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Expression Analysis of the Stem Cell Marker *Pw1/Peg3* Reveals a CD34 Negative Progenitor Population in the Hair Follicle

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ABSTRACT

***Pw1/Peg3* is a parentally imprinted gene expressed in adult stem cells in every tissue thus far examined including the stem cells of the hair follicle. Using a *Pw1/Peg3* reporter mouse, we carried out a detailed dissection of the stem cells in the bulge, which is a major stem cell compartment of the hair follicle in mammalian skin. We observed that PW1/Peg3 expression initiates upon placode formation during fetal development, coincident with the establishment of the bulge stem cells. In the adult, we observed that PW1/Peg3 expression is found in both CD34+ and CD34− populations of bulge stem cells. We demonstrate that both populations can give rise to new hair follicles, reconstitute their niche, and self-renew. These results demonstrate that PW1/Peg3 is a reliable marker of the full population of follicle stem cells and reveal a novel CD34− bulge stem-cell population.** STEM CELLS *2016; 00:000–000*

SIGNIFICANCE STATEMENT

This study uses a recently described pan stem cell marker to examine the described stem cells of the murine hair follicle. We show the existence of a previously unrecognized population of CD-34 negative bulge cells that are fully competent but display altered cell cycle properties.

INTRODUCTION

Adult stem cells are critical for proper tissue homeostasis and regeneration. Many tissues contain adult stem cells that remain quiescent for prolonged periods and are mobilized in response to injury or tissue turnover [1]. In the case of mammalian skin, which can heal in response to injury as well as undergo continuous hair follicle replacement, stem cells undergo repeated cycles of growth (anagen), apoptosis driven retraction (catagen), and rest (telogen) [2, 3]. During each cycle, stem cells are recruited into the cell cycle to generate differentiated cells and replace the stem cell compartment [4]. The major source of multipotent stem cells resides in an anatomically discrete structure called the bulge [5, 6]. Bulge stem cells were first identified by their label-retaining capacity [5–9]. Recent years have seen the combinatorial use of markers to purify bulge stem cells that are multipotent and clonogenic, giving rise to all epidermal lineages following engraftment [10–13] including the hematopoietic stem cell marker, CD34, combined with α6-integrin [11, 14], keratin 15 (K15) [15], and Leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) [16].

We demonstrated previously that the parentally imprinted gene *Pw1/Peg3* (hereafter referred to as *Pw1*) is expressed in a wide array of adult stem cells including blood, skeletal muscle, bone, CNS as well as the bulge cells of skin [17] and that *Pw1* functions in several lineages to confer stem cell competence [18]. While the expression of PW1 marks adult stem cells, we note that PW1 is widely expressed during early development upon gastrulation and becomes highly restricted to a smaller subset of cells during late fetal development [19]. However, it remains unclear if PW1 expression is initiated in tissues during development upon the establishment of the stem cell niche. In order to better characterize the utility of using PW1 expression as a marker of adult stem cells, it is critical to explore its expression in a tissue, for which a stem cell lineage is well-characterized during development and in the adult. In this study, we set out to determine whether PW1 expression can be used to follow the skin stem cells upon the establishment of the bulge and whether all

PW1-expressing cells share stem cell capacity. During murine hair follicle morphogenesis, we observed that PW1 expression is initiated in the developing skin at E15.5, when stem cells begin to be specified [20], and its expression is maintained throughout postnatal life. Using the PW1 reporter mouse model that we generated previously [17], we observe that there are two distinct PW1+ populations of bulge cells that differ in their expression of CD34. These populations display the same levels of clonogenicity and give rise to all hair follicle structures following engraftment but differ in behavior during the telogen-anagen transition, where we observed that the PW1+ bulge stem cells were uniquely activated and that the two populations displayed different cell cycle kinetics. These differences in cell-cycle behavior were also reflected in specific differences in cell-cycle regulatory gene expression.

Materials and Methods

Mice

Murine models: Tg(*Pw1*^{IRESnLacZ}) reporter mice [17]; C57Bl/6J and *Foxn1*^{nu} *(nude) mice (Elevage Janvier, Le Genest-Saint-Isle, France); Tg(HIST1H2BB/EGFP)1Pa/J (Jackson Laboratories, Bar Harbor, Maine), in which nuclear GFP is expressed under the chicken β-actin promoter.* Mouse studies were carried out according to the French and European guidelines.

Histological Analyses

Mouse back skin was either directly embedded in OCT (Tissue Tek) or fixed first in 4% paraformaldehyde (for tissue collected from GFP-expressing mice) before freezing on dry ice. Longitudinal sections (8-9 μm) and cultured cells were fixed for 10 minutes at room temperature with 4% Paraformaldehyde (PFA) before X-Gal staining [21], or processed for immunofluorescence as described previously [17, 22–24]. Tail epidermal whole mounts were prepared and immunolabeled as described previously [9].

Primary antibodies: PW1 [19]; CD34 and CD49f (a6-integrin, BD Pharmingen, San Jose, California); b-galactosidase (b-gal, Promega, Madison, Wisconsin); Ki67 (Vector Laboratories, Burlingame, California); Keratin 14 (K14) and Vimentin (Santa Cruz Biotechnology, Santa Cruz, California); Keratin 15 (Thermo Scientific, Waltham, MASS); Keratin K75 (keratin 6; Progen); P-Cadherin (Life Technologies, Carlsbad, California, USA); Lrig1 and Sox9 (R&D systems, Minneapolis, MN); BrdU (AbD Serotec, Hercules, California); Lgr5 (Abgent, San Diego, California); Keratin 5, AE15, AE13, and GFP (Abcam, Cambridge, UK). Antibody binding was revealed using species-specific secondary antibodies coupled to Alexa Fluor 488 (Molecular Probes, Eugene, Oregon), Cy3, or Cy5 (Jackson Immunoresearch). Nuclei were counterstained with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) or nuclear fast red (Sigma-Aldrich, Munich, Germany).

For quantitative analyses, at least 250 cells or 50 hair follicles from randomly chosen fields were counted from at least three independent experiments per group.

Values represent the mean ± SEM and statistical analyses were performed using Student's *t* test from at least three independent experiments.

RNA Isolation, Semiquantitative PCR, and Quantitative PCR

RNA was isolated from whole skin using RNeasy Fibrous Tissue Midi kit (Qiagen, Hilden, Germany) and from primary cells using RNeasy Micro kit (Qiagen). Reverse transcription was obtained using the SuperScript First-Strand (Invitrogen, Carlsbad, California, USA). Quantitative PCR analysis was performed using the Light Cycler 480 SYBR Green I master (Roche, Basel, Switzerland) and the LightCycler 480 II system (Roche). Samples were normalized to *Hprt* using the ΔCt method. Semiquantitative PCR and quantitative PCR were performed with the primer pairs in Supporting Information Table 1.

BrdU Experiments

Ten-day-old wild-type or Tg(*Pw1*^{IRESnLacZ}) reporter mice were injected with BrdU at a dose of 50 μg/g of body weight, twice a day, for three consecutive days. Four weeks later, tails were processed for whole-mount immunofluorescence as previously described [9].

Fluorescence-Activated Cell Sorting (FACS) Analysis

Seven- to eight-week-old mouse back skin was processed as previously described [16, 17, 25]. Briefly, mice were killed, dorsal hair was clipped, and dorsal skin was dissected free and placed into calcium- and magnesium-free Hank's balanced salt solution (HBSS; Sigma-Aldrich). Subcutaneous tissue and fat were removed by scraping, and the skin was incubated overnight at 4°C in trypsin (0.25%). The epidermis was gently scraped into S-minimal essential medium (Sigma-Aldrich) supplemented with 0.2% Bovine Serum Albumin (BSA) and 0.02% soybean trypsin inhibitor (Life Technologies, Carlsbad, CA). The cell suspension was filtered through a 70 μm followed by filtration through a 40 μm cell strainer (Corning). Cells were stained with 10 ng/ml of rat anti-mouse P-Cadherin-APC (R&D Systems, Minneapolis, MN) or P-Cadherin-PE-Cy5 (Interchim), rat anti-mouse CD34-Pacific blue (eBioscience, San Diego, California), and rat anti-mouse CD49f-PE (α6-integrin, BD Biosciences, San Jose, CA). To detect nuclear β-gal activity, FDG [26] or DDAO staining kits [27] (Molecular Probes, Eugene, Oregon) were used according to manufacturer's instructions. Flow cytometry was performed on a FACS Aria (Becton Dickinson, San Jose, CA).

