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PTEN Mediates Activation of Core Clock Protein BMAL1 and Accumulation of Epidermal Stem Cells

Chiara Zagni,[1,5] Luciana O. Almeida,[1,5] Tarek Balan,[3] Marco T. Martins,[1] Luciana K. Rosselli-Murai,[1] Petros Papagerakis,[3,4] Rogerio M. Castilho,[1,2] and Cristiane H. Squarize[1,2,*]

[1]Laboratory of Epithelial Biology, Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor, MI 48109-1078, USA
[2]Comprehensive Cancer Center, University of Michigan, Ann Arbor, MI 48109, USA
[3]OPD, University of Michigan School of Dentistry, Ann Arbor, MI 48109-1078, USA
[4]Center for Organogenesis, University of Michigan, Ann Arbor, MI 48109-2200, USA
[5]Co-first author
*Correspondence: csquariz@umich.edu
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SUMMARY

Tissue integrity requires constant maintenance of a quiescent, yet responsive, population of stem cells. In the skin, hair follicle stem cells (HFSCs) that reside within the bulge maintain tissue homeostasis in response to activating cues that occur with each new hair cycle or upon injury. We found that PTEN, a major regulator of the PI3K-AKT pathway, controlled HFSC number and size in the bulge and maintained genomically stable pluripotent cells. This regulatory function is central for HFSC quiescence, where PTEN-deficiency phenotype is in part regulated by BMAL1. Furthermore, *PTEN* ablation led to downregulation of BMI-1, a critical regulator of adult stem cell self-renewal, and elevated senescence, suggesting the presence of a protective system that prevents transformation. We found that short- and long-term PTEN depletion followed by activated BMAL1, a core clock protein, contributed to accumulation of HFSC.

INTRODUCTION

Epidermal stem cells (SCs) are localized in several well-defined cellular compartments with the best-studied being an anatomical area of the hair follicle (HF) known as "bulge" (Blanpain and Fuchs, 2009; Cotsarelis et al., 1990; Jaks et al., 2010). This unique anatomical site maintains SCs in a quiescent stage that is only interrupted by growth signals during new hair cycles or following tissue injury (Alonso and Fuchs, 2003; Morris et al., 2004). In either case, SCs exit the quiescent phase by migrating out of the bulge to initiate a new hair cycle or to repopulate a wounded site (Ito et al., 2007; Langton et al., 2008; Mascré et al., 2012). Therefore, tissue integrity requires the maintenance of a quiescent, yet responsive, population of SCs. New insights into the mechanism by which hair follicle stem cells (HFSCs) remain quiescent was revealed with the identification of NFATc1, a protein that controls cell-cycle progression (Horsley et al., 2008), the canonical BMP (bone morphogenetic protein) pathway, and, most recently, the core clock protein BMAL1 (Botchkarev and Sharov, 2004; Andl et al., 2004; Kobielak et al., 2003, 2007; Plikus et al., 2008; Lin et al., 2009; Janich et al., 2011). Tight control of SC quiescence ensures a sufficient supply of SCs for prolonged maintenance of tissue homeostasis and prevents tumor development by reducing DNA mutations caused by endogenous stress (Mohrin et al., 2010).

As powerful controllers of the cell cycle, tumor-suppressor genes may have an important role in the maintenance of SC quiescence (Sherr, 2004). Increasing evidence suggests that the PTEN (phosphatase and tensin homolog) tumor suppressor controls SC function in distinct tissue types, including the hematopoietic and mammary systems (Lee et al., 2010). Specifically, *Pten* controls hematopoietic SCs by preventing their release from the bone marrow niche, thereby reducing cell proliferation, self-renewal capacity, and the development of myeloproliferative disease (Zhang et al., 2006). In addition, reduced PTEN levels enrich normal and malignant human mammary SCs by activating the Wnt/β-catenin signaling pathway (Korkaya et al., 2009). Therefore, *PTEN* may control SC function in multiple organs and systems. We and others have previously reported that compromised *Pten* function contributes to epithelial transformation and tumor progression upon oncogenic stimulus (Squarize et al., 2008, 2013; Suzuki et al., 2003; Backman et al., 2004; Segrelles et al., 2014; White et al., 2014). These studies, using targeted deletion of *Pten* in the epidermis, suggest a broader regulatory role for PTEN in skin homeostasis and potentially in HFSC maintenance. Indeed, the role of PTEN in the biology of HFSCs remains poorly understood.

In this report, we investigated the role of *Pten* deficiency in HFSC maintenance and skin homeostasis. We found that monoallelic and biallelic excision of *Pten* led to delayed HF cycle. *Pten* deficiency induced uncontrolled accumulation of HFSC, as evidenced by an enlarged SC niche and increased number of SCs. We further observed that deregulation of *Pten* caused constitutive activation of the core circadian molecule BMAL1. Furthermore,

