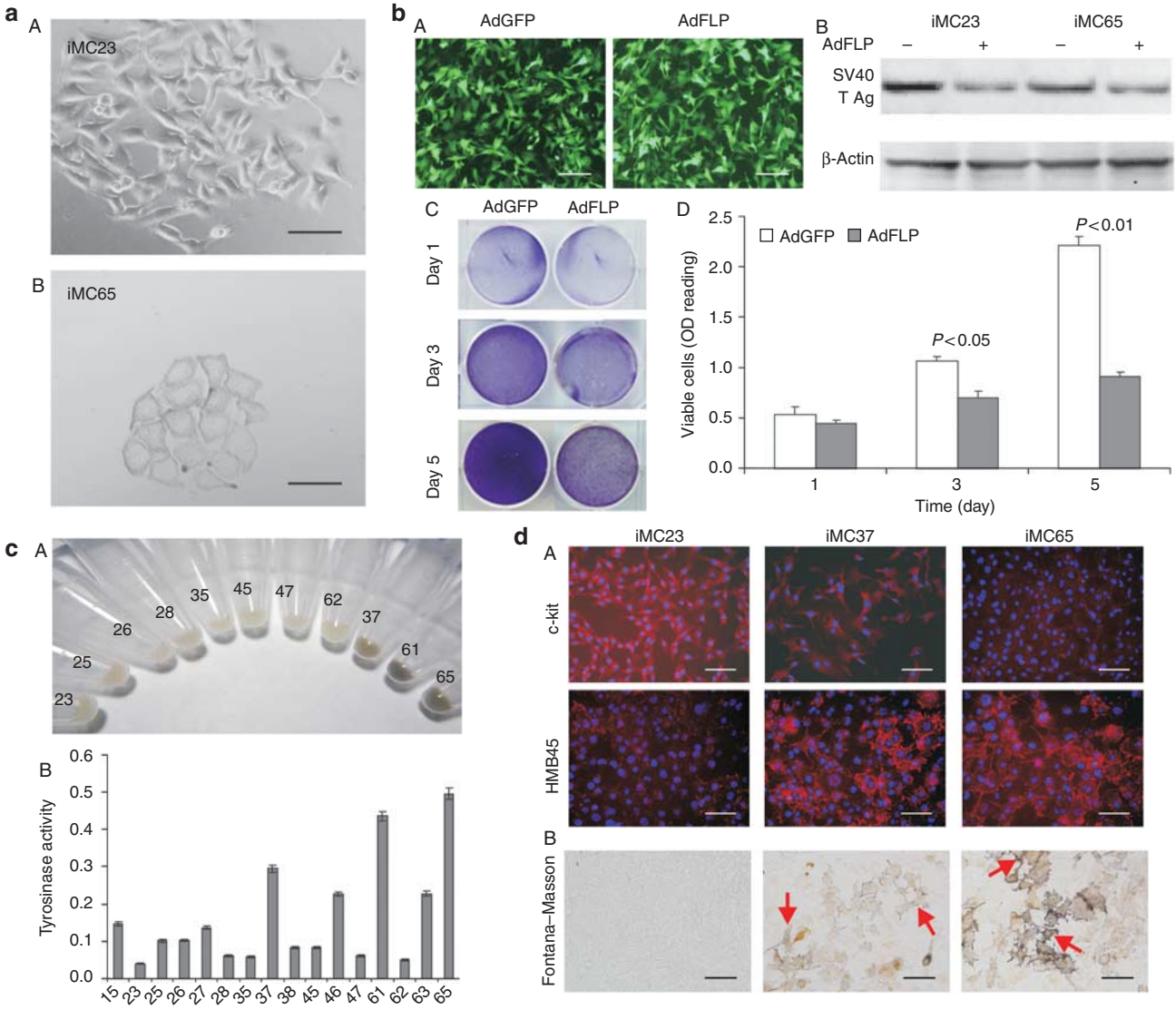


**Figure 1. Characterization of SV40T-immortalized melanocytes (iMCs).** (**a**) iMC23 (**a**A) and iMC65 (**a**B) were seeded at low density and photographed at day 5. (**b**A) iMCs were infected with AdGFP or AdFLP. Green fluorescent protein signal was detected at 24 hours. (**b**B) iMCs were infected with AdFLP (+) or AdGFP (−) for 48 hours. Total cellular proteins were analyzed by anti-SV40T western blotting (β-actin blotting for loading). (**b**C, **b**D) iMC23 cells were infected with AdFLP or AdGFP, stained with Crystal violet, and the absorbance was quantitatively measured at 560 nm. (**c**A) iMCs were collected by pelleting. Pigmentation of cell pellets was photographed. (**c**B) iMCs were collected for endogenous tyrosinase enzymatic assays. (**d**A) iMCs were immunostained with c-kit and HMB45 antibodies. (**d**B) iMCs were subjected to Fontana–Masson staining of melanin (arrows). OD, optical density. Bar = 50 μm.

Thus, these clones may represent different stages of melanogenic differentiation.

We further analyzed the melanogenic potential of iMC23. When iMC23 was treated with dexamethasone, tyrosinase activity increased in a dose-dependent manner (Figure 2aA). Dexamethasone induced tyrosinase and TRP-1 expression (Figure 2aB). Dose-dependent melanin production was visible in culture and cell pellet (Figure 2bA and bB), which was further confirmed by Fontana–Masson staining (Figure 2bC). These results indicate that iMC23 exhibits melanogenic potential.

Finally, we determined whether iMC immortalization phenotypes were reversible *in vivo*. iMC23 and iMC65 tagged with firefly luciferase were infected with AdFLP or AdGFP, collected for subcutaneous injection into athymic mice, and monitored by Xenogen bioluminescence imaging (Caliper Life Sciences, Hopkinton, MA). AdGFP-transduced iMC23 yielded a stronger signal than that of AdFLP-transduced iMC65, whereas within the same lines AdFLP-transduced cells produced no signal compared with the AdGF-transduced iMC65 (Figure 2cA), suggesting that removing SV40T may reduce proliferation and survival of iMCs *in vivo*. Pigmentation of injected iMCs was visible through skin, as iMC65 exhibited a higher level of pigmentation than iMC23. AdFLP-transduced iMC23 yielded more pigmentation than