Cell Culture and Clonogenicity Assay

Freshly sorted keratinocytes were cultured as previously described [16, 17]. Cells were grown in defined keratinocyte serum-free medium (DK-SFM; Invitrogen, Carlsbad, Callifornia, USA) at 8,000 cells per cm^2 on mitomycin-treated NIH3T3 feeders.

For colony-forming assays, 3,000 freshly sorted cells were plated on 6-well plates without coating. After 16 days in culture, colonies were fixed with 4% PFA, stained with 1% Rhodamine, and counted. To assess formation of secondary colonies, clones were trypsinized 16 days after plating, and 3,000 keratinocytes were plated and grown for an additional 16 days. Qualitative and quantitative analyses were performed from at least three independent experiments. Statistical analyses were done using the Student's *t* test.

Transplantation

Six independent transplantations (5×10^5 cells) were performed as previously described [17, 28]. Cell contribution was determined by counting the percentage of GFP+ follicles. Three or four weeks after engraftment, the engrafted area of 3 recipient mice per group was analyzed for immunostaining or injured by depilation.

Cell Cycle Analysis

Freshly sorted cell populations (see FACS analysis) were washed with PBS, and fixed in 70% ethanol. The cells were then treated with RNaseA and stained with propidium iodide (PI) (Sigma-Aldrich). Twenty thousand events were analyzed on an LSRFortessa SORP flow cytometer (Becton-Dickinson, San Jose, California), and cell cycle distribution was analyzed by the BD FACSDiva program.

Results

PW1/Reporter Gene Expression is Detected in the Establishment of the Stem Cell Niche

We used the Tg(*Pw1*^{IRESnLacZ}) reporter mouse [17] to track both PW1/reporter activity and concomitantly follow PW1 protein expression in the skin from embryonic day 15.5 (E15.5) up to the first anagen phase of the hair follicle at postnatal day 14 (P14) (Fig. 1A, 1O). At E15.5, the majority of PW1/reporter-expressing cells were detected in the dermal condensates, the dermis, and the interfollicular epidermis (IFE) as defined by the expression of the K14 basal cell marker (Fig. 1B, Supporting Information Fig. S1A). To better characterize the PW1+ cells at this early stage, we examined Sox9 expression, which previously has been shown to mark the embryonic label-retaining cells that persist in the adult bulge [20]. We observed that Sox9 expression was restricted primarily to a vertical line of 4-5 cells in the center of the hair peg and almost all Sox9+ cells coexpressed the PW1/reporter (Fig. 1C). A similar pattern of PW1/reporter expression was observed at E18.5 in the dermis and the IFE (Fig. 1D, 1E, Supporting Information Fig. S1B), including expression of the PW1/reporter expression in the K14+ population (Fig. 1A, 1D, 1E, Supporting Information Fig. S1C). We observed a decrease in the number of cells coexpressing Sox9 and the PW1/reporter (Fig. 1F), that may reflect the emergence of a distinct subpopulation of PW1+/Sox9- cells.

The pattern of PW1/reporter expression observed at E18.5 was also detected from P0 to P10 (Fig. 1G-1N, Supporting Information Fig. S1D-S1G). As the nascent hair grows, the K14+ population extends distally from the presumptive bulge to the hair follicle bulb (Fig. 1A). From P5 to P10, PW1/reporter expression was detected in the presumptive bulge stem cells and the developing outer root sheath (ORS) (Fig. 1I, 1L, Supporting Information Fig. S1E, S1F), whereas no PW1/reporter expression was observed in the K14+ matrix cells of the growing hair follicle or in the sebaceous glands (Fig. 1J, 1K, 1M, 1N, Supporting Information Fig. S1E, S1G, and data not shown). At P5 and P10, PW1/reporter expression remained detectable in the IFE (Supporting Information Fig. S1H, S1I) and in all dermal papilla compartments regardless of hair types (guard, awl, auchene, or zigzag) (Fig. 1J, 1L). Taken together, these data show that PW1/reporter expression is spatially and temporally coincident with early stem cell specification during the establishment and subsequent formation of the adult bulge as well as the dermal papilla and IFE.

We have shown previously that the bulge and dermal papilla express high levels of PW1/reporter during the telogen phase of the adult hair follicle [17]. In the current study, we observed that this adult pattern of expression is established by P10, once morphogenesis of the skin is complete. Following P10, postnatal hair undergoes repeated cycles composed of three phases, referred to as anagen (active growth), catagen (regression), and telogen (rest) (Fig. 1O). Specifically, a secondary hair germ is established following the first cycle, during which new pools of follicle stem cells emerge [29]. Examination of the first cycle revealed that PW1/reporter expression was dispersed along the ORS and detected in the dermal papilla during anagen. During catagen the expression became concentrated in the bulge as well as in secondary hair germ, dermal papilla, and in the degenerating area of hair follicle (Fig. 1P, 1Q). In telogen, we observed that PW1/reporter expression was restricted to the dermal papilla, bulge, and the nascent secondary hair germ (Fig. 1R). Taken together, we conclude that PW1 expression is primarily restricted to stem cells and the dermal papilla during postnatal skin development and during the first hair cycle.

PW1 Expression Remains Restricted to Stem Cells in the Adult Hair Follicle

In adult anagen (Fig. 2A-2E), we observed that half of the PW1/reporter expressing cells in the bulge coexpressed CD34 (Fig. 2A, 2A') and Keratin 5 (K5) (Fig. 2B, Supporting Information Fig. S1J) whereas the remaining PW1+ bulge population did not colocalize with any other marker presently used for bulge cells such as K14, K15, or α6-integrin (Supporting Information Fig. S1K-S1M). It has been demonstrated previously that Lgr5 does not colocalize with CD34+ cells in the bulge during anagen [16]. We confirmed this previous observation (Fig. 2A, 2A') and noted that many of the PW1+ cells also expressed Lgr5 (Fig. 2A', 2E) and K5 (Fig. 2B) in the upper ORS. We further analyzed the PW1+ population by using the keratin 6 (K6) marker that has been shown to define progenitor cells [30]. K6 expressing cells showed almost no PW1 reporter expression (Fig. 2C). As K6 has been shown to label the companion layer (CL), we conclude that PW1 expression is primarily restricted to the ORS and that very few PW1 positive cells are found in the CL. We noted that PW1/reporter expression was not detected in the cells located near the bulb, which are highly proliferative (data not shown, Fig. 2E), or in other compartments of the hair follicle, including the inner-root-sheath, hair shaft, cortex, or junctional zone indicated by the absence of colocalization of PW1/reporter with AE15 [31] (Fig. 2D), AE13 [32] (Supporting Information Fig. S1N), and Lrig1 (Leucine-rich repeats and immunoglobulin-like domains protein 1) [33] (Supporting Information Fig. S1O) expression, respectively. We observed that PW1/reporter gene activity was no longer detectable in the adult IFE (Supporting Information Fig. S1P, S1Q).

We conclude that PW1/reporter expression marks primarily CD34+ and a smaller population of CD34− cells in the anagen bulge compartment, several progenitor cells in the

