BLIMP1 Is Required for Postnatal Epidermal Homeostasis but Does Not Define a Sebaceous Gland Progenitor under Steady-State Conditions

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SUMMARY

B-lymphocyte-induced nuclear maturation protein 1 (BLIMP1) was previously reported to define a sebaceous gland (SG) progenitor population in the epidermis. However, the recent identification of multiple stem cell populations in the hair follicle junctional zone has led us to re-evaluate its function. We show, in agreement with previous studies, that BLIMP1 is expressed by postmitotic, terminally differentiated epidermal cells within the SG, interfollicular epidermis, and hair follicle. Epidermal overexpression of c-Myc results in loss of BLIMP1+ cells, an effect modulated by androgen signaling. Epidermal-specific deletion of Blimp1 causes multiple differentiation defects in the epidermis in addition to SG enlargement. In culture, BLIMP1+ sebocytes have no greater clonogenic potential than BLIMP1− sebocytes. Finally, lineage-tracing experiments reveal that, under steady-state conditions, BLIMP1-expressing cells do not divide. Thus, rather than defining a sebocyte progenitor population, BLIMP1 functions in terminally differentiated cells to maintain homeostasis in multiple epidermal compartments.

INTRODUCTION

Mammalian epidermis is maintained by stem cells that self-renew and give rise to the differentiated cells of the interfollicular epidermis (IFE), sebaceous glands (SGs), hair follicles (HF), and sweat glands (Kretzschmar and Watt, 2014). Several different epidermal stem cell pools have been identified, including multiple HF stem cell populations. Under steady-state conditions, stem cells in different regions of the epidermis only give rise to the differentiated cells appropriate for their location, but when the epidermis is damaged or genetically modified, individual stem cells exhibit a broader ability to differentiate into all epidermal lineages (Watt and Jensen, 2009).

Within the epidermis, the differentiated cells of the SG produce sebum that lubricates and waterproofs the skin surface (Zouboulis et al., 2008). The specialized SGs of the eyelid (meibomian gland) and male genitals (preputial gland) contribute to the composition of the tears and secrete pheromones, respectively (House et al., 2010). SG dysfunction results in benign conditions, such as acne and sebaceous cysts, and also in a range of different tumor types. In vivo lineage tracing by retroviral transduction has established that the SG can be maintained by a population of long-lived progenitors (putative stem cells) that are distinct from the stem cells of the HF (Ghazizadeh and Taichman, 2001). The only specific marker of sebocyte progenitors to be described is B-lymphocyte-induced nuclear maturation protein 1 (BLIMP1) (also known as PR domain zinc finger protein 1 [PRDM1]; Horsley et al., 2006).

First identified as a gene upregulated during, and capable of promoting, terminal differentiation of B lymphocytes (Turner et al., 1994), BLIMP1 was subsequently characterized in many other tissues, mainly as a transcriptional regulator of terminal differentiation (Bikoff et al., 2009; John and Garrett-Sinha, 2009). During embryonic skin development, BLIMP1 expression was identified in the upper differentiated layers of the IFE and in differentiated cells of the HF inner root sheath (Chang et al., 2002). It was subsequently reported that BLIMP1 is also expressed in terminally differentiated cells of the IFE and SG of postnatal human and mouse skin and is upregulated in differentiating sebocytes in culture (Cottle et al., 2013; Lo Celso et al., 2008; Magnúsdóttir et al., 2007; Sellheyer and Krahl, 2010). In addition, by employing a range of experimental strategies, including immunohistochemistry, genetic lineage tracing, and cell culture, Fuchs and coworkers described BLIMP1 to be a marker of sebocyte progenitors (Horsley et al., 2010).
et al., 2006). In view of the importance of the SG in skin biology and new reports that cells expressing leucine-rich repeats and immunoglobulin-like domain protein 1 (LRIG1) or leucine-rich repeat-containing G-protein-coupled receptor 6 (LGR6) are SG progenitors (Jensen et al., 2009; Page et al., 2013; Snippert et al., 2010), we have revisited the function of epidermal BLIMP1.