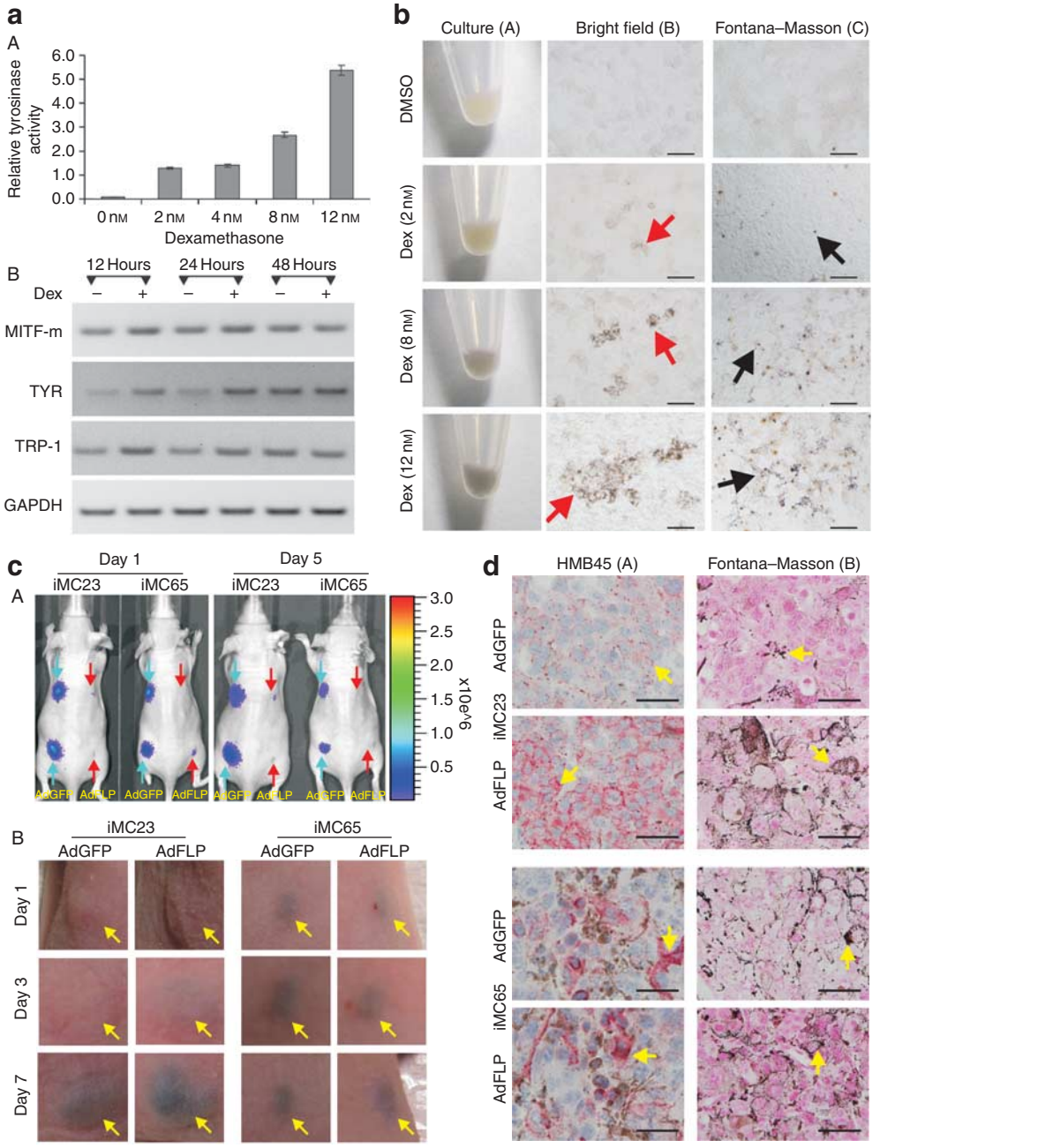


**Figure 2. Melanogenic potential of immortalized melanocytes (iMCs).** (**a**A) iMC23 cells were treated with dexamethasone (Dex) for 5 days and collected for tyrosinase enzymatic assay. (**a**B) iMC23 was stimulated with 12 nM Dex. Gene expression was determined by reverse transcriptase–PCR (RT-PCR; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control). (**b**) iMC23 was treated with 12 nM Dex for 5 days. Melanin production was visualized (**b**A) in cell pellet, (**b**B) in culture under the microscope, or by (**b**C) Fontana–Masson staining. Melanin-positive cells are indicated with arrows. Bar = 100 μm. (**c**A) Luciferase-tagged iMC23 and iMC65 infected with AdFLP or AdGFP were injected subcutaneously into flanks of athymic mice (arrows) and subjected to Xenogen imaging. (**c**B) Visual pigmentation at injection sites (arrows). (**d**) Injected sites were retrieved at day 7 and subjected to (dA) HMB45 immunostaining (HRP-AEC) and (**d**B) Fontana–Masson staining. Melanin-positive cells are indicated with arrows. Bar = 25 μm.

AdGFP-transduced iMC23 (Figure 2cB). Histologically, iMC23 injection sites had higher cellularity than iMC65 sites, whereas in both lines cellularity was lower in AdFLP-transduced cells (Supplementary Figure S3C online). Production of melanin was more pronounced in AdFLP-transduced iMCs as indicated by HMB45 immunostaining and Fontana–Masson staining (Figure 2dA and dB). Overall, the *in vivo* results are consistent with these clones' *in vitro* features.

SV40T-immortalized MCs are non-tumorigenic under our experimental conditions. Interestingly, melanoma phenotypes have been reported by enabling neoplastic transformation of primary human MCs with SV40 early region, which encodes both SV40 T and t antigens, in conjunction with hTERT (Gupta *et al.*, 2005). Nevertheless, we established a

repertoire of conditionally immortalized MCs with varied melanogenic potential, ranging from melanoblast-like to well-differentiated phenotypes. Such repertoire of iMCs should be useful for understanding MC biology and raveling molecular pathogenesis of pigment cell disorders, including melanoma.