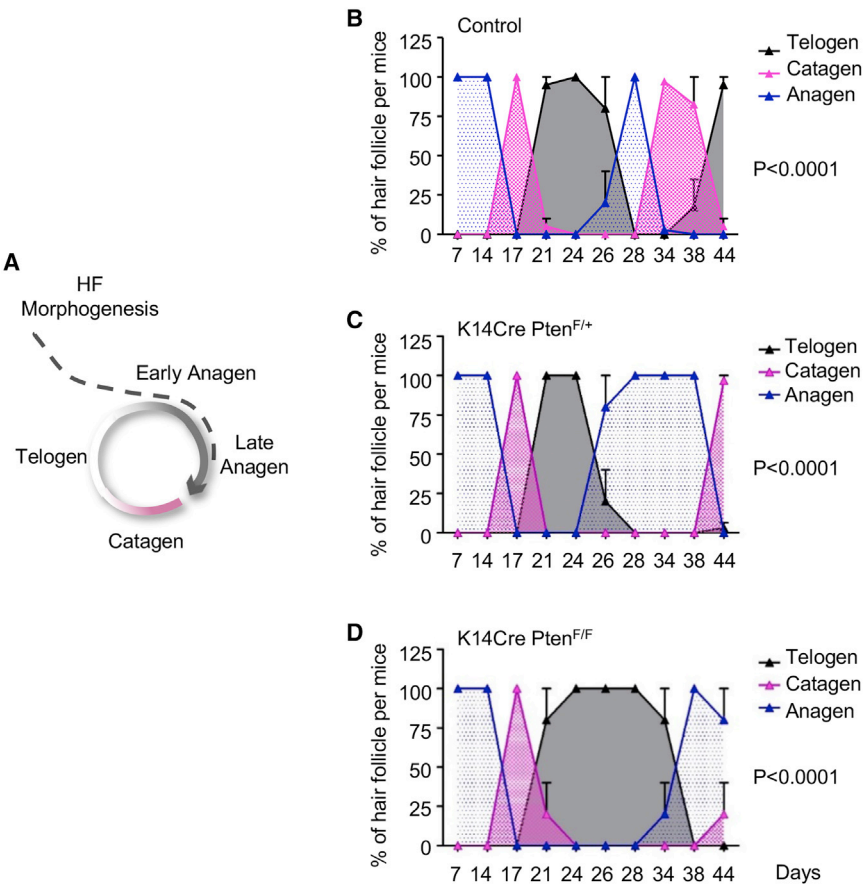

Figure 1. Lack of PTEN Disrupts the Synchronous Hair Follicle Cycle and Prolongs the Resting Phase

(A) Cyclic cycles during hair follicle (HF) morphogenesis comprise early and late anagen, catagen, and telogen phases.

(B–D) Graphic representation of the histological analyses of the synchronous hair cycle phases. As represented in control animals (B), the hair cycle follows a cyclic pattern in which the growth phase (anagen, blue), the involution phase (catagen, pink), and the resting phase (telogen, gray) are shown paired with the animals' age (days). (C) K14Cre-Pten$^{F/+}$ mice display a prolonged anagen in the second hair cycle. (D) K14Cre-Pten$^{F/F}$ mice have longer telogen phase. Data are presented as mean percentage ± SEM; n = 5 mice for each genotype.

loss-of-function assays performed in vivo revealed that expression of BMAL1 participates in the maintenance of the *Pten*-induced phenotype.

RESULTS

PTEN Regulates HF Cycling and Quiescence

To evaluate the biological effect of PTEN depletion in HSFCs and skin homeostasis in vivo, we generated epithelial-specific *Pten* conditional knockout mice by crossing mice harboring a floxed *Pten* allele (*Pten*^{tm1Hwu} or Pten$^{F/F}$) with mice that express the Cre recombinase driven by the K14 promoter (K14Cre) (Squarize et al., 2010). SCs at the bulge have alternating periods of dormancy and activation, which are reflected in the HF cycling (Fuchs, 2009; Nowak et al., 2008). The first two HF cycles are known to follow a fairly synchronous schedule in control mice (Paus et al., 1999a, 1999b). Each hair cycle comprises growth (anagen), regression (catagen), and quiescent (telogen) phases (Figure 1A), which are well characterized in wild-type mice (Figures 1B and S1A; Dry, 1926; Müller-Röver et al., 2001; Paus et al., 1999a, 1999b). In telogen, the SCs remain dormant (quiescent) as they wait for growth signals and progression to the anagen phase (Morris et al., 2004). First, we analyzed the abnormalities on the hair cycle and histomorphometry, which are strongly indicative of altered SC function. We found that single allele deletion of *Pten* (K14Cre-Pten$^{F/+}$) resulted in an extended anagen phase during the second hair cycle (Figures 1C and S1A). The alteration of the hair cycle upon PTEN deletion was enhanced with the biallelic excision of *Pten* (K14Cre-Pten$^{F/F}$), which resulted in extended telogen or quiescent phase (Figures 1D and S1A). These findings indicate that PTEN plays an important role in the hair cycle, which suggests a critical role in the control of SC function and quiescence. Additional histological analysis of the skin revealed that epithelial cell from K14Cre-Pten$^{F/F}$ mice displayed increased enlargement of the (interfollicular) basal cell layer, which is the cell layer harboring the interfollicular SCs, as well as increased skin keratinization (Figure S1B). Signs of cellular differentiation of HF cells (CK10 expression) were not different from the ones observed in control mice (Figure S1C). Nevertheless, the CK6 positive outer root sheath (ORS) cells were enlarged (Figure S1D), further suggesting the accumulation of SCs given that the HFSC