RESULTS

BLIMP1 Is Expressed by Terminally Differentiated Cells of the IFE, HF, and SG

We stained back skin sections of wild-type mice and transgenic mice expressing enhanced GFP (EGFP) under the control of the Blimp1 promoter (Blimp1EGFP) (Ohinata et al., 2005) from different postnatal stages for endogenous BLIMP1 (Figure 1 and Figure S1 available online). In agreement with previous publications, BLIMP1 was localized to cell nuclei (Horsley et al., 2006; Magnúsdóttir et al., 2007; Robertson et al., 2007). Specific cells within all epidermal compartments (IFE, HF, and SG) expressed BLIMP1 (Figures S1A–S1D). As reported previously (Coulombe and Bernot, 2004; Coulombe et al., 1989), the entire SG expressed keratin 14 (K14) (Figure S1D). Cells double positive for BLIMP1 or Blimp1EGFP and the marker of differentiated sebocytes, fatty acid synthase (FAS), were found in the upper SG (Figures 1A–1D). BLIMP1 expression by FAS+ sebocytes was evident as soon as the SG began to develop at postnatal day (P)2 (Figures S1A–S1D). BLIMP1+ involucrin (IVL)+ cells as well as Blimp1EGFP+ IVL+ (Figures 1C–1F) were found in the sebaceous duct, which sits like a cap atop the SG and is an elongation of the HF infundibulum/junctional zone (Cottle et al., 2013). In the IFE, BLIMP1+ cells were absent from the K14+ basal layer and were found in the terminally differentiated, IVL+ cells of the granular layers (Figures 1E, 1F, and S1A–S1D). We confirmed the existence of a population of BLIMP1+ cells in the upper HF adjacent to the SG. BLIMP1+ cells in that region coexpressed IVL and the HF shaft differentiation marker K31, indicating that they were undergoing terminal differentiation (Figures 1G and 1H). The location of BLIMP1-expressing cells in the epidermis is summarized in Figure 1I.

We also stained sections of murine meibomian glands and preputial glands, which are specialized SGs (Figures S1E–S1H) (House et al., 2010). BLIMP1+ cells were found in the center of the meibomian glands, where the most highly differentiated sebocytes reside; they expressed FAS (Figures S1E and S1F), as reported previously (Cottle et al., 2013). Cells expressing BLIMP1 in preputial glands were also FAS+, confirming that they are indeed lipid-producing, differentiated sebocytes (Figures S1G and S1H). BLIMP1+ cells were also detected in the supporting HF ductal structures, coexpressing IVL (Figure S1H). Consistent with the observations in mouse skin, in human skin BLIMP1 was expressed in terminally differentiated, IVL+ epidermal cells in IFE, SG, and HF (Figures S1I–S1K). In human sebaceous tumors, BLIMP1 was expressed by the most differentiated (IVL+ or weak FAS+) cells in the center of the neoplasm rather than in the periphery, where the most proliferative cells reside (Figures S1L–S1P). BLIMP1 is expressed in papillary dermal fibroblasts during normal skin development (Driskell et al., 2013; Lesko et al., 2013) and was also expressed in tumor stromal cells (Figures S1L–S1P).

Modulation of BLIMP1 Expression by MYC and Androgens

BLIMP1 has previously been shown to bind and negatively regulate the c-Myc promoter (Horsley et al., 2006), and epidermal c-Myc overexpression, like Blimp1 deletion, can lead to SG enlargement (Horsley et al., 2006; Berta et al., 2010; Cottle et al., 2013). To determine the effect of c-Myc overexpression on Blimp1 expression in the epidermis, we first confirmed that, in wild-type epidermis, BLIMP1+ cells did not express proliferating cell nuclear antigen (PCNA), a marker of proliferation (Figures 2A and 2B). When MYC was activated by high-dose 4-hydroxy-tamoxifen (4-OHT) treatment in K14c-MycER T transgenic mice (Berta et al., 2010; Cottle et al., 2013), proliferation in the SG was stimulated and there was a reduction in BLIMP1+ SG cells (Figure 2C). In contrast, when MYC-induced SG proliferation was inhibited by the androgen testosterone and sebocyte terminal differentiation was stimulated by the antiandrogen bicalutamide (Cottle et al., 2013), there was an increase in the number of BLIMP1+ differentiated sebocytes (FAS+) and BLIMP1+ cells in the upper layers of the IFE (Figure 2D). These observations indicate that accumulation of BLIMP1+ cells in the SG is correlated with terminal differentiation rather than proliferation of SG progenitors.