An efficient method to isolate and expand cutaneous MCs is needed, as it enables us to better understand melanogenesis. Here we demonstrate that SV40T-mediated immortalization of MCs is simplistic, effective, and reversible. This approach should be considered an important alternative to the isolation and characterization of melanogenic stem cells.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

## ACKNOWLEDGMENTS

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### Author contributions

KY: conception and design; manuscript writing; data analysis and interpretation; collection and/or assembly of data. J Chen and WJ: provision of study materials/reagents; collection and/or assembly of data. EH: provision of study materials/reagents; other (animal experiments and histology). J Cui: provision of study materials/reagents; collection and/or assembly of data. SHK: provision of study materials/reagents; data analysis and interpretation. NH, HL, WZ, RL, XC, YK, JZ, JW, and LW: provision of study materials/reagents. JS: others (histology); data analysis and interpretation. HHL and RCH: conception and design; manuscript writing. XL: conception and design; manuscript writing; data analysis and interpretation. TY: conception and design; manuscript writing; data analysis and interpretation; administrative support. T-CH: conception and design; manuscript writing; data analysis and interpretation.

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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# Conditional Immortalization Establishes a Repertoire of Mouse Melanocyte Progenitors with Distinct Melanogenic Differentiation Potential

**Short Title**: **Melanogenesis of Immortalized Melanocytes**

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# SUPPLEMENTARY MATERIALS

## MATERIALS AND METHODS

### Cell culture and chemicals

HEK-293 cells were from ATCC (Manassas, VA) and maintained in completed DMEM as described (Cheng *et al.*, 2003; Haydon *et al.*, 2002; Luo *et al.*, 2008a; Peng *et al.*, 2003; Tang *et al.*, 2009). Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

### Establishment of reversibly immortalized stable melanocyte progenitors (iMCs)

Cutaneous melanocytes were isolated from newborn C57BL/6 mice (Bennett *et al.*, 1987). Skin was dissected and digested in 1.0 ml 4% dispase II for 1h at 37°C. Epidermal portions were dissected from dermis and incubated in 0.25% trypsin/1mM EDTA with occasional agitations for 5min at 37°C, followed by centrifuge for 5 minutes at 1,000 rpm. The dissociated cells were suspended and grown in the complete DMEM containing 200nM TPA (12-O-tetradecanoylphorbol-13-acetate) and 200pM cholera toxin at 37°C. The isolated melanocytes were infected with packaged retrovirus SSR #41 (Westerman and Leboulch, 1996). The infected cells were plated into 96-well plates and selected with 0.3 mg/ml hygromycin B (Invitrogen). Numerous clones were recovered, and 31 clones were scaled up for further characterization. Stable cell pools were designated as iMC-pool.

### Recombinant adenovirus expressing FLP

Recombinant adenoviruses were generated using AdEasy technology (Cheng *et al.*, 2003; He *et al.*, 2010; He, 2004; He *et al.*, 1999; He *et al.*, 1998a; He *et al.*, 1998b; Kang *et al.*, 2009; Kang *et al.*, 2004; Luo *et al.*, 2007). The coding region of Flipe (FLP) recombinase was PCR amplified, subcloned into a shuttle vector and subsequently used to generate recombinant adenovirus in HEK-293 cells. The resulting adenovirus was designated as AdFLP, which also expresses GFP. Analogous adenovirus expressing only GFP (AdGFP) was used as control (He *et al.*, 2010; He *et al.*, 1999; He *et al.*, 1998a; He *et al.*, 1998b; Luo *et al.*, 2007; Luo *et al.*, 2004; Peng *et al.*, 2004; Sharff *et al.*, 2009; Si *et al.*, 2006; Tang *et al.*, 2009).