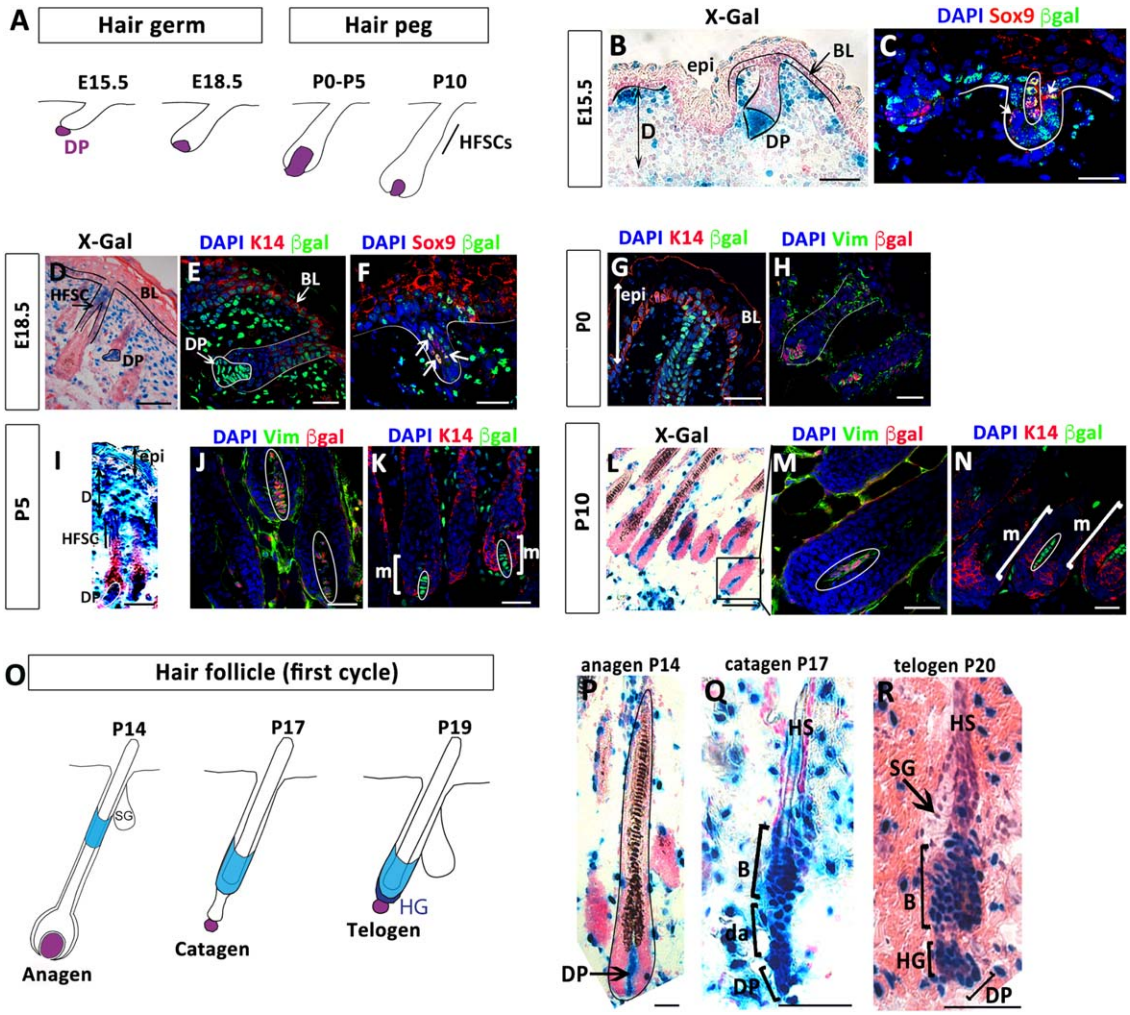

Figure 1. PW1/reporter gene expression in the forming stem cell niches during perinatal and postnatal skin development. **(A)**: Schematic representation of hair follicle development from E15.5 to 10 postnatal days (P10). DP (purple). **(B-N)**: Histochemical (X-Gal) and immunofluorescent (βgal) staining of back skin longitudinal sections of E15.5 (B, C), E18.5 (D-F), P0 (G, H), P5 (I-K), and P10 (L-N) Tg(*Pw1*^{IRESnLacZ}) reporter mice for early stem cell niche marker (Sox9; C, F), basal cell marker (K14; E, G, K, N) and mesenchymal cells (Vimentin; H, J, M). Nuclei were counterstained with DAPI. **(O)**: Schematic representation of hair follicle development during the first cycle from P14 to P19. DP (purple); Bulge (light blue); HG (dark blue). **(P-R)**: Histochemical staining of longitudinal hair follicle sections during the first hair cycle (P14-P20). Lines define the basal layer of the interfollicular epidermis (B-D) or demarcate the hair germ (C, E, F) or the hair peg (H). Circles: dermal papilla (B, E, M, N). Nuclei are counterstained with DAPI or nuclear fast red. Scale bars: 10 μm (B, C); 30 μm (P-R); 50 μm (D, I, K, L); 70 μm (E-H, J, M, N). Abbreviations: B, bulge; BL, basal layer; D, dermis; da, degenerating area; DP, dermal papilla; epi, epidermis; HFSC, developing hair follicle stem cells; HG, secondary hair germ; HS, hair shaft; m, matrix cells; ORS, outer-root-sheath; SG, sebaceous gland.

upper portion of the ORS and the dermal papilla cells (Fig. 2E). Taken together, these results show that PW1 expression marks several stem cell compartments in the hair follicle, including a previously undescribed population from the bulge that does not express CD34.

In order to confirm that the pattern of PW1 expression remains consistent following several hair follicle cycles, we examined reporter expression in telogen at P49. At this stage, we confirmed PW1/reporter activity in the bulge stem cells, as shown by colocalization with K15 (Fig. 2F), CD34, and Lgr5 (Fig. 2G) expression. The bulge compartment, originating from the ORS, is composed of two layers of cells: the CD34 + Lgr5+ cells constitute the external layer (Fig. 2H, 2H', 2K), whereas the CD34-Lgr5- cells reside in the internal layer (white stars; Fig. 2H, 2H', 2K). We detected PW1/reporter expression in a subset of CD34+/Lgr5+ stem cells, and we also identified a subset of CD34+/Lgr5+ cells that did not express PW1/reporter in the external layer (yellow arrows; Fig. 2G, 2H, 2H', 2K). We also observed weak PW1/reporter expression in a subset of CD34−/Lgr5− cells in the internal layer (white stars; Fig. 2H, 2H', 2K). Taken together, PW1/reporter expression defines two distinct populations of cells in the telogen bulge: one population, which partially overlaps with the well described CD34+/Lgr5+ [15, 16] stem cell populations, and one that resides in a subset of CD34− cells (Fig. 2K). In addition to the bulge, PW1/reporter expression is restricted to the hair germ, as shown by colocalization with P-Cadherin and Lgr5 expression (Fig. 2I-2K), and to the dermal papilla (Fig. 2F, 2I, 2K) of the telogen hair follicle.