Epidermal Loss of Blimp1 Causes Multiple Epidermal Deficiencies, Including Sebaceous Gland Enlargement

There are conflicting reports about the consequences of epidermal loss of Blimp1 (Blimp1 conditional knockout [cKO]). In one report, there were no obvious defects in the IFE or HF and specific SG abnormalities were found, including SG enlargement and hyperplasia in the skin, the meibomian glands, and the preputial glands (Horsley et al., 2006). In contrast, Magnúsdóttir et al. (2007) found that epidermal-specific deletion of Blimp1 caused not only SG enlargement but also IFE hyperplasia, abnormal expansion of the granular layer, and a hyperkeratotic HF infundibulum. The IFE hyperplasia was absent in Blimp1-deficient epidermis in mice older than 15 days, and only SG enlargement and hyperkeratinization of the HF infundibulum persisted into adulthood (Magnúsdóttir et al., 2007).
BLIMP1 Regulates Epidermal Homeostasis

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Both studies used the same K14Cre mouse strain; however, Magnúsdóttir et al. (2007) used a floxed Blimp1 strain that deletes exons 6–8 upon recombination (Prdm1tm1Clme; Figure 3A) (Shapiro-Shelef et al., 2003) whereas Horsley et al. (2006) used an exon 5 floxed Blimp1 strain (Prdm1tm2Masu; Ohinata et al., 2005).

In order to delete Blimp1 selectively in adult, rather than developing, epidermis and therefore evaluate whether a BLIMP1+ progenitor population does indeed govern cellular input to the SG, we crossed Prdm1tm1Clme mice with KSCreER1 transgenic mice (Liang et al., 2009) and induced Cre-mediated deletion of Blimp1 in adult mice by injecting tamoxifen (Figures 3A and 3B) (Chiang et al., 2013). Deletion of Blimp1 in the entire epidermis was confirmed as reported previously (Figure S2) (Chiang et al., 2013). Hematoxylin and eosin (H&E) staining of neck skin sections collected 2, 3, and 6 months after tamoxifen injection revealed clear differences between control and Blimp1 cKO epidermis, with epidermal loss of Blimp1 resulting in SG enlargement, IFE thickening, accumulation...
of cornified layers, and hyperkeratosis of the HF infundibulum (Figures 3C and 3D). Although our analysis was restricted to neck skin, other studies have shown no evidence for differences between neck and back skin (Magnúsdóttir et al., 2007; Chiang et al., 2013). The phenotype resembled that described by Magnúsdóttir et al. (2007) in neonatal Blimp1−/− epidermis rather than a SG-specific phenotype (Horsley et al., 2006).

Quantification of H&E-stained sections showed that Blimp1 deletion resulted in a gradual increase in SG size over time (Figure 3E). Sections of Blimp1 cKO tissue stained for FAS showed no obvious changes in expression within sebocytes (Figure 3F). Blimp1-deficient IFE was significantly thickened (Figure 3G) and showed a dramatic increase in Ki67+/PCNA+ basal layer cells in the IFE and HF infundibulum (Figures 3H and 3I). K14, a marker
that is usually restricted to the basal layer in the IFE, expanded into the suprabasal layers (Figure 3H), as reported in neonatal cKO skin (Magnúsdóttir et al., 2007). K10 and IVL, markers of IFE differentiation, were expressed in the suprabasal layers, but the strong overlap of those markers with K14 indicates perturbed IFE differentiation (Figures 3J and 3K). We also found that the infundibulum of adult Blimp1 cKO skin displayed increased proliferation and was hyperkeratotic, thickened, and elongated (Figures 3D and 3L).

Markers typical of epidermal hyperproliferation, namely K6, the retinoic acid (RA)-signaling molecule fatty-acid-binding protein, FABP5, and cellular RA-binding protein, CRABP2 (the latter two being also sebocyte markers in normal skin; Collins and Watt, 2008), were also dramatically increased in Blimp1 cKO epidermis (Figures 3M–3O). These changes are consistent with a defective epidermal barrier (Magnúsdóttir et al., 2007) and would explain the massive inflammatory infiltrate in the dermis (Figure 3D) (Chiang et al., 2013). Staining for the hair shaft marker K31 did not reveal any abnormalities in the lower, cycling portion of the HF (Figure 3P) (Horsley et al., 2006; Magnúsdóttir et al., 2007).

We conclude that epidermal loss of Blimp1 expression in adult epidermis causes multiple epidermal defects, including SG enlargement, hyperplasia, and perturbed differentiation of the IFE and HF infundibulum.

**All Sebocytes Retain Proliferative Potential in Culture**

BLIMP1+ epidermal cells have the ability to proliferate at clonal density and give rise to lipid-filled sebocytes (Horsley et al., 2006). In order to compare the proliferative ability of BLIMP1+ sebocytes and other epidermal cells, we first incubated adult back skin keratinocytes from Blimp1EGFP mice with LipidTOX dye (Life Technologies) to label lipid-producing sebocytes. We then flow sorted four populations of keratinocytes: (1) EGFP+ lipid+ sebocytes; (2) EGFP+ lipid- sebocytes; (3) EGFP+ lipid- cells (comprising BLIMP1+ cells of IFE, HF, and sebaceous duct); and (4) EGFP+ lipid+ cells (comprising primarily undifferentiated cells and BLIMP1+ differentiated cells; Figures 4A–4C). Sorted cells were cultured on a feeder layer, and colony-forming efficiency (CFE) was analyzed 2 weeks later.