**Semi-quantitative RT-PCR**

Total RNA was isolated using TRIZOL Reagents (Invitrogen). Reverse transcriptase-PCR was carried out as described (Chen *et al.*, 2010; Luo *et al.*, 2010; Luo *et al.*, 2008a; Luo *et al.*, 2008b; Sharff *et al.*, 2009; Su *et al.*, 2011). 10μg RNA was used to generate cDNA templates by reverse transcription with hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Inc). PCR primers were listed in **Supplemental Table 1**. Semi-quantitative RT-PCR reactions were carried out using a touchdown protocol: 94°C×20″, 68°C ×30″, 70°C×20″ for 13 cycles, with 1°C decrease per cycle, followed by 25–30 cycles at 94°C×20″, 55°C×30″, 70°C×30″. PCR products were resolved on 1.5% agarose gels. All samples were normalized by endogenous level of GAPDH.

**Tyrosinase promoter-driven Gaussia luciferase (GLuc) reporter and GLuc reporter assay**

2.5 kb genomic DNA immediately upstream exon-1 of mouse *tyrosinase* was PCR amplified and subcloned into retroviral reporter vector pSEB-GLuc to drive GLuc (New England Biolabs, Inc), resulting in pSEB-Tyr-GLuc, which was used to make stable iMCs via retroviral transduction. For GLuc assays, exponentially growing iMCs were seeded in 24-well plates and treated with various concentrations of dexamethasone (Dex) or DMSO. Culture medium was collected at 72h for GLuc assays as described (Bi *et al.*, 2009; Huang *et al.*, 2009; Tang *et al.*, 2009; Zhu *et al.*, 2009). Each assay condition was performed in triplicate.

**Crystal violet staining**

Exponentially growing cells were plated in 12-well culture plates and infected with AdGFP or AdFLP. The infected cells were stained with crystal violet at indicated time-points to visualize cell viability and quantified as described (He *et al.*, 2010; He *et al.*, 2011; Su *et al.*, 2011).

**Tyrosinase enzymatic assay**

Tyrosinase enzymatic activity was assessed by measuring the rate of L-DOPA oxidation (Bellei *et al.*). Cells were lysed by freezing-thawing cycles in 300μl PBS containing 1% Triton X-100. Lysates were cleared by centrifugation. Each assay contained 80μl cell lysate and 20μl of 5 mM L-DOPA. After incubation for 60min at 37°C, samples were

subjected to absorbance reading at 490nm. Each assay condition was done in triplicate. Total cellular proteins were determined to normalize samples.

**Immunofluorescence and immunohistochemical staining**

Stains were performed as described (Bi *et al.*, 2009; Huang *et al.*, 2009; Tang *et al.*, 2009; Zhu *et al.*, 2009). Cells were fixed with methanol, permeabilized with 1% triton-X100, and blocked with 10% donkey serum, followed by incubating with anti-c-kit or HMB45 antibody (Santa Cruz Biotechnology). Cells were then incubated with Texas Red-labeled secondary antibody (Jackson ImmunoResaerch Laboratories, Inc). Immunofluorescence stains were recorded under a fluorescence microscope. For HMB45 immunohistochemical staining, positive staining was visualized by using HRP-AEC detection system. Stains without primary antibodies or with control IgG were used as negative controls.

**Western blotting analysis**

Cells were lysed in Laemmli buffer, denatured by boiling, and subjected to 10% SDS-PAGE, followed by electro-transfer to Immobilon-P membranes. Membrane was blocked with SuperBlock Blocking Buffer and probed with anti-SV40 T antigen or anti-β-actin (Santa Cruz Biotechnology), followed by incubation with HRP-conjugated secondary antibody. Proteins of interest were detected by using SuperSignal West Pico Chemiluminescent Substrate kit.

