BNIP3 Plays Crucial Roles in the Differentiation and Maintenance of Epidermal Keratinocytes

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Transcriptome analysis of the epidermis of *Hes1*$^{-/-}$ mouse revealed the direct relationship between Hes1 (hairy and enhancer of split-1) and BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3), a potent inducer of autophagy. Keratinocyte differentiation is going along with activation of lysosomal enzymes and organelle clearance, expecting the contribution of autophagy in this process. We found that BNIP3 was expressed in the suprabasal layer of the epidermis, where autophagosome formation is normally observed. Forced expression of BNIP3 in human primary epidermal keratinocytes (HPEKs) resulted in autophagy induction and keratinocyte differentiation, whereas knockdown of BNIP3 had the opposite effect. Intriguingly, addition of an autophagy inhibitor significantly suppressed the BNIP3-stimulated differentiation of keratinocytes, suggesting that BNIP3 plays a crucial role in keratinocyte differentiation by inducing autophagy. Furthermore, the number of dead cells increased in the human epidermal equivalent of BNIP3 knockdown keratinocytes, which suggests that BNIP3 is important for maintenance of skin epidermis. Interestingly, although UVB irradiation stimulated BNIP3 expression and cleavage of caspase3, suppression of UVB-induced BNIP3 expression led to further increase in cleaved caspase3 levels. This suggests that BNIP3 has a protective effect against UVB-induced apoptosis in keratinocytes. Overall, our data provide valuable insights into the role of BNIP3 in the differentiation and maintenance of epidermal keratinocytes.

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INTRODUCTION

The skin epidermis is a stratified epithelium. Stratification is a key process of epidermal development. During epidermal development, the single layer of basal cells undergoes asymmetric cell division to stratify, and produce committed suprabasal cells on the basal layer. These suprabasal cells are still immature and sustain several rounds of cell divisions to form fully stratified epithelia. Recent studies have identified numerous molecules involved in epidermal development, although how these molecules coordinate to induce proper stratification of the epidermis remains to be elucidated. Previously, by integrating both loss- and gain-of-function studies of Notch receptors and their downstream target Hes1 (hairy and enhancer of split-1), we demonstrated the multiple roles of Notch signaling in the regulation of suprabasal cells (Moriyama *et al.*, 2008). Notch signaling induces differentiation of suprabasal cells in a Hes1-independent manner, whereas Hes1 is required for maintenance of the immature status of suprabasal cells by preventing premature differentiation. In light of the critical role of Hes1 in the maintenance of spinous cells, exploration of the molecular targets of Hes1 in spinous layer cells may lead to the discovery of the molecules required for differentiation of spinous layer cells to granular layer cells. Because Hes1 is thought to be a transcriptional repressor (Ohtsuka *et al.*, 1999), loss of Hes1 is expected to cause aberrant upregulation of genes that are normally repressed in spinous layer cells. To identify these genes, we previously conducted comparative global transcript analysis using microarrays and found several candidates that may play a crucial role in regulating epidermal development (Moriyama *et al.*, 2008). One of the genes that was highly expressed was *BNIP3* (*BCL2 and adenovirus E1B 19-kDa-interacting protein 3*), an atypical pro-apoptotic BH3-only protein that induces cell death and autophagy (Zhang and Ney, 2009).

The molecular mechanism through which BNIP3 induces cell death is not well understood; however, it has been reported that BNIP3 protein is induced by hypoxia in some tumor cells and that the kinetics of this induction correlate with cell death (Sowter *et al.*, 2001). In contrast,

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Abbreviations: 3-MA, 3-methyladenine; BNIP3, BCL2 and adenovirus E1B 19-kDa-interacting protein 3; ChIP, chromatin immunoprecipitation; Hes1, hairy and enhancer of split-1; HPEK, human primary epidermal keratinocyte; Q-PCR, quantitative PCR

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BNIP3-induced autophagy has been shown to protect HL-1 myocytes from cell death in an ischemia–reperfusion model (Hamacher-Brady *et al.*, 2007). Induction of autophagy by BNIP3 has a protective effect in some conditions, whereas in others it is associated with autophagic cell death. Recent evidence also suggests that BNIP3, through autophagy, is also required for the differentiation of chondrocytes under hypoxic conditions (Zhao *et al.*, 2012).