Lipid+ sebocytes, whereas not proliferative in vivo, were clonogenic at low frequency, confirming previous studies (Laurent et al., 1992). EGFP+ sebocytes did not show a significantly increased CFE compared to EGFP+ sebocytes (Figures 4D–4F). EGFP+ lipid- cells displayed a reduced CFE compared to EGFP+ lipid+ cells, although this was not statistically significantly (Figure 4E). Analysis of colony sizes revealed EGFP+ lipid+ sebocytes formed the largest colonies in comparison to the other three cell populations (Figure 4F). We conclude that BLIMP1+ cells, whether or not they are sebocytes, have a lower clonogenic capacity than BLIMP1+ cells, consistent with their differentiated status.

**BLIMP1+ Cells Do Not Give Rise to Proliferative and Differentiating Sebocytes In Vivo**

In order to understand whether a subset of BLIMP1+ cells gives rise to differentiated sebocytes, we performed genetic lineage-tracing experiments (Kretzschmar and Watt, 2012). We crossed Blimp1Cre transgenic mice (Ohinata et al., 2005; Horsley et al., 2006) with chicken β-actin promoter and cytomegalovirus (CMV) enhancer-chloramphenicol acetyltransferase-EGFP (CAGcatEGFP) transgenic (Kawamoto et al., 2000) or Rosa26tdTomato (Ai9 line) gene trap (Madsen et al., 2010) reporter mice (Figure 5A). By constitutively expressing Cre recombinase under the control of the Blimp1 promoter, the floxed STOP cassette of the reporter construct is removed and the fluorescent reporter (EGFP or tdTomato) expressed in BLIMP1+ cells and their descendants (Figure 5A). If these Blimp1-expressing cells are indeed sebocyte progenitors, labeled progeny should be found throughout the SG in cells at all stages of differentiation.

We collected tissue from Blimp1Cre × CAGcatEGFP and Blimp1Cre × Rosa26tdTomato mice at weaning age (P21) and adult (P56) and stained tail epidermal whole mounts (Braun et al., 2003) with antibodies against the respective reporter and the sebocyte marker, FAS (Cottle et al., 2013). Neither EGFP nor tdTomato was significantly detected in FAS+ sebocytes (Figures 5B–5I and 5L). Clones expressing either reporter were found in differentiated cells of the inner bulge, as well as in the matrix of anagen HFs (Figures 5B–5I). Figure 5D shows that rare EGFP+ clones, containing a small number of cells, were present in the HF junctional zone and sebaceous duct, but not in basal or FAS+ sebocytes. These lineage-tracing results largely mirror the expression of endogenous BLIMP1 and Blimp1EGFP in all epidermal lineages (Figures 1, 5J, and 5K). Labeled progeny adjacent to the SG were mainly found in the inner layers of the HF junctional zone, and only one EGFP+ traced cell in the epidermis of all mice examined expressed FAS (Figures 5M and 5N). Similar data were obtained in horizontal whole mounts of back epidermis, which also show EGFP+ lineage traced clones in the differentiated layer of IFE (overlapping with IVL) and HF (Figure S3). Dermal labeling is also observed in line with recent studies on the role of BLIMP1 in dermal fibroblast subpopulations (Driskell et al., 2013; Lesko et al., 2013).

In Blimp1EGFP mice, EGFP is expressed by cells that are expressing endogenous BLIMP1, regardless of whether they are derived from BLIMP1+ cells (Lesko et al., 2013).
contrast, in Blimp1Cre × Rosa26tdTomato mice, tdTomato expression (and likewise Blimp1Cre × CAGcatEGFP mice EGFP expression) is restricted to cells that are BLIMP1+ and their progeny. The cells that give rise to the BLIMP1+ cells of the IFE and SG were BLIMP1−/C0 (e.g., IFE basal layer) and so were labeled with EGFP and not tdTomato. Of note, cells in the upper layers of the IFE cannot be visualized in tail epidermal whole mounts, because antibodies cannot penetrate deeper than the first suprabasal layer of the fixed tissue. However, in horizontal whole mount sections, EGFP and tdTomato labeling in the IFE and SG was clearly visible (Figure S3; data not shown). The lack of tdTomato labeling in terminally differentiated sebocytes may reflect the short half-life of the cells. The lack of EGFP- or tdTomato-labeled basal cells and early sebocytes supports the conclusion that BLIMP1 does not define a sebocyte progenitor population.