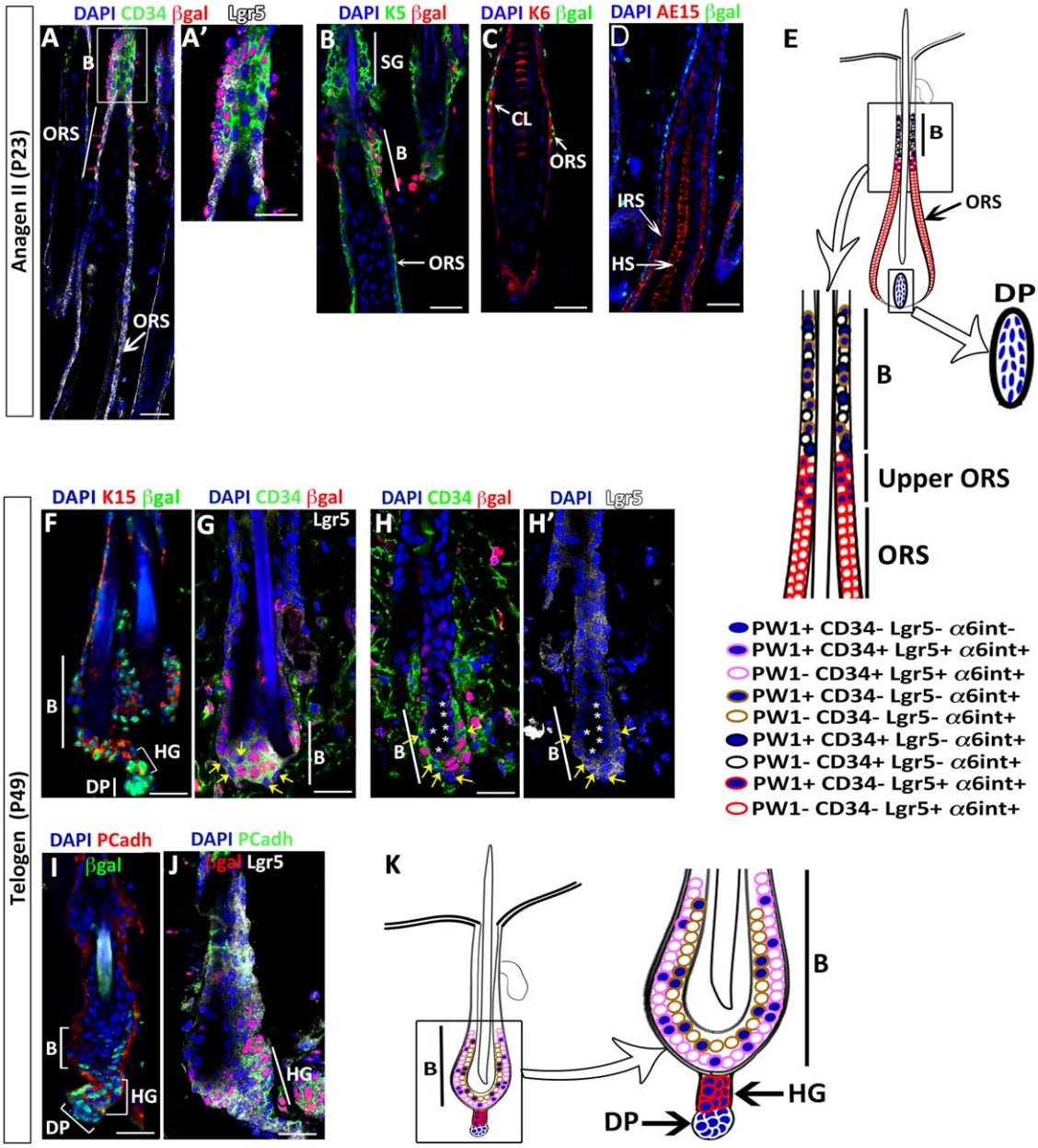

Figure 2. PW1 expression is restricted to stem cell niches in the adult hair follicle. **(A-K)**: Longitudinal cryosections from P23 (anagen; A-D) and P49 (telogen; F-K) Tg(*Pw1*^{IRESnLacZ}) mouse hair follicles immunostained for β-galactosidase and bulge cell markers (CD34, Lgr5; A, A', G-H', J), basal cell markers (K5, K6, AE15, K15; B-D, F) or HG marker (P-Cadherin; I-J). Nuclei were counterstained with DAPI. Rectangle in (A) indicates the bulge at higher magnification in (A'). Yellow arrows indicate CD34+Lgr5+ cells negative for PW1 (G-H'). PW1-expressing cells in the internal layer of the bulge are designated with stars (H, H'). (E, K) Schematic of the PW1 expressing cells in the stem cell niches of the anagen (E) and telogen (K) follicle. During anagen (E), PW1 is expressed in the bulge (B), upper ORS and DP. In the bulge, PW1+ cells are found in CD34+Lgr5-α6integrin+ and CD34-Lgr5-α6integrin+ cells. A subset of PW1 expressing cells resides in the Lgr5+ cells of the upper ORS (B). Rectangles in (E) indicate the bulge and DP shown at higher magnification. During telogen (K), ~50% of the PW1+ cells reside in the external layer of the bulge containing the CD34+Lgr5+α6integrin+ cell population whereas ~50% of the PW1+ cells are found in the bulge in the internal layer of α6int+ cells (negative for CD34 and Lgr5 expression). PW1 and Lgr5 are coexpressed in the HG while PW1 marks the DP cells (K). Rectangles in (K) indicate the bulge, HG, and DP shown at higher magnification. Scale bars: 10 μm (E-I); 30 μm (A, A'); 50 μm (B, C, D); 70 μm (J). Abbreviations: B, bulge; CL, companion layer; DP, dermal papilla; HG, secondary hair germ; HS, hair shaft; IRS, inner-root-sheath; ORS, outer-root-sheath; SG, sebaceous gland.

PW1/Reporter-Expressing Cells Comprise a Larger Population than Label-Retaining Cells

We next wished to determine the correspondence of PW1 expression with cells that are label-retaining. Therefore, we used a previously established protocol, in which Bromodeoxyuridine (BrdU) was injected at P10, and then BrdU LRCs were visualized histologically in the adult [5, 34]. Using whole-mount immunostaining for BrdU and PW1 in the adult tail epidermis 4 weeks following BrdU exposure, we found that LRCs were confined primarily to the bulge (Fig. 3A) and that

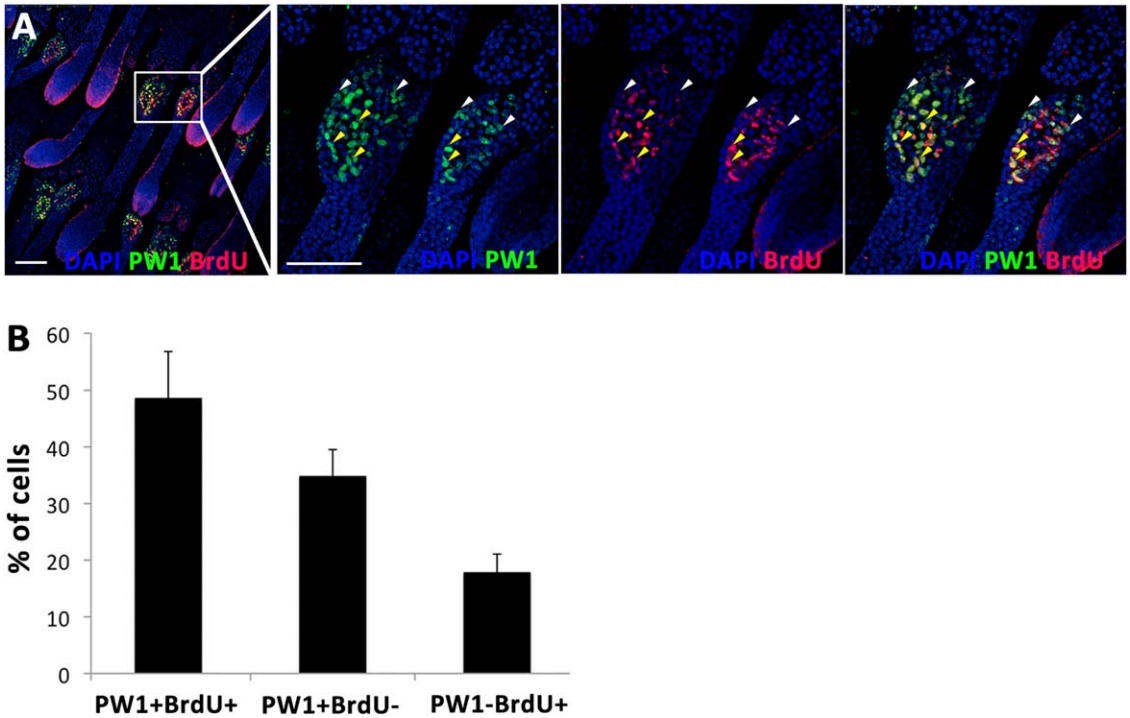

Figure 3. Half of the PW1/reporter expressing cells are label retaining. **(A)**: Photomicrograph of 5-week-old wild-type mouse tail epidermis stained for PW1 and BrdU. White arrows indicate the PW1+ cells. Yellow arrows show the PW1+BrdU+ cells. Nuclei were counterstained with DAPI. **(B)**: Schematic representation of the percentage (%) of cells labeled for PW1 and BrdU in the bulge, 4 weeks following BrdU injection in 10-day-old wild-type mice. 48.5% ± 8.2 of BrdU+ cells express PW1. Data are represented as mean ± SEM from at least three independent experiments. Scale bars: 70 μm.

~50% of the PW1-expressing cells were label retaining and ~82% of LRCs expressed PW1 (Fig. 3A, 3B). Coupled with the observations PW1 is expressed with many stem cell markers and largely overlaps the label retaining population, these observations raise the possibility that PW1 is not restricted completely to stem cells in the skin.

Isolation of the PW1+ Cells from the Bulge

In order to determine the stem cell capacity of all the PW1-expressing populations isolated from the bulge, we used flow cytometry to isolate ß-galactosidase-expressing cells from the bulge of 7- to 8-week-old Tg(*Pw1*^{IRESnLacZ}) mice [16]. Following harvesting of mouse dorsal skin keratinocytes, we performed FACS analysis. Secondary hair germ cells were excluded by eliminating P-Cadherin-expressing cells (PCadh+) (Fig. 4A). The P-Cadherin negative fraction (PCadh−) was then sorted for reporter gene expression (βgal). Since our histological analyses revealed the presence of CD34+/PW1+ and CD34−/PW1+ populations, we used antibodies for α6-integrin and CD34 to identify bulge stem cells as demonstrated previously. [11, 14]. First, we obtained PCadh-βgal+α6int$_{High}$ (PCadh-βgal+) cells that were further analyzed for the expression of CD34 (Fig. 4A). These analyses revealed that ~10% of PCadh-βgal+ cells were CD34 positive (Fig. 4A) whereas ~40% of this population were negative for CD34. We also noted that α6-integrin was detected in both the CD34− and CD34+ cell fractions (Supporting Information Fig. S2A-S2C) with more than 70% and 40% of α6int$_{High}$ cells in the PCadh-βgal + CD34+ (named βgal + CD34+) and PCadh-βgal + CD34− (named βgal + CD34−) fractions, respectively (Supporting Information Fig. S2C). In addition, we also isolated the CD34+α6int$_{High}$ bulge stem cells that did not express PW1 (named βgal^{NEG}CD34+; Fig. 4A).