Autophagy was initially described based on its ultrastructural features of the double-membraned structures that surrounded the cytoplasm and organelles in cells, known as autophagosomes (Mizushima *et al.*, 2010). To date, only microtubule-associated protein light chain 3 (LC3), a mammalian homolog of yeast Atg8, is known to be expressed in autophagosomes and, therefore, it serves as a widely used marker for autophagosomes (Kabeya *et al.*, 2000; Mizushima *et al.*, 2004). Autophagy is an evolutionarily conserved catabolic program that is activated in response to starvation or changing nutrient conditions. Recently, autophagy was shown to be involved in differentiation of multiple cell types, including erythrocytes, lymphocytes, adipocyte, neuron, and chondrocyte (Srinivas *et al.*, 2009; Mizushima and Levine, 2010).

Epidermal cornification, the process of terminal keratinocyte differentiation, requires programmed cell death in a similar but different pathway from apoptosis (Lippens *et al.*, 2005). Cornification is also accompanied by activation of lysosomal enzymes and organelle clearance. Moreover, some researchers have reported that autophagy may play a role in epidermal differentiation (Haruna *et al.*, 2008; Aymard *et al.*, 2011; Chatterjea *et al.*, 2011). Therefore, it is likely that BNIP3 is involved in cornification through cell death or autophagy.

In this study, transcriptome analysis of *Hes1*$^{-/-}$ mouse epidermis revealed that Hes1 could directly suppress BNIP3 expression in epidermal keratinocytes. We also found that BNIP3 was expressed in the suprabasal layer of the human skin epidermis, where autophagosome formation was observed. BNIP3 was also sufficient to promote cornification through induction of autophagy. Finally, we found that BNIP3 had a protective effect against UVB-induced apoptosis in keratinocytes *in vitro*. Our data thus indicate that BNIP3, an inducer of autophagy, is involved in the terminal differentiation and maintenance of epidermal keratinocytes.

RESULTS

Hes1 directly represses BNIP3 expression in epidermal cells and keratinocytes

We previously performed a microarray analysis with epidermal RNAs isolated from wild-type and *Hes1*$^{-/-}$ mice (Moriyama *et al.*, 2008) and found that BNIP3 was preferentially overexpressed in *Hes1*$^{-/-}$ epidermis. The upregulation of *Bnip3* in the *Hes1*$^{-/-}$ epidermis was confirmed by quantitative PCR (Q-PCR) and immunofluorescent staining (Figure 1a and b). As Hes1 is thought to be a transcriptional repressor (Ishibashi *et al.*, 1994), it might play a repressive role in the regulation of BNIP3 expression. In accordance with this hypothesis, BNIP3 expression in *Hes1*$^{-/-}$ epidermis at embryonic day 15.5 was observed in the suprabasal layers (Figure 1b), where Hes1 has been reported to be expressed in wild-type epidermis at the same age (Blanpain *et al.*, 2006; Moriyama *et al.*, 2008). To confirm whether Hes1 suppresses BNIP3 expression, an adenoviral vector expressing Hes1 was used to infect human primary epidermal keratinocytes (HPEKs) and, subsequently, the expression level of BNIP3 was quantified by Q-PCR and western blot analysis. The BNIP3 protein was detected as multiple bands between 22 and 30 kD as previously reported (Vengellur and LaPres, 2004; Walls *et al.*, 2009; Mellor *et al.*, 2010; Sassone *et al.*, 2010). We found that Hes1 induced a substantial reduction of BNIP3 expression in HPEKs at the mRNA and protein levels (Figure 1c and d), demonstrating that Hes1 is involved in the repression of BNIP3. To determine whether Hes1 directly regulates *BNIP3* expression, we performed chromatin immunoprecipitation (ChIP) assays. We identified at least 5 Hes1 consensus binding sites 1 kb upstream of the transcription initiation site of the human *BNIP3* gene, and subsequent Q-PCR analysis revealed that a DNA fragment located at -247 to -87 was slightly amplified from crosslinked chromatin isolated by Hes1 immunoprecipitation (Figure 1e). We also found an additional site between -212 and $+22$ that was strongly amplified. These data clearly show that Hes1 specifically binds to the promoter region of *BNIP3* and directly suppresses its expression.