LGR6+ and LRIG1+ Stem Cells Contribute to Maintenance of the Sebaceous Gland

LRIG1 and LGR6 have previously been shown to be markers of epidermal stem cells in the HF junctional zone that produce progeny in the SG (Figures 6A and 6B) (Jensen et al., 2009; Page et al., 2013; Snippert et al., 2010). We stained tissue sections of adult Lrig1EGFPiresCreERT2 (Page et al., 2013) and Lgr6EGFPiresCreERT2 (Snippert et al., 2010) knockin (KI) mice with antibodies against EGFP (to label the respective stem cell pools) and endogenous BLIMP1. In agreement with the earlier findings, we found that BLIMP1 was not coexpressed with LRIG1 or LGR6 (Figures 6A–6D).

To confirm that our lineage-tracing strategy was capable of labeling the SG lineage and to rule out any possible effects of using the CMV and CAG promoter versus Rosa26 promoters or different fluorescent reporters, we crossed our Rosa26tdTomato strain (Madisen et al., 2010) with Lrig1 KI and Lgr6 KI mice (Figure 6E). Offspring positive for either of the two EGFPiresCreERT2 cassettes and the tdTomato reporter were treated with 1.5 mg 4-OHT at 7–9 weeks of age, and back skin was examined 4 days and

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**Figure 4. Clonogenic Potential of BLIMP1+ Sebocytes**

(A) Schematic of experimental setup.

(B) Epidermal tail whole mount of Blimp1EGFP mouse stained with GFP antibody and LipidTOX to visualize lipid-producing cells; counterstained with DAPI (blue). Dashed line outlines HF and SG. Numbers correspond to sorted populations in (C).

(C) Flow cytometry plot showing sorted cell populations.

(D) Phase contrast images (top) and stained dishes (bottom) of mouse epidermal cells after 14 days in culture (after removal of feeder layer).

(E and F) Colony-forming efficiency (E) and average colony size (F; whisker plot) of sorted populations. Error bars in (E) show SEM. In (F), vertical lines show 25% confidence intervals; means are indicated by crosses and medians by dashed lines. (E) and (F) data are biological replicates (n = 3 mice). Asterisks indicate significance of differences between cell populations (unpaired two-tailed Student’s t test; p values: ***p < 0.001). The scale bar represents 100 μm.
4 weeks later (Figure 6F). This dose of 4-OHT achieved maximal labeling of the stem cell pools. Paraffin sections were stained for tdTomato, BLIMP1, and FAS.

Four days after 4-OHT application, tdTomato+ LRIG1 stem cell progeny were found in the periphery and lower SG, as well as in some sebaceous duct cells. However, BLIMP1+ sebocytes were negative for the reporter (Figure 6G). As Lrig1 is also expressed in the dermis (Driskell et al., 2013; Gomez et al., 2013), tdTomato+ dermal LRIG1 progeny were also observed. TdTomato+ LGR6 stem cell progeny were also found in the lower SG, but again tdTomato labeling was absent from BLIMP1+ sebocytes (Driskell et al., 2013; Figure 6H). Four weeks after 4-OHT treatment, some BLIMP1+ sebocytes were also tdTomato+ (Figures 6I and 6J), establishing that, between 4 days and 4 weeks after labeling, progeny of LRIG1+ and LGR6+ stem cells underwent terminal differentiation into BLIMP1+ sebocytes. Of note, even with the high dose of 4-OHT applied, SGs of Lgr6 KI × Rosa26tdTomato mice were frequently labeled in the absence of isthmus labeling (Figure S4), strongly suggesting the existence of sebocyte progenitor residing within the SG, as proposed previously (Page et al., 2013).

In conclusion, our results confirm that the SG lineage is indeed derived from LRIG1+ and LGR6+ stem cells (Jensen et al., 2009; Page et al., 2013; Snippert et al., 2010) and that BLIMP1+ SG cells are terminally differentiating sebocytes (Cottle et al., 2013).

**DISCUSSION**

Although the concept that BLIMP1 is a marker of sebocyte progenitor cells has become established in the literature (Beck and Blanpain, 2012; Blanpain and Fuchs, 2014; Niemann and Horsley, 2012; Solanas and Benitah, 2013; Zhang et al., 2011), our studies suggest that BLIMP1 is primarily a marker of terminal differentiation in SG, IFE, and HF and that BLIMP1+ cells do not divide in undamaged postnatal epidermis. Nevertheless, genetic ablation of BLIMP1 confirms its importance in epidermal homeostasis, and clonogenic assays demonstrate that BLIMP1+ sebocytes, whereas nonproliferative in vivo, can divide in vitro.