**Subcutaneous implantation of melanogenic progenitors and Xenogen bioluminescence imaging**

Animal experiments were carried out according to the guidelines approved by Institutional Animal Care and Use Committee. Firefly luciferase-tagged iMC23 and iMC65 cells were infected with AdGFP or AdFLP for 24h, and collected for subcutaneous injection ($10^6$ cells/injection) into the flanks of athymic nude (nu/nu) mice (n=5/group, 4-6wk, female, Harlan Sprague Dawley). Animals were sacrificed one week later. Implantation sites were retrieved for histologic evaluation and other stains. Mice were anesthetized with isoflurane and imaged with Xenogen IVIS 200 system (Caliper Life Sciences, Hopkinton, MA) after subcutaneous implantation. At 10min prior to imaging, mice were injected intraperitoneally with D-luciferin sodium salt (Gold Biotechnology, St. Louis, MO) at 100mg/kg bw. Pseudo images were obtained by

superimposing emitted light over the grayscale photographs. Quantitative analysis was done with Xenogen Living Image V2.50.1 software as described (He *et al.*, 2010; He *et al.*, 2011; Luo *et al.*, 2008a; Rastegar *et al.*, 2010; Su *et al.*, 2011).

**Histologic evaluation and Fontana-Masson melanin staining**

Animals were euthanized. Dorsal skin biopsies were kept in 10% buffered formalin until paraffin embedding and sectioning. Serial sections were stained with H & E. Fontana-Masson staining for melanin was performed (Warkel *et al.*, 1980; Zappi and Lombardo, 1984). Sections were deparaffinized and rehydrated. Slides were fixed in 10% formalin and washed with distilled water. Samples were incubated with Fontana silver nitrate working solution at 60°C for 1h and rinsed with distilled water.

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## FIGURE LEGENDS

**Figure S1. Immortalization and morphology of the SV40 T-immortalized mouse melanocytes (iMCs).** (**A**) Schematic representation of the retroviral vector SSR #41 for SV40 T-mediated immortalization of primary cells, as reported (Westerman and Leboulch, 1996). (**B**) Morphology of primary melanocytes and the iMC pool and clones. Cells were seeded at a low density and photographed at 48h (***a*** & ***c***), followed by consecutive five passages of 20% of the cells every four days (***b*** & ***d***). Representative images are shown.

**Figure S2. Marker expression of the iMC clones.** (**A**) Expression of melanoblast and melanocyte progenitor markers in the selected 31 clones. Subconfluent iMC clones (n=31) were seeded in 25cm$^2$ flasks in 1% fetal bovine serum medium for 24h. Total RNA was isolated and subjected to semi-quantitative RT-PCR analysis. The expression levels of melanocyte progenitor markers c-kit, Pax3, Sox10, and MITF-m were determined. (**B**) Expression levels of melanocyte differentiation markers Dopachrome tautomerase (Dct), tyrosinase, and TRP-1. The same set of RNA samples collected in A was used for RT-PCR analysis. (**C**) Expression of normalization marker GAPDH. The cDNA samples were normalized for GAPDH expression. RT-PCR assays were repeated in at least three independent batches of experiments. Representative three clones are boxed. Representative results are shown.

**Figure S3. Dex induces Tyr-GLuc reporter activity and malenogenic differentiation of iMCs in the presence of FLP recombinase.** (**A**) Schematic representation of the menocyte-specific reporter pTyr-GLuc. (**B**) iMCs stably expressing Tyr-GLuc were infected with AdGFP or Ad-FLP and treated with dexamethasone for 5d. GLuc was measured. Each assay condition was done in triplicate. (**C**) Histological evaluation of the subcutaneously injected iMCs. The injected cell masses were retrieved at day 7 (as described in **Figure 2C**) and subjected to histologic analysis with H & E staining, Melanin positive cells are indicated with arrows.