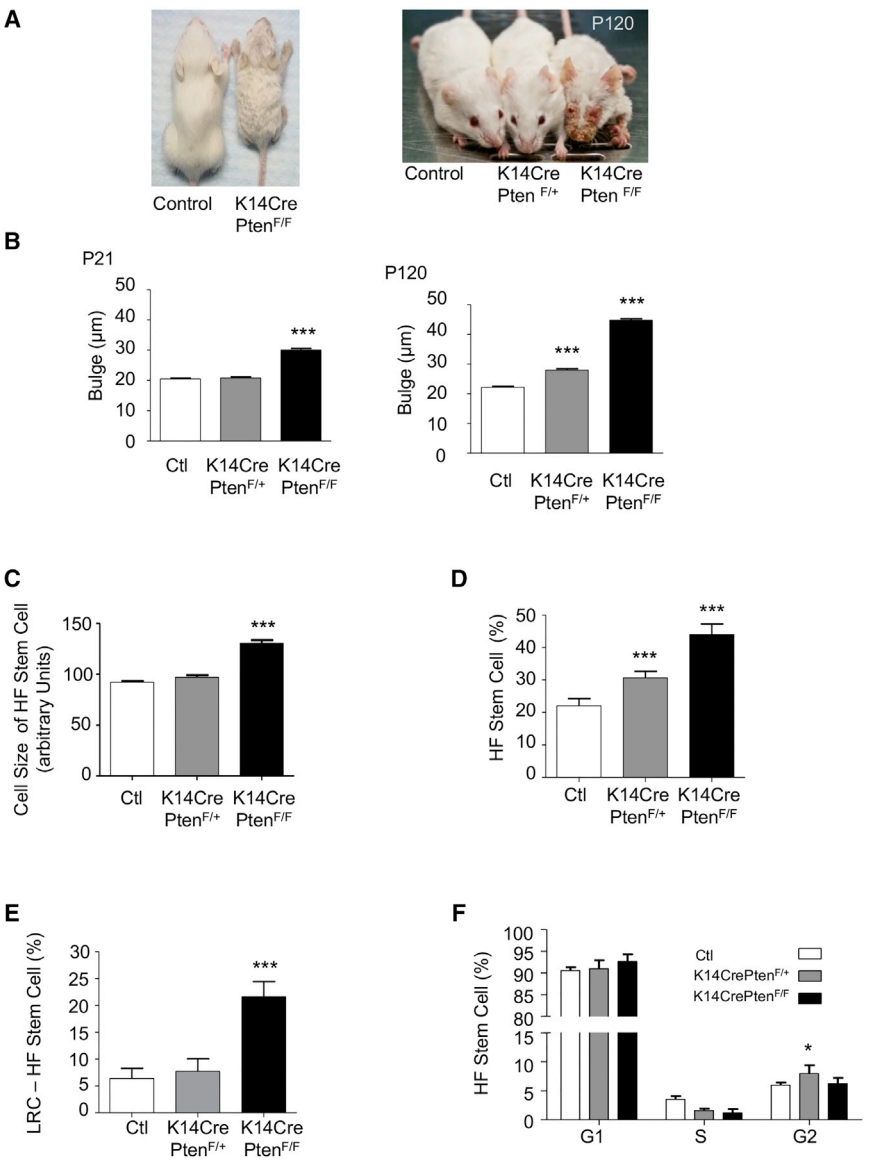

Figure 2. PTEN Ablation in the Epidermis Leads to Enlargement of the HFSC Niche and Accumulation of HFSCs

(A) Representative examples of K14Cre-Pten$^{F/F}$ mice displaying fur changes (disheveled appearance) from P12 through the remainder of their lives (n = 10 mice per genotype). As age progresses, K14Cre-Pten$^{F/F}$ mice display cutaneous changes, particularly in the vibrissae areas and eyelids as seen at P120.

(B) Bulge measurements for each phenotype (mean ± SEM; n = 20 for each genotype). ***p < 0.001.

(C) The cell size of HFSCs was calculated using FACS analysis (mean ± SEM, n = 10^4 cells per genotype). ***p < 0.001.

(D) Quantification of the number of HFSCs using FACS analysis. Note the increased number of stem cells in the K14Cre-Pten$^{F/+}$ and K14Cre-Pten$^{F/F}$ mice (mean percentage ± SEM, n = 10^4 cells per genotype). ***p < 0.001.

(E) Percentage of bromodeoxyuridine-label-retaining cells (LRC) in the hair follicle (HF) calculated by FACS analysis. Note that SC from K14Cre-Pten$^{F/F}$ mice had most LRC, indicating quiescent stem cells (mean percentage ± SEM, n = 10^4 cells per genotype). ***p < 0.001.

(F) FACS analysis of HFSCs showing the percentage of cells in G_1, S, and G_2 cell-cycle phases. Note that most stem cells are in G_1 arrest, especially the ones from K14Cre-Pten$^{F/F}$ mice. K14Cre-Pten$^{F/+}$ and K14Cre-Pten$^{F/F}$ displayed decreased DNA synthesis (S), although some stem cells from K14Cre-Pten$^{F/+}$ mice progressed to G_2 (mean percentage ± SEM, n = 10^4 cells per genotype). *p < 0.05.

niche is a specialized portion of the upper part of the ORS (Lavker et al., 2003). Independent of the PTEN status, the SCs were positive to the SC regulators β-catenin and pSMAD (Figures S1E and S1F). Altogether, our findings corroborate with the emerging concept that PTEN is involved in the SC function and hair cycle.

PTEN Deficiency Induces Expansion of the SC Niche and Increased Number of Quiescent SCs

We next analyzed the impact of *Pten* deficiency on skin homeostasis. Gene excision using the K14Cre-promoter in the follicular and interfollicular skin compartments that comprise the SC niche is well established (Nowak et al., 2008; Castilho et al., 2007; Merrill et al., 2001). To ensure the specificity of our findings, we used fluorescence-activated cell sorting (FACS) to identify the HFSCs (CD34$^+$α6-positive cells) (Trempus et al., 2003; Blanpain et al., 2004). Although newborn K14Cre-Pten$^{F/F}$ mice were mostly indistinguishable from control mice at birth, they had delayed hair coat growth, reduced body size, wrinkled flaky skin, and disheveled hair coat throughout their lifetimes (Figure 2A; postnatal day 21 [P21]). *Pten* mice also developed multiple epidermal alterations as they aged (Figure 2A; P120). Changes in the skin compartments were also accompanied by the enlargement of HFSC niche (Figure 2B; P21, p < 0.001). As the mice aged, bulges from K14Cre-Pten$^{F/+}$ (mean 27.94 ± 2.3 μm) and K14Cre-Pten$^{F/F}$ mice (mean 44.78 ± 2.27 μm) continued to expand