In order to verify the efficiency of our sorting purification prior to subsequent functional analyses, freshly sorted cells from each fraction were analyzed directly by immunostaining (Fig. 4B) for β-galactosidase, CD34, and Lgr5, and by RT-PCR (Fig. 4C) for P-Cadherin, PW1, CD34, and a6-integrin. Consistent with our histological analysis (Fig. 2H, 2H', 2K), CD34 and Lgr5 expression colocalized with reporter activity in the βgal + CD34+ cells, whereas the expression of these markers was completely absent in the βgal + CD34− and βgal^{NEG}CD34+ cell fractions (Fig. 4B). RT-PCR analysis confirmed the purity of the 3 sorted cell fractions showing a correlation between reporter and PW1 expression (Fig. 4C). All βgal+ bulge cells were negative for *P-Cadherin* but positive for *α6-integrin*, regardless of CD34 expression. *CD34* was completely absent in the βgal + CD34− fraction, but was highly expressed in the βgal + CD34+ and βgal^{NEG}CD34+ bulge cells (Fig. 4C). Further semiquantitative analysis for markers enriched in the bulge showed that *K15*, *Lgr5,* and *Nfatc1* were expressed in the βgal + CD34+ and βgal^{NEG}CD34+ cells whereas they were all absent in the βgal + CD34− fraction (Fig. 4D). These results show that markers commonly used to enrich bulge stem cells are expressed in the βgal + CD34+ fraction. However, we noted that expression levels of a more general stem cell marker, such as *Bmi1,* was similar in all sorted and unsorted cell fractions (Fig. 4D). In order to further verify that other stem cell compartments did not contaminate the bulge populations, expression of *Lrig1* and *Lgr6*, which define stem cell populations in the junctional zone [33] and above the bulge [35],

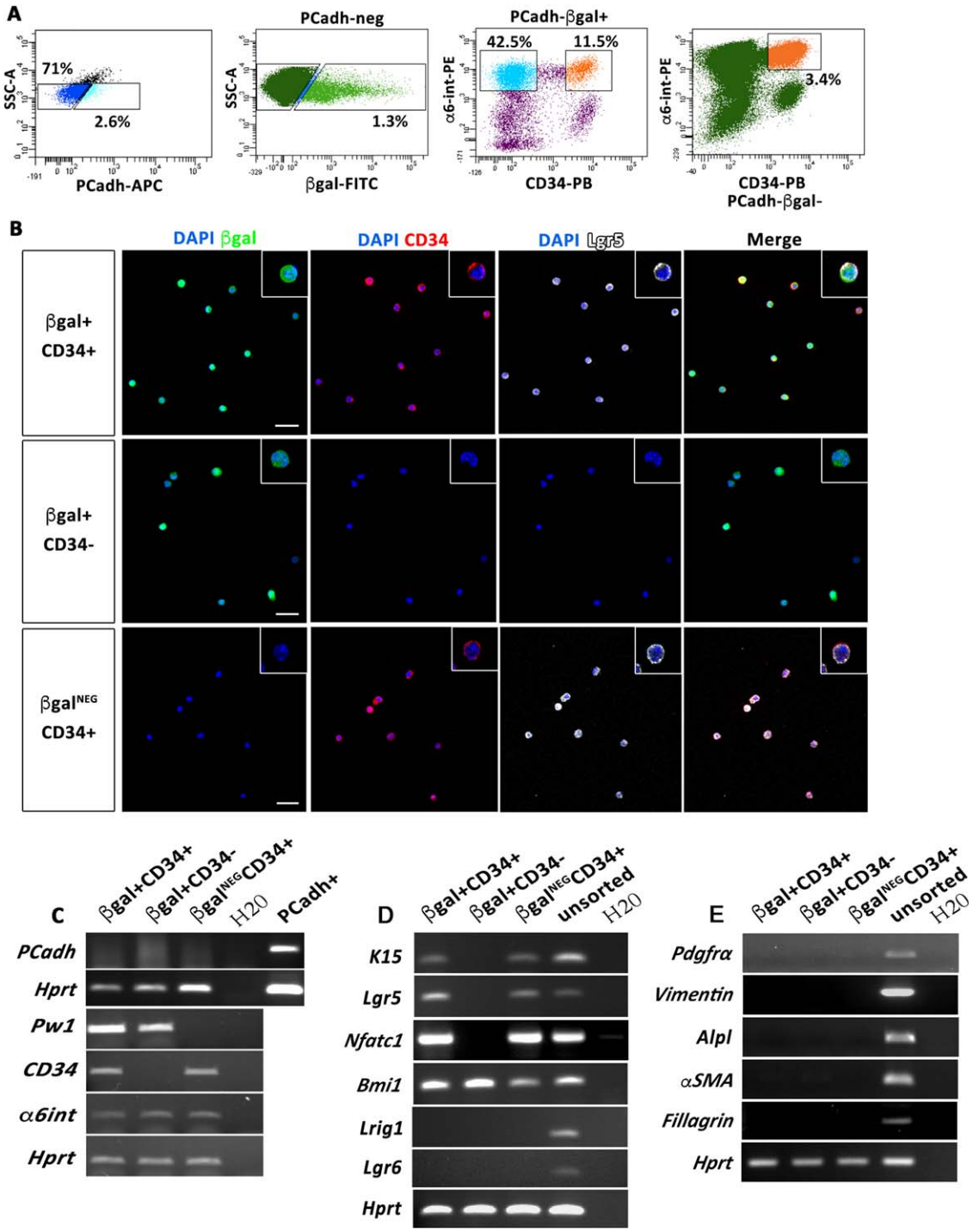

Figure 4. Isolation of a pure population of PW1+ cells from the bulge. **(A)**: Flow cytometric analyses of single cells from 7-week-old Tg(*Pw1*^{IRESnLacZ}) mouse back skin stained for P-Cadherin, β-galactosidase (using FDG treatment), CD34 and α6-integrin. The gates used to isolate Pcadh-βgal+CD34+α6int+ (βgal+CD34+), Pcadh-βgal+CD34-α6int+ (βgal+CD34−) and Pcadh-βgal-CD34+α6int$_{High}$ (βgal^{NEG}CD34+) cells are shown. **(B)**: Representative images of FACS isolated populations immunostained for β-galactosidase (βgal) and bulge stem cell marker (CD34 and Lgr5). Enlargement for one cell is shown for each picture. Scale bars, 20μm. **(C-E)**: RNA levels of each sorted and unsorted fractions collected in (A) were analyzed by semiquantitative PCR: for the expression of *PCadherin*, *Pw1*, *CD34*, *α6-integrin* to assess purity (C), for the expression of enriched markers in the bulge as *K15*, *Lgr5*, and *Nfatc1*, and general stem cell marker as *Bmi1* (D), as well as for the expression of *Lrig1* and *Lgr6* that marks stem cell populations outside the bulge (E). *Vimentin, Alkaline phosphatase, Pdgfrα*, and *Fillagrin* were expressed only in the unsorted fractions confirming the absence of dermal contamination and the undifferentiated state of the cells in both PW1+ fractions, respectively, (E). The PCadh+ fraction was isolated as a positive control for P-Cadherin expression (C) and *Hprt* was used as a constitutively expressed housekeeping gene (C-E).