We showed that specific deletion of Blimp1 caused defects in differentiation in multiple epidermal compartments, namely the IFE, SG, HF infundibulum, and juncional zone. Hyperplasia was not restricted to the SG and was more pronounced in the IFE and infundibulum. The aberrant IFE differentiation was suggestive of a barrier defect, which would explain the previously reported inflammatory skin phenotype (Chiang et al., 2013). The two previously published studies on epidermal deletion of Blimp1 in early development (Horsley et al., 2006; Magnusdottir et al., 2007) used a K14Cre mouse described by Vasioukhin et al. (1999) to target the epidermal basal layer but used different conditional alleles of Blimp1: Horsley et al. (2006) used an exon 5 floxed Blimp1 strain (Prdm1tm2Masu; Ohinata et al., 2005), whereas Magnusdottir et al. (2007) used a floxed Blimp1 strain with loxP sites between exons 6–8 (Prdm1tm1Cgime; Figure 3A) (Shapiro-Shelef et al., 2003). Both studies reported SG enlargement (Horsley et al., 2006; Magnusdottir et al., 2007), a phenotype that we also observed on epidermal deletion of Blimp1 in adult mice. In addition, Magnusdottir et al. (2007), Chiang et al. (2013), and the present study found thickening of the IFE and HF infundibulum, regardless of whether or not Blimp1 was deleted in the embryo or adult. Potential reasons for the divergent observations regarding whether or not Blimp1 has a selective role in the SG include differences in the genetic background of the mice, animal husbandry (diet, health status, and pathogens), or the presence of truncated BLIMP1 protein that is undetectable with current antibodies (Bikoff et al., 2009). Differences in Cre expression over time could also contribute, as suggested for other transgenic Cre lines (Kang et al., 2014).

BLIMP1 is a transcriptional repressor of c-Myc (Lin et al., 1997), and forced activation of MYC resulted in downregulation of BLIMP1 and increased proliferation within the SG (Cottle et al., 2013). We have previously observed that androgen receptor signaling modulates epidermal responses to MYC activation (Cottle et al., 2013), and consistent with this, stimulation of sebocyte differentiation was accompanied by an increase in the number of BLIMP1+ cells. BLIMP1 repression of c-Myc may also contribute to the transition of IFE cells out of the granular layer (Honma et al., 2006). Consistent with BLIMP1 being associated with terminal differentiation, BLIMP1 expression was downregulated in human SG tumors. Another negative regulator of BLIMP1 is miR-125b, which is upregulated in stem cells and progenitors in the HF and SG (Zhang et al., 2011).

For lineage-tracing experiments, we crossed Blimp1Cre transgenic mice with two different loxP-STOP-loxP fluorescent reporter strains. We found no evidence that BLIMP1+ cells gave rise to differentiated sebocytes or indeed any labeled progeny. Instead, subpopulations of Lgr6- and Lrig1-expressing cells founded the sebocyte lineage, as reported previously (Jensen et al., 2009; Page et al., 2013; Snippert et al., 2010). Although we used the same Blimp1Cre line as in the earlier lineage-tracing experiments (Horsley et al., 2006; Ohinata et al., 2005), we utilized different reporter lines, transgenic CAGcatEGFP and gene-trap Rosa26tdTomato (used in our study) compared to gene-trap Rosa26EYFP (used in the study by Horsley et al., 2006; Magnusdottir et al., 2007).
Figure 5. Lineage Tracing the Progeny of BLIMP1+ Cells

(A) Schematic of lineage-tracing experiments. Numbers correspond to labeling strategies in (B)–(I).

(B–F) Epidermal tail whole mounts collected from Blimp1Cre × CAGcatEGFP mice at P21 (B–D) and P49 (E) and control mice (F) stained with antibodies against GFP (green) and FAS (red) and counterstained with phalloidin (blue).

(G–I) Epidermal tail whole mounts collected from Blimp1Cre × Rosa26tdTomato mice at P49 (G and I) and control mice (H) stained with antibodies against tdTomato (red) and FAS (green) and counterstained with phalloidin.

(J and K) Epidermal tail whole mounts collected from Blimp1GFP mice at P49 (J) and control mice (K) stained with antibodies against GFP (green), FAS (red), and IVL (white).