BNIP3 is expressed in the granular layer of the epidermis, where autophagosome formation is observed

To determine the BNIP3 expression profile in the epidermis, we performed immunofluorescent staining in human skin epidermal equivalent. BNIP3 was expressed in the granular layer of epidermal equivalent 18 days (Figure 2a and b) or 24 days (Figure 2c and d) after exposure at the air–liquid interface. BNIP3 expression in the granular layer was also observed in the normal human skin epidermis (Figure 2g and h). Recent reports show that BNIP3 is expressed in mitochondria and that it induces autophagy (Quinsay *et al.*, 2010). In addition, some researchers have reported that autophagy may play a role in epidermal differentiation (Haruna *et al.*, 2008; Aymard *et al.*, 2011; Chatterjea *et al.*, 2011). We therefore investigated whether autophagy occurred in the epidermis, especially in the granular layers. To quantitate the level of autophagy, cytosol to membrane translocation of the autophagy marker EGFP-LC3 (Kabeya *et al.*, 2000) was monitored in a human skin equivalent model (Mizushima *et al.*, 2004). When autophagy is active, autophagosomes containing EGFP-LC3 are visible as fluorescent puncta (Kabeya *et al.*, 2000). As expected, EGFP-LC3 puncta were observed in the granular layers of the epidermal equivalent (Figure 2e). Moreover, endogenous LC3 dots were observed in the granular layers of normal human skin epidermis (Figure 2f). These data suggested that BNIP3 might be involved in the induction of autophagy in the granular layer of the epidermis.

BNIP3 is required for terminal differentiation of keratinocyte by induction of autophagy *in vitro*

To investigate the involvement of BNIP3 in the induction of autophagy, we transduced HPEKs stably expressing EGFP-LC3