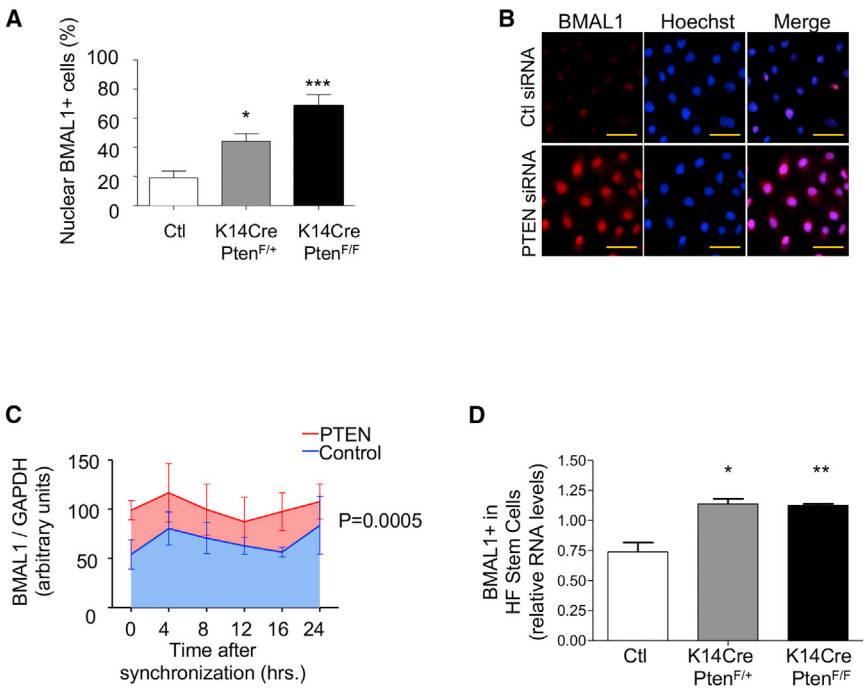

Figure 3. Loss of PTEN Induces Accumulation of Nuclear BMAL1 in the HFSCs

(A) Graphic shows the percentage of cells with active nuclear BMAL1 from K14Cre-Pten$^{F/+}$, K14Cre-Pten$^{F/F}$, and control mice (mean percentage ± SEM, n = 4 fields per three mice per genotype). *p < 0.05, ***p < 0.001.

(B) BMAL1 is activated and localized in the nucleus of PTEN-deficient human keratinocytes (NOK-SI cells). BMAL1 is activated in the nucleus of human keratinocytes following PTEN knockdown. Scale bars, 50 μm.

(C) Clock machinery follows a cyclic pattern. Densitometric analysis of protein bands on blots image of BMAL1 and GAPDH (control) from synchronized keratinocyte with siRNA control and siRNA for *PTEN*. Mean ± SEM, independent experiments in triplicates.

(D) Increased mRNA expression of *Bmail1* in HFSCs (isolated from mice using FACS sorting) (mean ± SEM; n = 6 per genotype). *p < 0.05; **p < 0.01.

(Figure 2B, p < 0.001). Collectively, these results show a continuous enlargement of the SC niche following PTEN excision. The niche expansion was a result of increasing SC number and size (Figures 2C and 2D). The SCs presenting PTEN biallelic excision became quiescent, as indicated by the increased numbers of label-retaining cells (LRCs) (Figure 2E) and delayed hair regrowth (Figures S2A and S2B, p < 0.01 and p < 0.001). The SCs from K14Cre-Pten$^{F/F}$ mice were mostly in G_1 arrest while SC with single allele deletion of *Pten* progressed to G_2 phase (Figure 2F).

Compromised *Pten* Function Results in the Accumulation of the Core Clock Protein BMAL1

Following the recent discovery that HFSC heterogeneity and quiescence may be affected by clock genes (Lin et al., 2009; Janich et al., 2011; Paus et al., 1999a, 1999b; Paus and Foitzik, 2004; Tanioka et al., 2009), we investigated whether the phenotype observed in *Pten*-deficient mice was associated with alterations in clock genes. We examined BMAL1 expression in the SCs and the follicular and interfollicular epidermis of *Pten* conditional knockout mice. Skin samples from control mice showed reduced nuclear localization of active BMAL1. K14Cre-Pten$^{F/+}$ and K14Cre-Pten$^{F/F}$ mice showed significant nuclear accumulation of BMAL1 (Figures 3A and S3A). To better characterize the *Pten* control on BMAL1, we used small interfering RNA (siRNA) technology to downregulate *PTEN* in human epithelial cells. Downregulation of *PTEN* resulted in nuclear accumulation of BMAL1 and increased levels of pAKT^{Ser473} (Figures 3B, S3B, and S3C; p < 0001). Notably, *PTEN* knockdown gene positively regulates BMAL1 expression in the epidermal cells, which maintained a cycling pattern (Figure 3C). Most importantly, *Bmal1* upregulation was confirmed in HFSCs of K14Cre-Pten$^{F/+}$ and K14Cre-Pten$^{F/F}$ mice (Figure 3D). We also analyzed whether upregulation of *Bmal1* affects the SCs. PER2 is a core negative transcriptional inhibitor of the circadian clock and controls BMAL1 and CLOCK (Fu et al., 2002). mPer2 knockout mice have constitutively active BMAL1 (Figure S3D). Depletion of Per2 from the epidermis resulted in the accumulation of HSFCs compared with control littermates (Figure S3E).