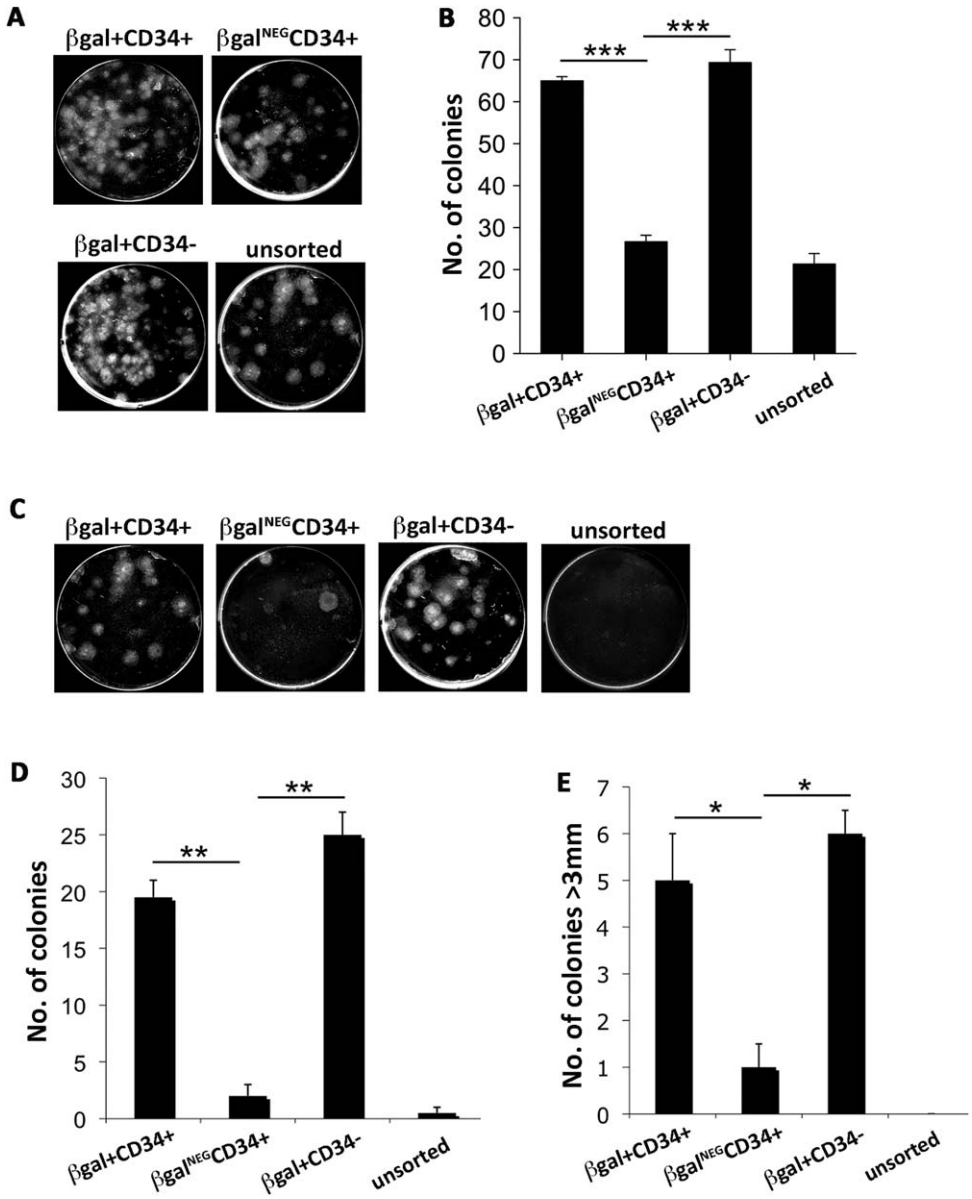

Figure 5. PW1-expressing bulge cells are potent stem cells in vitro regardless of CD34 expression. **(A-E)**: Freshly sorted- (βgal+CD34+, βgal+CD34−, βgal^{NEG}CD34+) and unsorted- epidermal cells were tested for primary (A, B) and secondary (C-E) clonogenicity assay. Quantification of the number of colonies formed by each population in primary (B) and secondary (D, E) clonogenicity assay. Values represent the number of colony ± SEM from at least three independent experiments. *t* test (***, $p < .001$) in (B), *t* test (**, $p < .01$) in (D) and *t* test (*, $p < .05$) in (E).

respectively, were absent in all sorted fractions. We also examined the sorted populations for expression of *Vimentin* and *Alpl*, which are markers of mesenchymal cells, specifically the dermal papilla in the skin (Fig. 4E). We observed *Vimentin* and *Alpl* expression only in the unsorted cells. Expression of dermal sheath marker *α-smooth muscle actin* (α-SMA) and of fibroblast marker platelet derived growth factor receptor alpha (*Pdgfrα*) were also detected only in the unsorted cells (Fig. 4E). Lastly, the absence of *Fillagrin* expression, a marker of terminally differentiated mammalian epidermis, confirmed the undifferentiated state of the sorted populations including the PW1+ cell fractions (Fig. 4E). Consistent with our histological analysis (Fig. 2F-2K), these data confirm that PW1 defines two distinct populations, namely a CD34+ and CD34− subpopulation, in the adult resting bulge.

PW1+ Bulge Cells are Fully Competent Stem Cells In Vitro and In Vivo Regardless of CD34 Expression

Since CD34 expression was shown to be a marker of competent bulge stem cells, we tested whether bgal + CD34− cells possessed stem cell capacity. Bulge stem cells display a robust clonogenic capacity that serves as one accepted in vitro measure of bulge stem cell potential [36]. Therefore, we sorted the PW1-expressing cells from the bulge, as described above into CD34+ and CD34− fractions, plated them at low density, and analyzed for colony formation after 16 days in culture, as previously described [16]. We found that the PW1+ cells were highly clonogenic, forming colonies of similar size regardless of CD34 expression (βgal + CD34+: 65 clones ± 0.7; βgal + CD34−: 69.3 clones ± 2.2) as compared to the

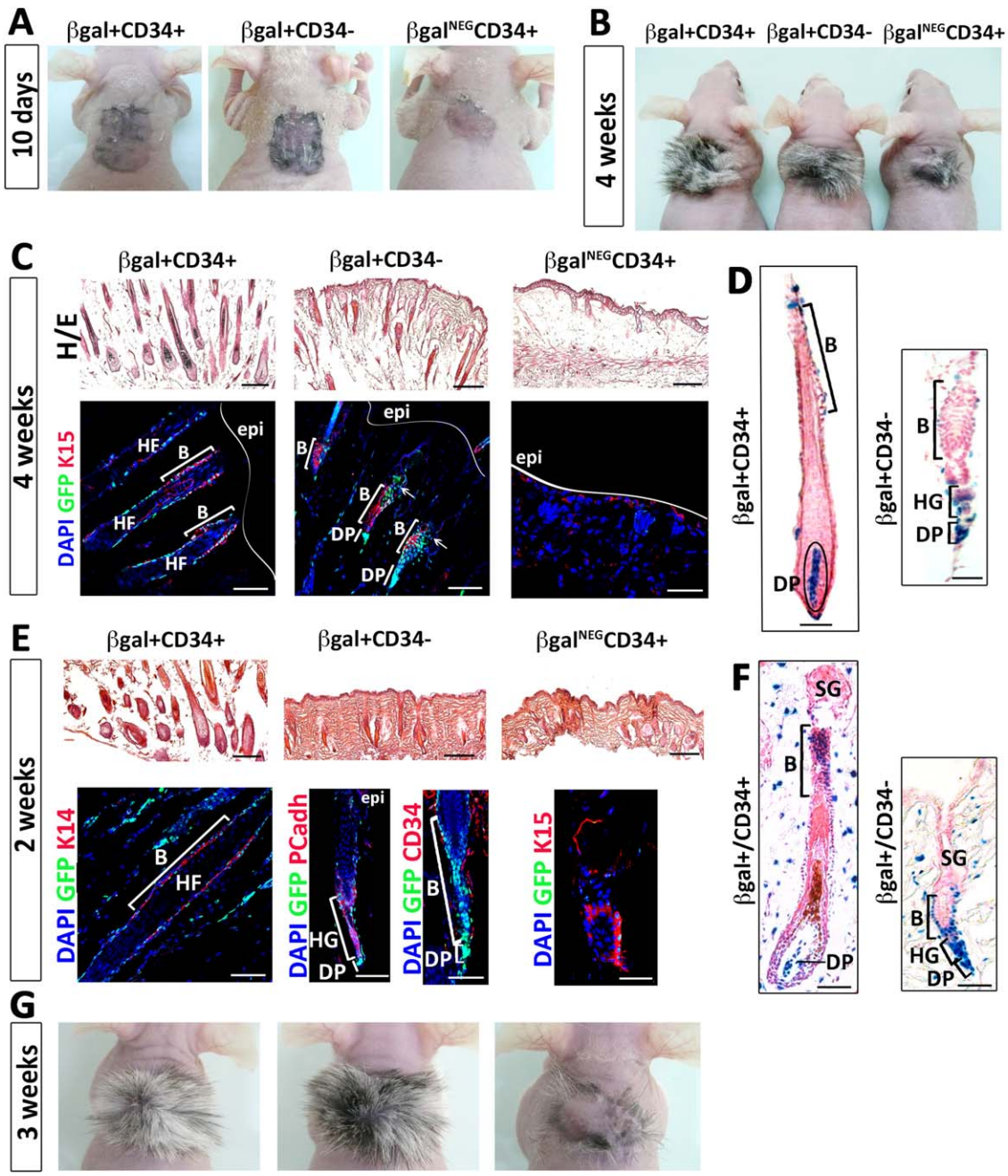

Figure 6. PW1-expressing cells contribute to hair follicle reconstitution regardless of CD34 expression. **(A, B)**: Hair growth resulting from βgal+CD34+, βgal+CD34-, βgal^{NEG}CD34+ cell grafts in 2-month-old nude mice, 10 days (A) and 4 weeks (B) after engraftment. **(C, D)**: Longitudinal sections of graft tissue 4 weeks after engraftment were stained for hematoxylin and eosin (H&E) ([C], upper panels) or X-Gal (D) and immunostained for K15 ([C], lower panels). GFP expression was detected from the H2BGFP+ engrafted cells. **(E, F)**: Two weeks after depilation, longitudinal sections of the grafts were stained for H&E ([E], upper panels) or X-Gal (F) and analyzed for expression of GFP and markers of basal cells (K14, K15), bulge (CD34) and secondary hair germ (Pcadh) ([E], lower panels). **(G)**: Hair regrowth of βgal+CD34+, βgal+CD34−, βgal^{NEG}CD34+ cell grafts 3 weeks after depilation. Only the βgal+ cells derived grafts show robust regeneration. Nuclei are counterstained with DAPI or nuclear fast red. Abbreviations: B, bulge; DP, dermal papilla; Epi, epidermis; HF, hair follicle; HG, secondary hair germ; SG, sebaceous gland. Scale bars: 70 μm (C-F).