**Table S1. Oligos sequences for qRT-PCR and cloning**

| | Forward primer | Reverse primer | Application |
|---|---|---|---|
| GAPDH | ACCCAGAAGACTGTGGATGG | CACATTGGGGGTAGGAACAC | RT-PCR |
| Pax3 | GCAGCTGGAGGAACTGGA | GGCGGTTGCTAAACCAGA | RT-PCR |
| SOX10 | AGCCCAGGTGAAGACAGAGA | ATAGGGTCCTGAGGGCTGAT | RT-PCR |
| DCT | AGCAGACGGAACACTGGACT | GCATCTGTGGAAGGGTTGTT | RT-PCR |
| Tyrosinase | GGGCCCAAATTGTACAGAGA | ATGGGTGTTGACCCATTGTT | RT-PCR |
| TRP1 | AAGTTCAATGGCCAGGTCAG | TCAGTGAGGAGAGGCTGGTT | RT-PCR |
| c-kit | GGGCTAGCCAGAGACATCAG | AGGAGAAGAGCTCCCAGAGG | RT-PCR |
| MITF-M | CTCGGGATGCCTTGTTTATG | GAGACACCGCAGACCACTTAG | RT-PCR |
| Tyrosinase promoter | cgcGGATCCttgctttgtagctacagaagcctt | ggcGAGCTCtacaaaacagccaagaacattttc | Gluc reporter |
| FLP recombinase | cgcggatccaccatgggcatgtcacaatttgatatattatgtaaaac | cggctcgagttatatgcgtctatttatgtagg | Cloning |
| eGFP | cccaagcttgccaccatggtgagcaagggcg | cgcggatccttacttgtacagctcgtccatgccg | Cloning |

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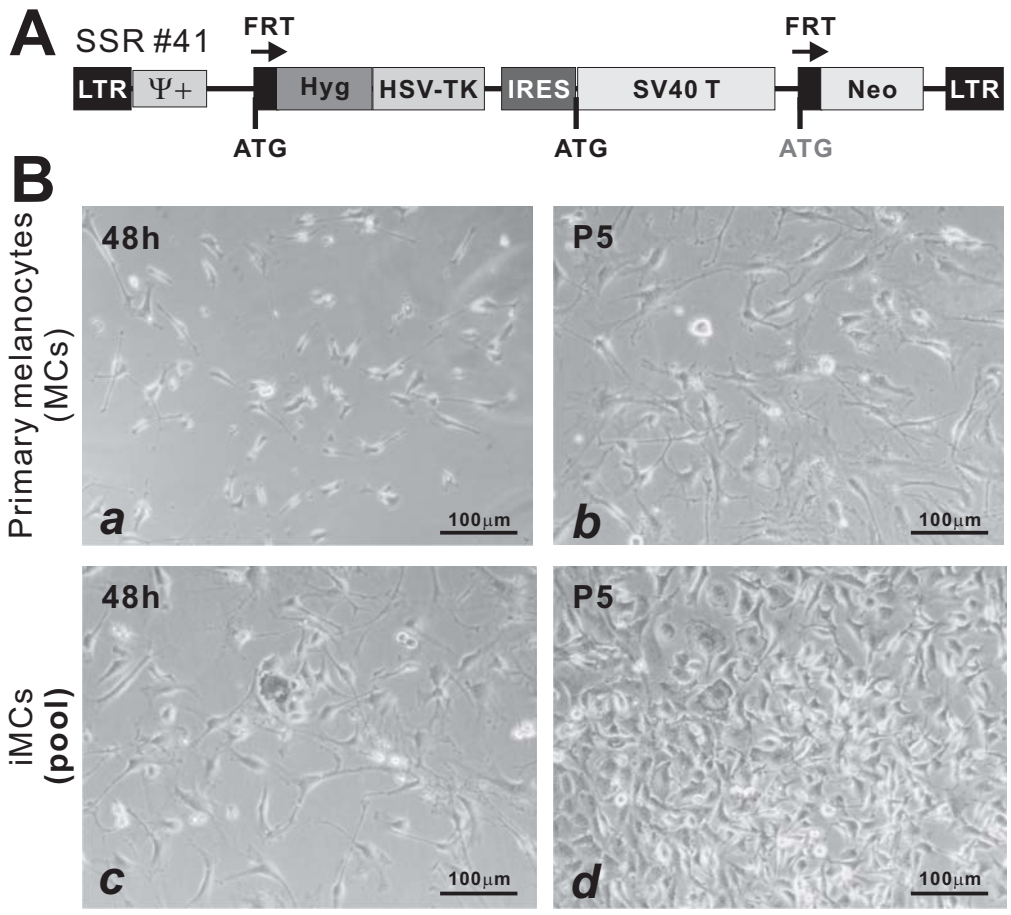