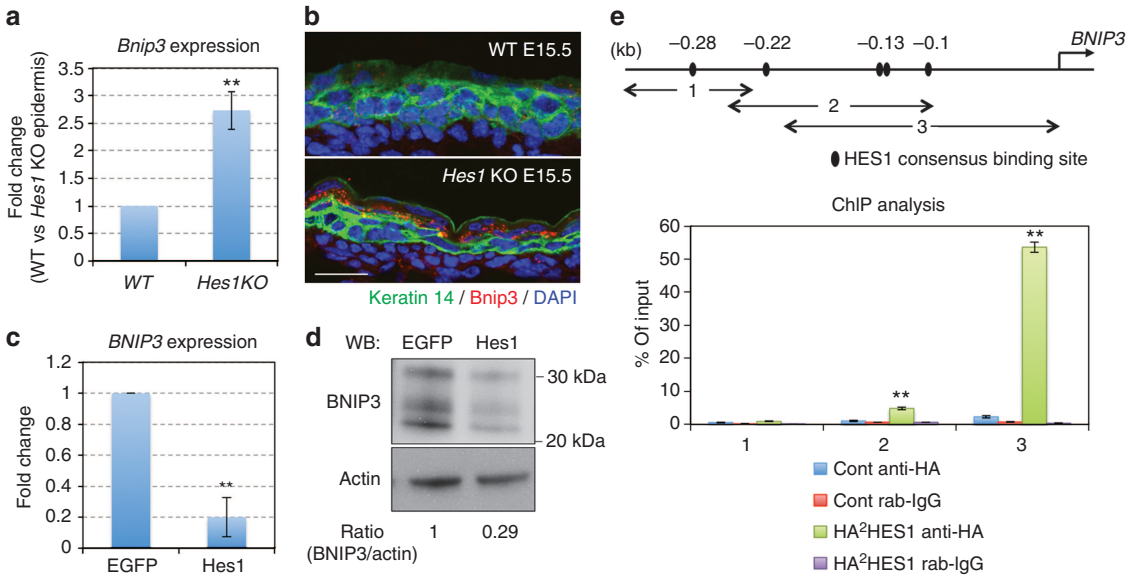

Figure 1. BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) is directly suppressed by HES1(hairy and enhancer of split-1). (**a**) Quantitative PCR (Q-PCR) analysis of *Bnip3* expression in dorsal skin epidermis from either wild-type (WT) or *Hes1* knockout (KO) embryo (embryonic day 14.5 (E14.5)). (**b**) Immunofluorescent analysis of Bnip3 expression in dorsal skin epidermis from either WT or *Hes1* KO embryo (E15.5). Keratin 14 staining is shown in green and Bnip3 staining is shown in red. The blue signals indicate nuclear staining. Scale bars = 20 μm. (**c**) Q-PCR and (**d**) western blot analysis of BNIP3 expression in human primary epidermal keratinocyte (HPEK) cells infected with adenoviruses expressing enhanced green fluorescent protein (EGFP) or Hes1. (**c**) Each expression value was calculated with the ΔΔCt method using *UBE2D2* as an internal control. (**d**) Numbers below blots indicate relative band intensities as determined by ImageJ software. (**e**) Specific binding of Hes1 to the *BNIP3* promoter. HPEK cells were infected with adenoviral constructs expressing hemagglutinin (HA)-tagged Hes1, and processed for chromatin immunoprecipitation (ChIP) with an anti-HA antibody and normal rabbit immunoglobulin G (Cont rab-IgG) as a nonimmune control. Q-PCR amplification of the region of the *BNIP3* gene described in the indicated map (upper panel; nucleotides −360 to −244 (1); nucleotides −247 to −87 (2); −212 to +22 (3)) was also performed. The amount of precipitated DNA was calculated relative to the total input chromatin. All the data represent the average of three independent experiments ± SD. $^{**}P < 0.01$.

with a BNIP3 adenoviral vector. BNIP3 expression was found to be sufficient to trigger the formation of EGFP-LC3 puncta that was significantly reduced by addition of 3-methyladenine (3-MA), an inhibitor of autophagy (Figure 3a and b). On the other hand, BNIP3 knockdown markedly decreased the punctuate distribution of EGFP-LC3 in differentiated HPEKs (Figure 3c and d). Furthermore, flow cytometry analysis using a green fluorescent probe used to specifically detect autophagy (Cyto-ID autophagy detection dye) (Chan *et al.*, 2012) also showed that BNIP3 was required for the autophagy induction (Figure 3c and f). These data indicate that BNIP3 is involved in the induction of autophagy in HPEKs. Intriguingly, these data also confirm the previous finding that autophagosome induction is accompanied by keratinocyte differentiation (Haruna *et al.*, 2008). We observed that the number of mitochondria was decreased in the granular layers, where BNIP3 expression and autophagosome formation was observed (Figure 4a). In addition, mitochondria were significantly decreased in the differentiated HPEKs *in vitro* (Figure 4b). Colocalizations of mitochondria and EGFP-LC3 dot were observed only in the differentiating keratinocytes (Figure 4c), suggesting the contribution of autophagy in the decrease of mitochondria. BNIP3 expression was also correlated with decreased mitochondria in HPEKs, whereas addition of 3-MA restored mitochondrial numbers (Figure 4d). Furthermore, we also observed colocalization of mitochondria and EGFP-LC3 dot in BNIP3-overexpressing HPEKs (Figure 4e). These data indicated that mitochondria were removed by BNIP3-induced autophagy. Next, we investigated the involvement of BNIP3 in the differentiation of epidermal keratinocytes. Western blot analysis and immunofluorescent staining revealed that BNIP3 expression increased during differentiation (Figure 5a and b). Knockdown of BNIP3 significantly suppressed keratinocyte differentiation when the cells were treated with differentiation medium (Figure 5c and d), indicating that BNIP3 is required for terminal differentiation of keratinocyte. On the other hand, forced expression of BNIP3 in HPEKs markedly stimulated loricrin expression (Figure 5e and f). To determine whether BNIP3-dependent keratinocyte differentiation was induced by autophagy, 3-MA was added to the cells transduced with BNIP3. As shown in Figure 5e and f, 3-MA notably abolished the keratinocyte differentiation induced by BNIP3, suggesting that BNIP3 is required for terminal differentiation of keratinocyte by induction of autophagy.