Pten-Deficient Mice Develop Small Wart-Like Lesion, i.e., Hamartoma, Characterized by Overexpression of BMAL1 and Activation of Cellular Senescence

Complete *Pten* ablation from the proliferative compartment of the epidermis resulted in increased wart formation on the face and limbs of mice (Figure 4A), in line with our previous report (Squarize et al., 2008). In particular, *Pten* was also associated with the development of progressive acanthosis of the epidermis (thickening of the skin) (Figure 4A) and the development of benign warts in aging mice (Figure 4B). Interestingly, benign warts (hamartomas) that originated on the skin of *Pten*-deficient mice were self-limited and the cells resembled the normal cellular component of the HF, including the sebaceous glands, ORS, and inner root sheath (Figures 4B and 4C).

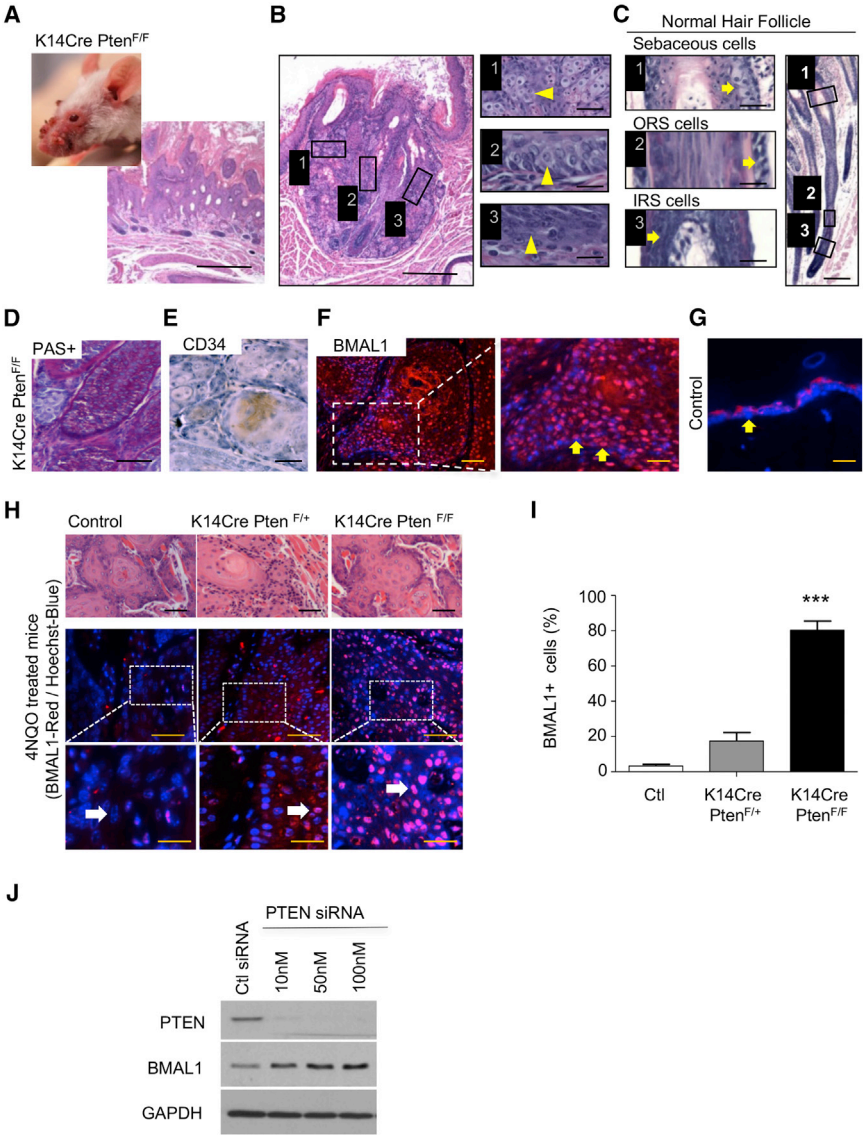

Figure 4. Sole Long-Term PTEN Depletion and Senescent Hamartomas

(A) Images of K14Cre-Pten$^{F/F}$ mice showing cutaneous warts on the face. H&E sections depicting epithelial acanthosis (larger spinous layer) and hyperkeratosis. Scale bar, 200 μm.

(B) Hamartomas are composed of cell types (yellow arrowheads) that closely resemble the internal structures of a normal hair follicle; yellow arrows, showing (1) sebaceous glands, (2) outer root sheath (ORS), and (3) inner root sheath (IRS). Scale bar, 200 μm; scale bars in insets, 25 μm.

(C) The normal hair follicle structures (from control mice) are composed of many epithelial layers and specialized cells including (1) sebaceous cells, (2) outer root sheath (ORS) cells, and (3) inner root sheath (IRS) cells that are also present in tumors (yellow arrows). Scale bar, 200 μm; scale bars in insets, 25 μm.

(D) Periodic acid-Schiff (PAS) staining depicting cells from hair follicle-derived ORS cells. Scale bar, 25 μm.

(E) Immunohistochemistry shows positive staining for the CD34 stem cell marker. Scale bar, 25 μm.

(F and G) Anti-BMAL1 staining of lesions from PTEN-deficient mice (F) showing numerous strong nuclear localizations (yellow arrows) compared with control epithelium (G). Scale bars, 100 μm; scale bar in inset, 25 μm.

(H–J) Chemically induced epithelial tumors with 4NQO (carcinomas) on PTEN-deficiency mice (H, I) and human samples (J) have increased expression of BMAL1. White arrows show nucleus in blue and BMAL1 in red. Scale bars in (H), 200 μm and (insets) 25 μm. Data in (I) represent mean percentage ± SEM, n = 4 per genotype; ***p < 0.001.