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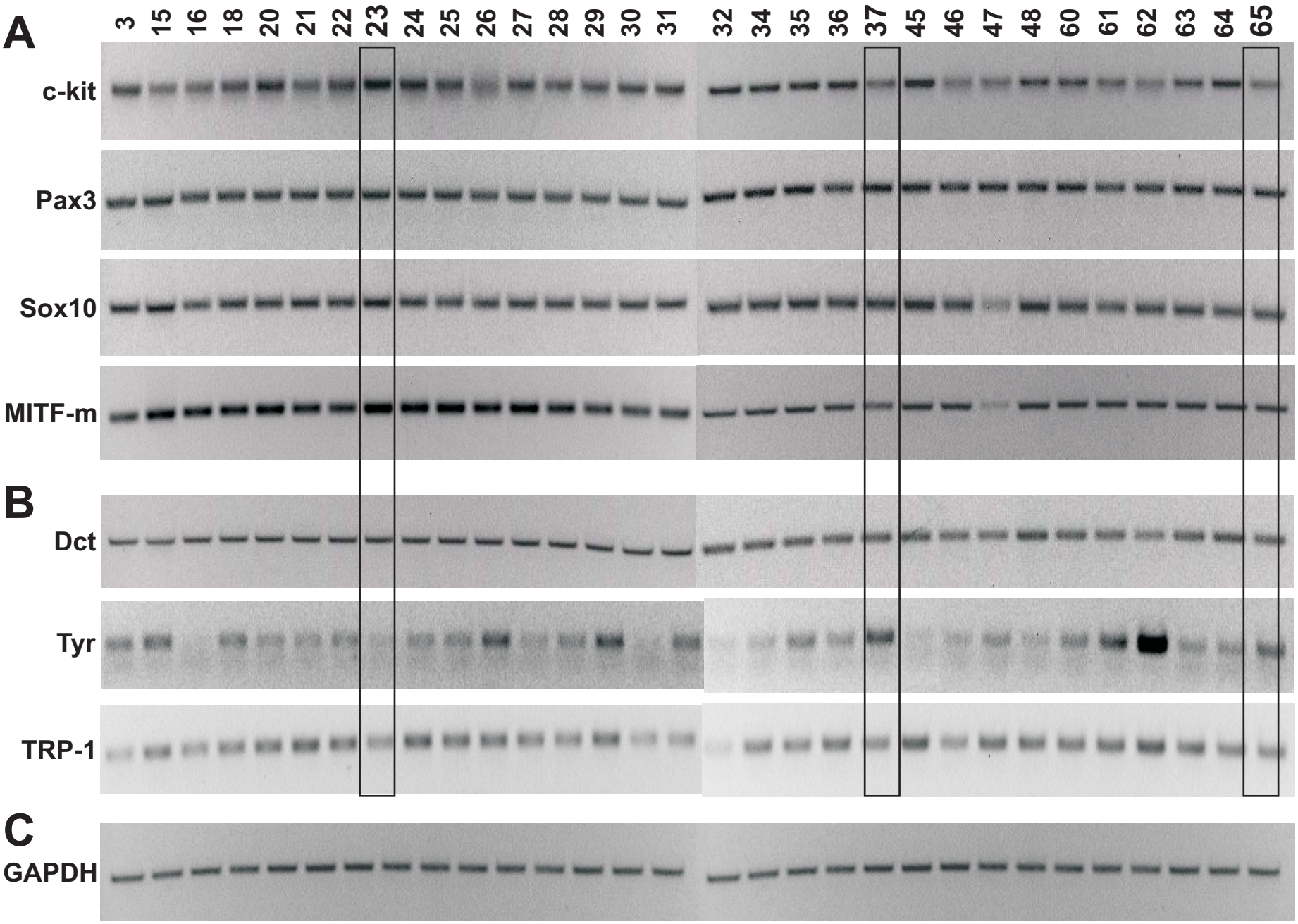

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