BNIP3 maintains epidermal keratinocytes

To further determine the roles of BNIP3 in epidermal differentiation, the human skin epidermal equivalent was reconstituted from HPEKs stably expressing a BNIP3 RNA interference (RNAi). Unfortunately, we did not observe drastic differentiation defects; however, we unexpectedly discovered

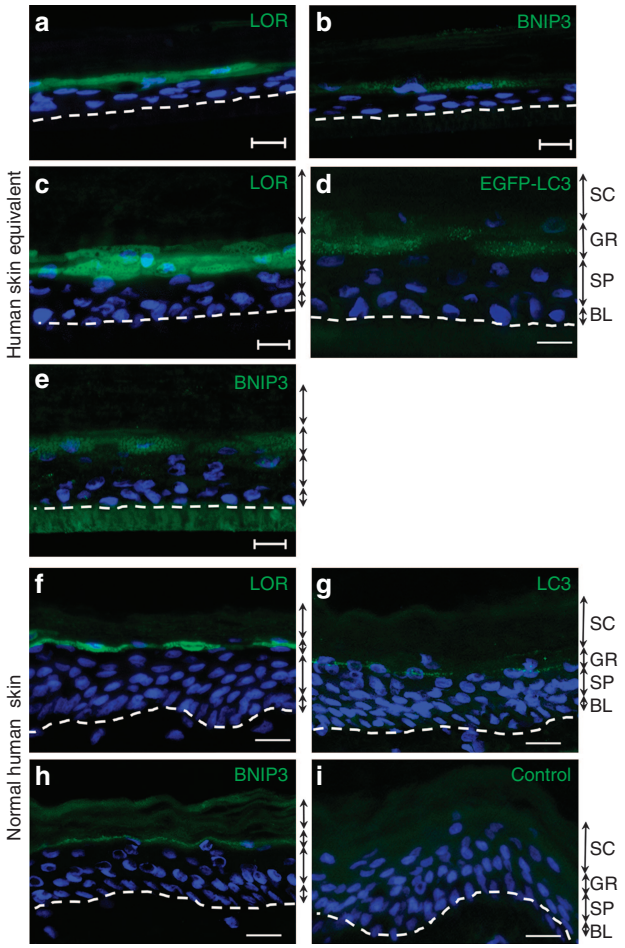

Figure 2. BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) is expressed in the granular layer of the human epidermis. (**a**–**e**) Human skin epidermal equivalents were constituted from (**a**–**d**) normal human primary epidermal keratinocytes (HPEKs) or (**e**) HPEKs transfected with EGFP-LC3 by lentiviral vector. Cells were grown for (**a**, **b**) 18 days and (**c**–**e**) 24 days after exposure at the air–liquid interface. (**f**–**i**) Normal human skin epidermis. (**a**, **c**, **f**) Expression pattern of loricrin (LOR). (**b**, **e**, **h**) Expression pattern of BNIP3. (**i**) Control staining without BNIP3 antibody is shown. (**d**) Autophagosome formation determined by EGFP-LC3 puncta. (**g**) Endogenous expression pattern of LC3. The blue signals indicate nuclear staining. The dotted lines indicate (**a**–**e**) the boundary between the epidermis and the membrane or (**f**–**i**) the boundary between the epidermis and the dermis. Scale bars = 20 μm. BL, basal layer; GL, granular layer; SC, stratum corneum (cornified layer); SP, spinous layer.