βgal^{NEG}CD34+ bulge stem (26.7 clones ± 1.1) and unsorted cells (21.3 clones ± 1.8) (Fig. 5A, 5B). Epidermal stem cells continue to grow after passaging [36]. Therefore, we picked large colonies from primary clones of each sorted population, dissociated, and plated them at the original conditions for 16 days. As shown in Figure 5C, the PW1+ cells consistently formed more colonies that were also markedly larger in size, as compared to controls, regardless of CD34 expression (Fig. 5D, 5E).

We next tested the capacities of the two PW1-expressing populations to reconstitute the epidermal lineages in vivo. We isolated bulge cells from resting hair follicles from the back skin of Tg(*Pw1*^{IRESnLacZ}) mice crossed to mice expressing nuclear GFP driven by the ubiquitous *histone2B promoter* (*H2B-*

eGFP) in order to follow the fate of the engrafted cells, as previously described [28]. We observed that the grafts obtained from PW1-expressing cells displayed robust hair growth regardless of CD34 expression, 10 days and 4 weeks after engraftment (Fig. 6A, 6B). In contrast, we observed that the PW1- derived grafts gave rise to fewer hairs (Fig. 6A, 6B). Histological analysis of GFP expression revealed an 8- to 10-fold higher number of hair follicles derived from PW1-expressing cells as compared to controls, 4 weeks after engraftment (Supporting Information Fig. S3A). Both PW1+ populations gave rise to all the structures of the hair follicles including the bulge, sebaceous gland, and dermal papilla 3 and 4 weeks after engraftment (Fig. 6C, 6D, Supporting Information Fig. S3B). All engrafted cell populations contributed to the epidermis (Supporting Information Fig. S4A). We noted that previous studies using engrafted bulge stem cells have not reported reconstitution of the dermal papilla, but those studies were performed on populations purified on the basis of both α6-integrin and CD34 expression [11, 15, 37]. Therefore, we purified CD34+α6int$_{High}$ bulge stem cells from the epidermis of 7-week-old *H2BeGFP* mice (Supporting Information Fig. S5A) and engrafted them onto the back skin of recipient nude mice. As reported by others [15, 37], we observed hair regrowth (Supporting Information Fig. S5B) 4 weeks after engraftment and histological analysis revealed GFP expression in all compartments of the hair follicles generated from the CD34+α6int$_{High}$ bulge cells, except in the dermal papilla (Supporting Information Fig. S5C). These results confirm that our experimental procedures for epidermal cell preparation and subsequent engraftment were reliable and did not show any dermal contamination. These data, coupled with the observation that the sorted PW1+ cells do not express *Vimentin*, *Alpl*, *Pdgfrα*, and *α-SMA* revealed that the PW1+ bulge cells have a higher degree of cell fate plasticity. In addition, PW1+ cells can also contribute to the dermal papilla. Lastly, our results show that PW1+ bulge cells, regardless of whether or not they express CD34, are fully competent to generate all hair follicle structures. However, while the hair follicles generated from the βgal + CD34+ cells were invariably in anagen as expected, the hair follicles derived from βgal + CD34− fraction had all progressed to catagen when analyzed 4 weeks after engraftment (Fig. 6C, 6D).

PW1+ Bulge Cells Undergo Efficient Self-Renewal

A critical feature of stem cells is their capacity to undergo self-renewal. We tested the self-renewal capacity of the grafts by depilation that triggers a regenerative response (Supporting Information Fig. S6A). Ten days after depilation, we observed new hair growth in PW1+ derived grafts whereas almost no hair growth was observed in the controls (Supporting Information Fig. S6B). Histological analyses confirmed that hair follicles were in anagen at this stage (Supporting Information Fig. S6B). The expression pattern of bulge (K14 and CD34) and secondary germ (PCadh) stem cell markers appeared normal 2 weeks after depilation (Fig. 6E). The GFP+βgal+ cells were restricted to the bulge, secondary germ, and the dermal papilla (Fig. 6E, 6F). Three weeks following depilation, the PW1+ cell-derived grafts showed robust regrowth (Fig. 6G) regardless of CD34 expression, whereas grafts resulting from the control stem cells (βgal^{NEG}CD34+) displayed almost no regeneration (Fig. 6G). Taken together, the PW1+ bulge cells, regardless of CD34 expression, give rise to differentiated epidermal cell fates, reconstitute the hair follicle stem cell niche, and self-renew in response to depilation.

Only PW1-Expressing Bulge Cells are Activated During Normal Hair Follicle Turnover and in Response to Injury

We have observed that the CD34+ and CD34− PW1-expressing cells displayed a difference in hair cycle progression, we therefore compared the cell cycle profiles for each population sorted in Figure 4A, in telogen (7 weeks; Fig. 7A, 7B), at telogen-anagen transition (hair follicle turnover at 10 weeks; Fig. 7D, 7E), and 48 hours after depilation (7 weeks; Fig. 7F, 7G) when the bulge cells start to proliferate [1, 30]. During telogen, when the bulge cells are expected to be in G0/G1, we noted that ∼98% of all the populations examined were noncycling (Fig. 7A, 7B). In contrast, we observed a small but consistent low level of cycling in the βgal + CD34− cells (Fig. 7A, 7B). We therefore analyzed regulatory cell cycle genes by semi-quantitative PCR for each population (Fig. 7C). We observed a low level of *Ki67* expression in βgal + CD34- cells purified from the telogen bulge concomitant with a decrease of the cyclin-dependent kinase inhibitor genes *p16* and *p57* as compared to the CD34+ cells (βgal + CD34+ and βgal^{NEG}CD34+ bulge cells). Consistent with a downregulation of *CyclinD1* expression in the purified βgal + CD34− cells, we found that the cyclin dependent kinase 4 (*CDK4*) expression level was markedly increased whereas *p21* expression was similar in both populations. (Fig. 7C). These variations cell cycle gene expression are consistent with the different cell cycle kinetics observed between the βgal + CD34− and βgal + CD34+ cell populations. A difference in cell cycle status was also detected during the telogen-anagen transition, in which the PW1+/CD34− stem cells were 5-6-fold higher in the G2/M. However, we noted that whereas the expression of CD34 is coupled with a different cell cycle behavior during the normal anagen induction (i.e., hair follicle turnover), we did not see any differences between the two populations in response to depilation during which both CD34+ and CD34− cells are equally present in all cell cycle phases (Fig. 7D-7G) thus, the functional significance of these cell cycle differences remains unclear.

We next wished to determine whether the PW1-expressing and nonexpressing bulge cells differed in their cell cycle behaviors. Consistent with the observation that PW1- bulge cells provide a low contribution to hair follicle structures following engraftment (Fig. 6), we found that only PW1-expressing cells are activated into the cell cycle in response to injury and normal hair follicle turnover (Fig. 7D-7G). Taken together, these results demonstrate that PW1+ bulge stem cells participate in the maintenance and regeneration of the hair follicle whereas the CD34+α6int$_{High}$ expressing cells that do not express PW1 are less competent.