Remarkably, even after long-term follow-up, malignant transformation of epidermal cells was not detected in our PTEN-deficient mice. As seen in Figure S4, *Pten* disruption on the skin activates cellular senescence, a biological process that enables critical protection against malignant conversion. Senescence was identified by well-established hallmarks of cellular senescence (Castilho et al., 2009; Liu et al., 2007; Dimri et al., 1995), including endogenous β-galactosidase (SA-β-Gal) activity at pH 6, accumulation of nuclear γ-H2AX, and p16^{ink4} (Figures S4A–S4C). Additionally mice develop trichilemmomas, which are also seen in patients with PTEN deficiency (Squarize et al., 2008). These benign lesions of the HF are characterized by cells derived from the ORS and by positive periodic acid-Schiff staining (Figure 4D). Notably, trichilemmomas are highly enriched with cells that express the CD34 SC marker, as observed in the skin from our animal model (Figure 4E). Human trichilemmomas also express CD34 (Sanders and Carr, 2007). These results indicate that alterations derived following complete ablation of *PTEN* have many of the morphological characteristics and molecular signatures of HFs and their SCs. The lesions derived from K14Cre-Pten$^{F/F}$ mice retained high levels of BMAL1 (Figure 4F) compared with control littermates (Figure 4G),

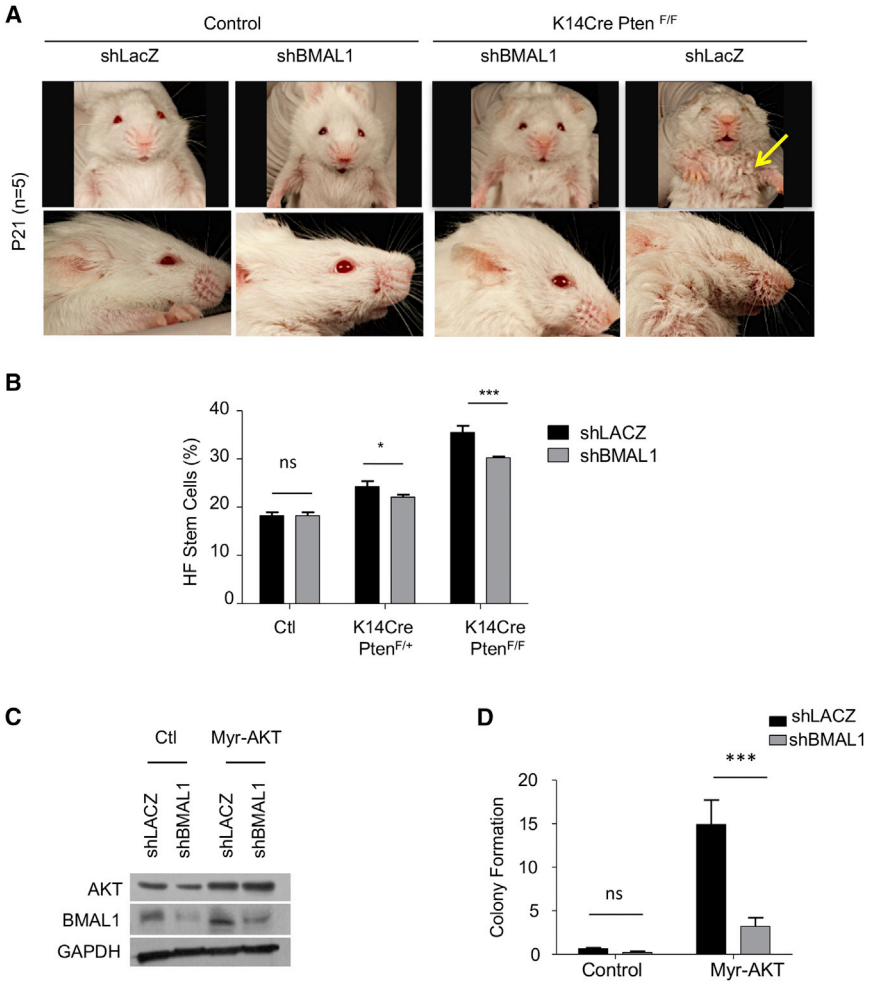

Figure 5. BMAL1 Ablation Reduces PTEN-Associated Stem Cell Accumulation

(A) Examples of control and PTEN-deficient mice after BMAL1 knockdown (Ad-shBmal1) or Ad-shLacZ. Note that K14CrePTEN mice displayed disheveled fur (yellow arrow). Rescue of the phenotype of K14CrePTEN mice with Ad-shBmal1 resembles controls animals (n = 5 mice per group).

(B) BMAL1 knockdown also reduced PTEN-driven stem cell accumulation (mean percentage ± SEM, n = 5 per genotype). *p < 0.05, ***p < 0.001.

(C) Immunoblot showing BMAL1 knockdown produced by Ad-shBMAL1 transduction.

(D) Graphic representation of colony formation assay depicting that BMAL1 knockdown decreases the number of individual colonies with AKT activation (Myr-AKT) (mean ± SEM, sextuplicates). ***p < 0.001.

suggesting that clock genes are consistently involved in the *PTEN*-driven phenotype. Interestingly, even after senescence is surpassed by chemical carcinogenesis (4-nitroquinoline 1-oxide [4NQO]), BMAL1 overexpression was found in PTEN-derived carcinomas (Figures 4H and 4I; Squarize et al., 2013), as well as in human carcinomas with PTEN knockdown (Figure 4J).