(L) Quantitation of percent HFs with fluorescent-reporter-labeled SGs. Quantification was performed on stained 0.5 × 0.5 cm epidermal tail whole mounts collected from three mice per strain and time point.

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et al., 2006), which have been shown to be more sensitive (Duffield and Humphreys, 2011; Kawamoto et al., 2000; Kretzschmar and Watt, 2012; Madisen et al., 2010). The fluorescent lineage tracers we used are expressed more strongly upon recombination, due to expression driven from the CAG promoter rather than the weaker Rosa26 promoter used by Horsley et al. (2006; Kawamoto et al., 2000; Soriano, 1999; Srinivas et al., 2001). EGFP and tdTomato also have the spectral advantage over EYFP when distinguishing true epifluorescence from highly autofluorescent structures such as the lipid-rich SG and sebaceous duct.

Based on our findings and the recent literature, we propose the following model of sebocyte differentiation (Figure 7). First, subsets of LGR6- and LRG1-expressing cells residing in the upper HF and periphery of the SG constitute the bona fide stem cells of the sebocyte lineage (Page et al., 2013; Snippert et al., 2010). This is in line with the observation that the SG can be maintained independently of the HF lineages (Ghazizadeh and Taichman, 2001). We do not rule out the existence of other SG stem cell compartments, and indeed, we believe this is likely, given the diversity of stem cells elsewhere in the epidermis (Kretzschmar and Watt, 2014). Second, we propose that MYC plays a role in proliferation of cells that are committed to undergo terminal differentiation. Third, upregulation of BLIMP1 promotes terminal differentiation by repressing c-Myc and inhibiting proliferation (Figure 7) (Berta et al., 2010; Cottle et al., 2013). Our revised model suggests that Blimp1 cKO mice exhibit SG hyperplasia directly because of derepression of c-Myc, as reported previously (Horsley et al., 2006), and indirectly by causing a barrier defect in the IFE (Chiang et al., 2013), which activates stem cells within the HF infundibulum/junctional zone and SG periphery to proliferate (Page et al., 2013).

In conclusion, our findings indicate that the role of BLIMP1 in the epidermis is to maintain homeostasis in multiple compartments, including the SG, but that it exerts its effects in terminally differentiated cells rather than in sebocyte progenitors.

**Experimental Procedures**

**Human Tissue**

Human skin and tumors were collected and diagnosed by Dr. Harald P. Gollnick and Dr. Sven R. Quist from the Clinic of Dermatology and Venereology, Otto-von-Guericke University Magdeburg, Germany, and Dr. Ken Natsuga from the Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan. Patient consent records and ethical review are retained by the respective institutions.

**Generation and Experimental Treatment of Mice**

Mouse experiments were subject to Cancer Research UK, University of Cambridge, King’s College London, and Institutional Animal Care and Utilization Committee of Academia Sinica ethical review and performed in accordance with the UK Government Animals (Scientific Procedures) Act 1986.

Blimp1EGFP (Ohinata et al., 2005) transgenic and F1 (CBA × B6) wild-type mice were used for the initial characterization of BLIMP1 expression. To obtain mice with epidermal deletion of Blimp1 (Prdm1), Blimp1<sup>Rbox/ROX</sup> (Prdm1<sup>tm1Clme</sup>; Shapiro-Shelef et al., 2003) and KSCreERT<sup>m</sup> mice (Liang et al., 2009) were crossed and tamoxifen treated as described previously (Chiang et al., 2013). For constitutive lineage-tracing experiments, Blimp1Cre mice (Ohinata et al., 2005) were either crossed with Rosa26tdTomato (Ai9 line) knockin (Madisen et al., 2010) or CAGcatEGFP transgenic (Kawamoto et al., 2000) reporter mice.

For 4-OHT (Sigma)-induced lineage-tracing, Lgr6EGFPiresCreERT<sup>2</sup> (Snippert et al., 2010) and Lrig1EGFPiresCreERT<sup>2</sup> (Page et al., 2013) knockin mice were bred to Rosa26tdTomato reporter mice. Their offspring were treated with one dose of 1.5 mg 4-OHT dissolved in 100 μl acetone applied to clipped back skin at 7–9 weeks of age, and tissue was collected 4 days and 4 weeks after 4-OHT application.