Discussion

Pw1 is a parentally imprinted gene that plays a role in mediating cell stress responses apoptosis [22, 23, 38, 39]. Using a reporter mouse model for PW1, we recently demonstrated

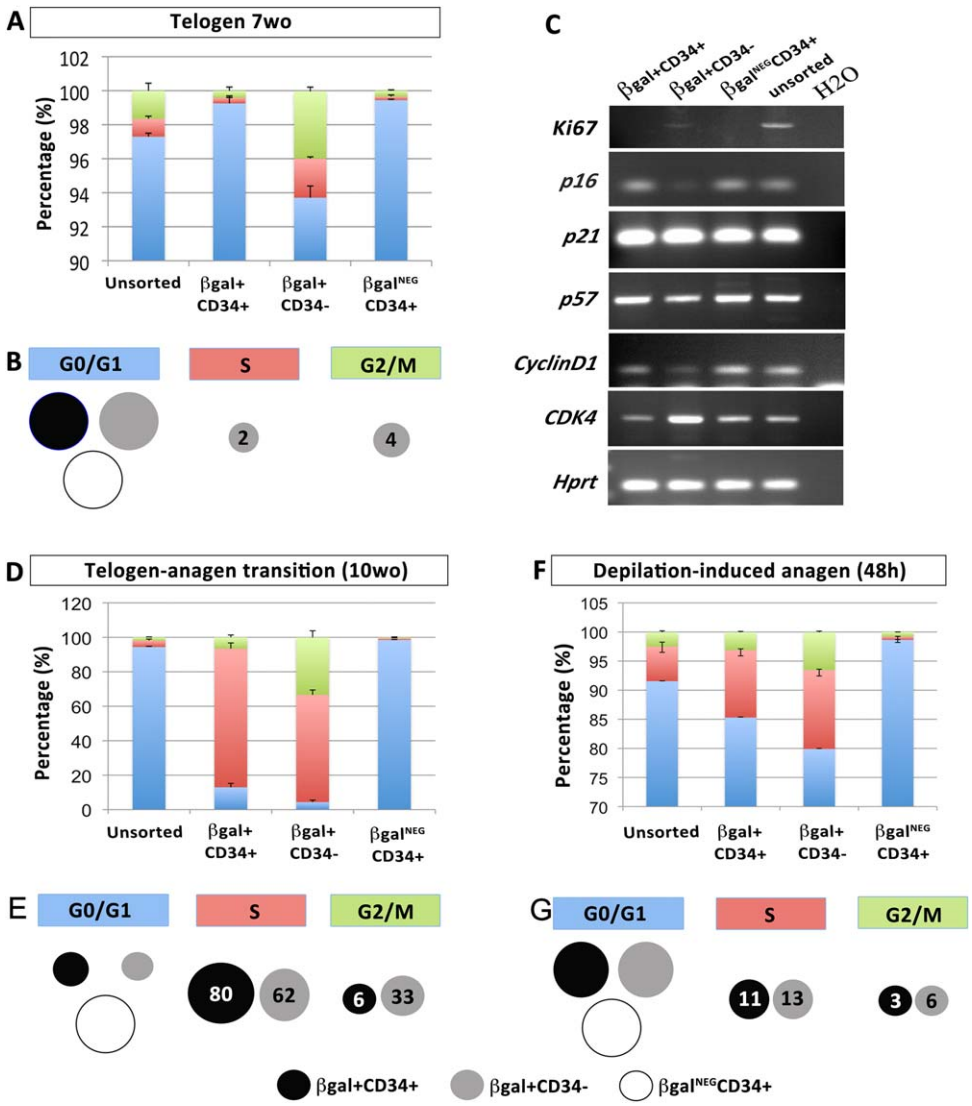

Figure 7. Only PW1 expressing bulge cells are activated during normal hair follicle turnover and in response to injury. **(A, D-G)**: Schematic representations of the percentage (%) of freshly sorted- (βgal+CD34+, βgal+CD34−, $βgal^{NEG}$CD34+) and unsorted- epidermal cells in G0/G1 (blue), S (red), and G2/M (green) phases of the cell cycle in three distinct conditions: telogen (7 week old; [A, B]), at telogen-anagen transition (hair follicle turnover at 10 week old; [D, E]), and 48 hours after depilation-induced anagen; (F, G). The mean percentage of cells is indicated in the center of the white ($βgal^{NEG}$CD34+), gray (βgal+CD34−), and black (βgal+CD34+) circles representing the populations of cells in G0/G1, S and G2/M phases (B, E, G). Values represent the mean percentage of cells ± SEM from at least three independent experiments. **(C)**: RNA levels of each sorted and unsorted fractions collected in (A) were analyzed by semiquantitative PCR for the expression of *Ki67* and the cyclin dependent kinase inhibitor genes including *p16, p21, p57,* the *cyclin D1 and the CDK4* genes. *Hprt* was used as a constitutively expressed housekeeping gene. Abbreviation: *CDK4,* cyclin dependent kinase 4.

that PW1 is expressed in adult stem cell niches in many tissues including the CNS, blood, bone, intestine, skin, and skeletal muscle [17]. Our recent analyses of skeletal muscle revealed that PW1 expression is present in the major muscle progenitor cells (satellite cells) as well as fibroadipogenic progenitors and a novel interstitial population of myogenic cells [40]. Parentally imprinted genes are implicated in the regulation of perinatal growth and control directly or indirectly cell proliferation in multiple tissues, [41–43]. Deletion of the *Pw1*-imprinting domain causes a global impact on growth rates although the mechanisms of action were not elucidated [44]. In addition, at least 10 parentally imprinted genes including PW1 are highly represented in a variety of adult stem cell populations [45]. In this study, we focused upon the bulge stem cell niche of the skin, which has been a topic of intense study in recent years [46, 47]. Using a reporter mouse for PW1, we showed that PW1 is expressed in the bulge and that PW1-expressing cells obtained from the bulge displayed all the hallmarks expected for bulge stem cells [17]; however, we did not fully explore the relationship between PW1-expressing cells in the bulge and previously described bulge stem cell populations or how PW1 was expressed during the establishment of the bulge.

Conclusion

We report here that PW1 is expressed in the skin throughout the entirety of perinatal and postnatal life. During embryonic development, we observed that PW1 is expressed and colocalizes with Sox9-expressing cells. Sox9 is a marker of the early

LRCs that are the direct precursors of the LRCs, which ultimately reside in the adult bulge [20]. While Sox9 and PW1 colocalize completely at E15.5, corresponding to a stage, during which stem cell identity is determined, we observed that the level of coexpression decreases to ~60% by E18.5 thereby providing the first sign that PW1 expression marks 2 distinct populations. We noted that several adult bulge markers including CD34 are first detected at later stages of hair follicle development [11, 14, 15, 34], placing the initiation of PW1 expression and Sox9 as the earliest markers corresponding to the establishment of a quiescent stem cell population in the bulge. In addition, CD34 in combination with α6-integrin have been widely used as markers for identifying and purifying adult bulge stem cells [11, 15]. We report here that PW1 expressing cells isolated from the bulge represent a larger population of cells including cells that do not express CD34. While the precise role of the PW1+/CD34− cells remains unclear, we demonstrate that these cells are highly clonogenic and capable of giving rise to all the cell fates of CD34+ bulge cells following engraftment. However, we noted that these cells have a different inherent cell cycle behavior such that they are less quiescent and respond more rapidly to the telogen-anagen transition in the adult. Previous studies have shown that there is a small number of cells present in the bulge that do not express CD34 [11, 14]. Whereas the CD34+/α6int$_{High}$ bulge stem cell population represents 7%-8% of the epidermal cells [14], PW1+ cells represent only 0.6% of the epidermal cell population [17]. Combined with the absence of other markers to detect the CD34− bulge population (i.e., 0.3%), it was impossible until now to neither track these cells nor determine their stem cell potential. In conclusion, PW1 expression is restricted to compartments identified to contain stem/progenitor cells [1, 11, 14–16, 48] and defines a novel population of cells that had previously escaped identification.

We show here that PW1 labels both the bulge and the dermal papilla. Recent studies reported that Prominin 1 is also expressed in both compartments [49, 50], so possible common markers could be used to label cells in the bulge and the dermal papilla. We report here that both the CD34-expressing and nonexpressing populations of PW1 cells are able to repopulate the dermal papilla. To date, while it has been proposed that the dermal papilla is more representative of a mesodermal lineage [51, 52], no definitive proof has emerged to support this hypothesis. We can rule out that our preparations of PW1-expressing bulge cells were contaminated by dermal components as well as cells from dermal papilla based upon markers used to exclude the dermal papilla upon sorting and PCR-based verification of gene expression of each cell fraction. While the dermal papilla cells do not directly contribute to new skin cells during regeneration, this population has nonetheless been demonstrated by others to possess a robust stem cell capacity comparable to cells isolated from the bulge [48, 53]. Insomuch that PW1 expression appears to be a robust maker of stem cells in multiple tissues from multiple germline origins (i.e., mesoderm, ectoderm, endoderm; [17]), these data suggest that the bulge and dermal papilla may play a more interchangeable role than previously anticipated.

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Author Contributions

V.B. and S.K.: Performed the experiments; V.B., S.K., G.M., and D.S.: Analyzed the results and wrote the manuscript. P.J. and S.A.B.: Performed the whole mount immunofluorescence on mouse tails.

Disclosure of Potential Conflicts of Interest

A patent has been filed for the Tg(*Pw1*^{IRESnLacZ}) mouse model but it is available to any and all academic laboratories for noncommercial use through Jackson Laboratories. The authors indicate no competing financial interests.

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