PTEN/AKT-Induced Accumulation of SCs Is Partially Regulated by BMAL1

We have shown that PTEN deletion in the skin triggers accumulation of SCs and activation of BMAL1. To test whether BMAL1 is actively involved in the accumulation of SCs upon PTEN excision, we deleted *Bmal1* in vivo using short hairpin (sh) technology (shBmal1 or shLacZ control, n = 5/group). Interestingly, we found that administration of shBMAL1 resulted in the partial rescue of the Pten-induced phenotype, including attenuation of the disheveled fur phenotype compared with control mice receiving shLacZ (Figure 5A). Furthermore, decreased Bmal1 expression reduced the accumulation of SCs in PTEN-deficient mice (Figure 5B). Because it is well known that the loss of PTEN activates the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway and that the phenotypic alterations driven by PTEN loss involve AKT activation (Sun et al., 1999; Squarize et al., 2008), we investigated whether BMAL1 knockdown would interfere with AKT-induced effects. Here we found that BMAL1 knockdown was able to interfere with the biological effects following AKT activation (Myr-AKT), which resulted in decreased numbers of colony formation (Figures 5C and 5D). Altogether, these results suggest that BMAL1 may participate in PTEN molecular signaling.

DISCUSSION

Molecular signals involved in the control of SCs are often associated with the PI3K pathway, which is activated in

neural, mammary, HF, and hematopoietic SCs (Korkaya et al., 2009; Kobielak et al., 2007; Groszer et al., 2006; Pietras et al., 2011; Polak and Buitenhuis, 2012; Segrelles et al., 2014). PI3K signaling is regulated by the *PTEN* tumor suppressor, a phosphatase that targets the second-messenger molecule phosphatidylinositol 3,4,5-trisphosphate and is an emerging regulator of normal SC function (Zhang et al., 2006; Fukuyama et al., 2006; Yilmaz et al., 2006). The *Pten*-deficient mouse phenotype, which includes striking alterations in the hair coat, suggested dysregulation of HFSC function. Indeed, under homeostatic conditions, deletion of *Pten* in the epidermis resulted in a progressive accumulation of SCs in the niche (bulge). The requirement for PTEN in HFSC homeostasis was further emphasized by the $CD34^{+}\alpha6$ cell accumulation following single and double allele deletion of *Pten*, indicating that *Pten* deficiency is sufficient to interfere with HFSC homeostasis. The accumulation of SCs in control mice occurs as age progresses (Doles et al., 2012), and this accumulation is increased by PTEN deletion.

Further characterization of the downstream molecular mechanism involved in the *Pten*-driven accumulation of HFSCs resulted in the identification of activation of the core circadian molecule BMAL1. BMAL1 regulates the timing of the HF cycle and promotes activation of HFSCs following external signals, such as transforming growth factor β and Wnt (Lin et al., 2009; Janich et al., 2011). Aligned with our results, in vivo studies on animals lacking BMAL1 in the epidermis generate epidermal SCs with reduced clonogenic potential, delayed anagen, and fewer proliferative cells, and therefore with constant proportion of bulge SCs in adulthood (Lin et al., 2009; Janich et al., 2011; Plikus et al., 2013). Similarly, constitutive activation of BMAL1 in the Per1/2 knockout mice resulted in more clonogenic epidermal SCs (Janich et al., 2011). These molecular changes affected hair growth (Plikus et al., 2013; Geyfman et al., 2012; Gaddameedhi et al., 2011; Mitra, 2011). Indeed after shaving, older adult PTEN mice displayed a halt in hair growth.

Loss of tumor-suppressor genes is involved in increased tumor predisposition in leukemogenesis and brain, prostate, intestinal, breast, and skin tumors (Squarize et al., 2008, 2013; Yan et al., 2002; Li et al., 2002; Fukuda et al., 2005; Guldberg et al., 1997; He et al., 2007; Ming et al., 2011). However, the biological features underlying such mechanisms are still poorly understood. Interestingly, K14Cre-$Pten^{F/F}$ mice did not spontaneously develop malignant tumors in the skin; on the contrary, we found hamartomas (benign lesions) characterized by the presence of well-differentiated HF cells and increased levels of the $p16^{ink4}$ senescence marker. Activation of cellular senescence often occurs during the development of benign lesions/tumors and restrains tumor size and malignant transformation (Dimri et al., 1995). Our findings are similar to those of White et al. (2014), who could not identify tumor formation upon short-term depletion of *Pten*. Although our promoter strategy (K14) differs from those used by White et al. (2014) (K15 and Lgr5), in both reports *Pten* deletion comprised the SC compartment of the epidermis. Interestingly, while we showed that the long-term accumulation of DNA damage preceded senescence and tumor formation in the presence of a carcinogen (4NQO), White et al. (2014) were able to induce tumor formation through the activation of Kras oncogene, a strategy to induce and expedite tumor formation. Of note, the development of malignant epidermal tumors was only observed upon the combined deletion of two tumor-suppressor genes (*Pten* and p53) along with constitutive activation of the Kras oncogene. It is also noteworthy that if a chemical carcinogen is applied, the resulting tumor with PTEN loss displays overexpresses BMAL1. This same correlation is seen in human tumors, and the BMAL1 expression is also dependent in PTEN and mTOR signaling (Matsumoto et al., 2017).

In humans, the autosomal dominant PTEN-deficient disorder Cowden's syndrome displays the development of multiple mucocutaneous hamartomas (Squarize et al., 2008). The mucocutaneous lesions in patients with Cowden's disease take years to develop (Hobert and Eng, 2009). In mouse models, the mucocutaneous lesions also have age-related penetrance (Squarize et al., 2008), which indicates that an accumulative effect of the PTEN deficiency is important for the phenotype development in vivo. Consistent with these clinical and experimental findings, short-term deletion of PTEN (e.g., over a period of 3–4 weeks) during adulthood is not enough to trigger hair cycle alterations and tumor development phenotype (White et al., 2014; Squarize et al., 2013; Chen et al., 2011; Kalaany and Sabatini, 2009), and cellular senescence usually occurs (White et al., 2014; Collado et al., 2005; Chen et al., 2005; Nardella et al., 2011).