K14-c-MycERT<sup>2</sup> (2184 C.1 line) transgenic mice (Arnold and Watt, 2001) were treated once with 1.5 mg 4-OHT and then subsequently with 2 mg testosterone and 2 mg bicalutamide or carrier only (acetone) for 4 days and analyzed 4 days after the first treatment (Cottle et al., 2013). At the start of every experiment, all the mice were 7–9 weeks old and therefore in the resting phase (telogen) of the hair cycle (Stenn and Paus, 2001). Wild-type littermates and acetone-only-treated transgenic mice were used as controls. At least three mice were treated per condition.

**Mouse Keratinocyte Isolation, Flow Cytometry, and Clonogenic Assays**

Keratinocytes were isolated from telogen back skin of adult Blimp1EGFP mice using trypsin (Life Technologies), as previously described (Jensen et al., 2010). Isolated keratinocytes were incubated with LipidTOX dye (Life Technologies) diluted 1:500 in PBS for 20 min. Flow sorting was carried out using a MoFlo high-speed sorter (Dako Cytomation) or a FACSAria II cell sorter (BD Biosciences). Sorted keratinocytes were plated onto a J2 3T3 feeder layer in six-well plates and cultured for 14 days as previously described (Jensen et al., 2010). Feeders were removed from the keratinocyte colonies prior to fixation with 2% paraformaldehyde.
Figure 6. Lineage Tracing the Progeny of LRIG1\(^+\) and LGR6\(^+\) Stem Cells

(A and B) Genetic elements of the Lrig1 knockin (KI) and Lgr6 KI.
(C and D) Paraffin sections of adult Lrig1 KI (C) and Lgr6 KI (D) back skin stained with antibodies against GFP (green) and BLIMP1 (red), counterstained with DAPI (blue). Boxed area is shown at higher magnification.
(E and F) Schematic of experimental breeding and procedures.
(G–J) Paraffin sections of Lrig1 KI × Rosa26tdTomato (G and I) and Lgr6 KI × Rosa26tdTomato (H and J) collected 4 days (G and H) and 4 weeks (I and J) after one dose of 4-OHT and stained for BLIMP1 (green, left panels) or FAS (green, right panels) and tdTomato (red). Arrowheads indicate BLIMP1\(^+\) cells. Dashed lines indicate epidermal-dermal boundary. Scale bars represent 100 μm.

See also Figure S4.
and staining with 1% Nile red and 1% rhodamine B blue (all Sigma). Images of stained colonies were taken on a Molecular Imager Gel Doc XR+ imaging system (BioRad).

Histology and Immunohistochemistry
Tissue samples for sections were fixed overnight in 4% paraformaldehyde (Sigma) and embedded in paraffin wax. Five micrometer sections were prepared and stained with H&E.

Tail epidermal whole mounts and back skin horizontal whole mounts were prepared as described previously (Braun et al., 2003; Driskell et al., 2013). Immunohistochemistry on paraffin wax sections was performed as described elsewhere (Niemann et al., 2002). Primary antibodies used were: rat anti-BLIMP1 (1:100; eBioscience 14-5963-82), rabbit anti-CRABP2 (1:100; Proteintech 10225-1-AP), goat anti-FABP5 (1:100; R&D Systems AF1476), mouse anti-FAS (1:100; Santa Cruz Biotechnology sc-48357), rabbit anti-GFP (1:500; Life Technologies A11122), chicken anti-GFP (1:500; Abcam ab13970), goat anti-GFP (1:200; Abcam ab6673), rabbit anti-involucrin (1:800; ERLI-3, in-house), rabbit anti-K6 (1:500; Covance RBP-169P), mouse anti-K14 (1:1,000; LL002; in-house), rabbit anti-K14 (1:1,000; Covance PRB-155P), chicken anti-K14 (1:1,000; Covance SIG-3476), guinea pig anti-K31 (1:100; Progen GP-hHa1), rabbit anti-Ki67 (1:100; Abcam ab16667), mouse anti-PCNA (1:100; Millipore CBL407), and rabbit anti-RFP (1:1,000; recognizing tdTomato; Rockland 600-401-379).

Antibody staining was visualized using appropriate species-specific secondary antibodies conjugated to Alexa 488, Alexa Fluor 555, or Alexa Fluor 647 (1:300; Life Technologies). LipidTOX dye (1:500; Life Technologies) was used in some cases to label lipids. Slides were mounted using ProLong Gold antifade reagent (Life Technologies) containing DAPI (Sigma) as nuclear counterstain.

All fluorescent sections were analyzed on a TCS SP5 confocal microscope (Leica) or an A1 confocal microscope (Nikon). All images of H&E-stained sections were taken on images of H&E-stained vertical tissue sections collected from three Blimp1 cKO and three control mice. Statistical analysis was performed using the unpaired two-tailed Student’s t test.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.08.007.

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