Searching for a possible mechanism to explain the activation of cellular senescence, we found that silencing BMI-1 and the presence of $p16^{ink4}$ precede (and protect against) the development of skin tumors in K14Cre-$Pten^{F/F}$ mice. BMI-1 is a transcriptional repressor that is involved in cell-cycle arrest, SC longevity, and replicative senescence (Itahana et al., 2003; Lessard and Sauvageau, 2003; Park et al., 2004; Barker et al., 2008; Liu et al., 2009). Loss of BMI-1 leads to premature cellular senescence and increased $p16^{ink4}$ and $p19^{arf}$, proteins encoded by the *ink4a* gene (Jacobs et al., 1999a). In contrast, BMI-1 overexpression triggers an oncogenic effect mediated by $p16^{ink4}$ suppression and subsequent pRB and p53 signaling activation (Jacobs et al., 1999a, 1999b).

Following oncogene activation, such as *Ras*, cellular transformation results from Bmi-1 activation or $p16^{ink4}$ suppression (Kamijo et al., 1997; Serrano et al., 1996), suggesting a balance between Bmi-1 and $p16^{ink4}$ expression during the control or prevention of neoplastic occurrence.

In addition, our challenge was to identify and characterize the hierarchical interactions between PTEN and BMAL1. Evidence of this possible interplay of BMAL1 and PTEN deficiency came from the observation that *Bmal1* knockout mice have reduced the burden of squamous cell carcinoma and reduced $CD34^{+}\alpha6^{HI}$ (also referred as α6bright/$CD34^{+}$ tumor-initiating cells) (Janich et al., 2011). Furthermore, we challenged our *Pten*-deficient mice with the knockdown of BMAL1 before the development of the phenotype (day 3). We found that in vivo delivery of Ad-shBMAL1 was sufficient to rescue the skin phenotype, and the accumulation of HFSCs in both heterozygous and homozygous *Pten*-deficient mice. Interestingly, the foci formation driven by overexpression of the PI3K/AKT pathway was rescued upon downregulation of BMAL1. Our findings indicate that *Pten*-induced phenotype may be in part regulated by the clock core gene BMAL1 to induce accumulation of HFSC, which depicts a unique integration of PI3K and the clock gene signaling pathways during epidermal homeostasis.

EXPERIMENTAL PROCEDURES

Mice

All animal studies were carried out according to the University of Michigan approved protocols and in compliance with the Guide for the Care and Use of Laboratory Animals. Epithelial-specific *Pten* knockout mice were obtained by crossing $Pten^{F/F}$ mice (*Pten*^{tm1Hwu}) with K14Cre- (KRT14-cre) mice as described by Squarize et al. (2008). *mPer2* knockout mice (*Per2*^{tm1Drw}) were genotyped as previously described (Bae et al., 2001; Hoogerwerf et al., 2010). Mice were entrained to restricted 12-hr light/dark cycles. Where indicated, LRC was performed by injecting mice with bromodeoxyuridine (50 μg/g body weight) as described previously (Bickenbach and Chism, 1998; Braun and Watt, 2004). For further details see Supplemental Experimental Procedures.

FACS Analysis, Normal Keratinocyte Cell Line, and Immunohistochemistry/Immunofluorescence

SCs from the skin of 21-day-old and 17-week-old littermate mice were stained for CD34-FITC (BD Pharmingen, #553733) and CD49f-PE/Cye5 (BD Pharmingen, #551129) for FACS analysis as previously described (Castilho et al., 2009). Isotype controls were used as negative controls. HN13, NIH3T3, and NOK-SI cells were cultivated as described by Castilho et al. (2010). Immunohistochemistry or immunofluorescence was conducted with BMAL1 (Novus Bioscience, NB100-2288) overnight at 4°C (see Supplemental Experimental Procedures for details).

siRNA, SA-β-Gal, Immunoblotting, Adenoviruses, and Statistical Analysis

Cellular senescence was detected using an SA-β-Gal detection kit (Roche) at pH 6.0. Knockdown of PTEN or BMAL1 was achieved in NOK-SI and HN13 cells using siRNA. BMAl1 shRNA in vivo and in vitro experiments were conducted with Ad-shBmal1 or Ad-shLacZ-control, which was kindly provided by Dr. L. Yin (University of Michigan). Where indicated, Ad-shLacZ control or Ad-shBmal1 shRNA adenoviruses were injected into mice (age P3) subcutaneously at a dose of 1×10^{12} plaque-forming units (Zhang et al., 2014; Liu et al., 2007). The targeting sequence for mouse Bmal1 is 5′-CAT CGA TAT GAT AGA TAA CG-3′. All the adenoviruses were produced in 293AD packaging cells (Agilent) after Lipofectamine-mediated transfection, and concentrated after ultracentrifuge in cesium chloride gradient solutions. Mice were euthanized at P21, and SCs from skin were isolated and quantified by FACS analyses (UM Flow Cytometry Core). See Supplemental Experimental Procedures for additional details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.05.006.

AUTHOR CONTRIBUTIONS

C.Z., L.O.A., and R.M.C. designed and performed the assays; T.B. and P.P. contributed to mPER2 in vivo experiments; M.T.M. contributed to in vivo experiments; R.M.C. performed bioinformatics analyses; L.K.R.-M. performed BMI-1 and p16 experiments; R.M.C. and C.H.S. analyzed and interpreted the data, wrote the manuscript, and designed and supervised the study.

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