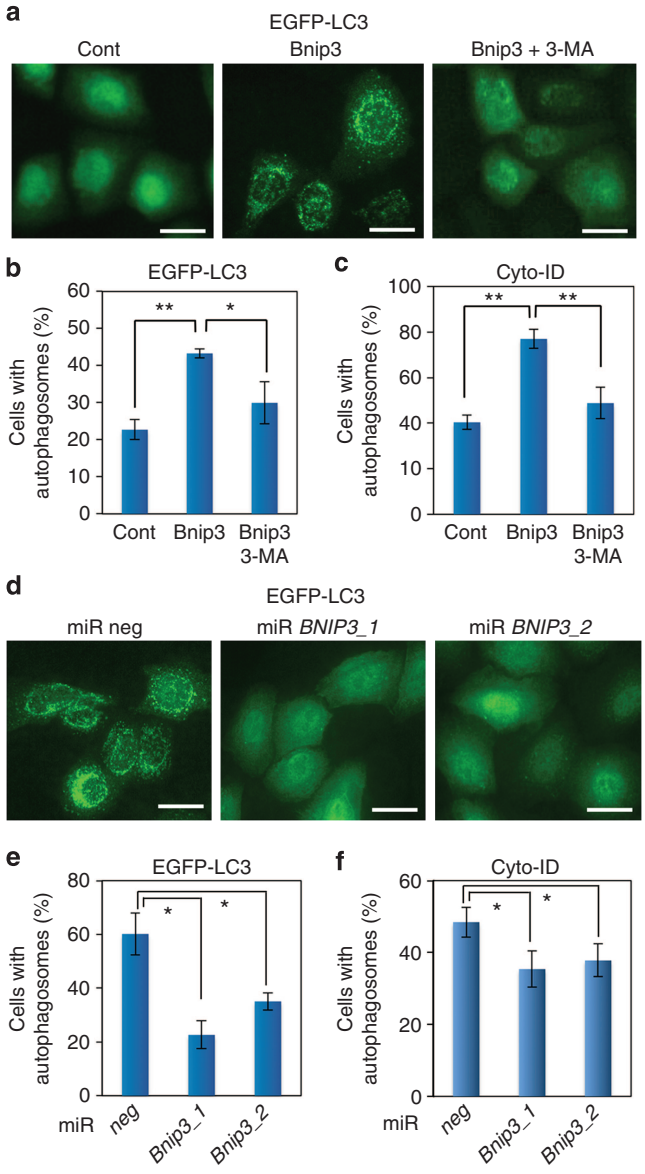

Figure 3. BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) stimulates autophagy. (**a**, **b**) EGFP-LC3-expressing human primary epidermal keratinocytes (HPEKs) were transduced with DsRed (Cont) or BNIP3. As an inhibitor of autophagy, 3-methyladenine 3-MA (5 mM) was added. Cells were then stained with anti-EGFP at 24 hours after transduction. (**a**) EGFP-LC3 staining is shown in green. Scale bars = 20 μm. (**b**) The percentage of EGFP-LC3-positive cells with more than five puncta were quantified and are presented as the mean of three independent experiments ± SD. (**c**) HPEKs were transduced with DsRed (Cont) or BNIP3. As an inhibitor of autophagy, 3-MA (5 mM) was added. Autophagy induction was determined by Cyto-ID staining and quantified by flow cytometry. (**d**, **e**) EGFP-LC3-expressing HPEKs were transduced with miR *neg*, miR *BNIP3_1*, or miR *BNIP3_2* and induced to differentiate. Cells were then stained with anti-EGFP at 8 hours after differentiation induction. (**d**) EGFP-LC3 staining is shown in green. Scale bars = 20 μm. (**e**) The percentage of EGFP-LC3-positive cells with more than five puncta were quantified and are presented as the mean of three independent experiments ± SD. (**f**) HPEKs were transduced with miR *neg*, miR *BNIP3_1*, or miR *BNIP3_2* and induced to differentiate. Autophagy induction was determined by Cyto-ID staining and quantified by flow cytometry. All the data represent the average of three independent experiments ± SD. $^{**}P<0.01$; $^{*}0.01<P<0.05$.

that ''sunburn-like cells'' existed in BNIP3 knockdown epidermal equivalent (Figure 6a and b). We therefore hypothesized that BNIP3 might play a key role in the survival of epidermal keratinocytes. To evaluate this hypothesis, HPEKs were irradiated with 20 mJ cm^{-2} UVB. UVB irradiation triggered the formation of autophagosome that was significantly reduced by BNIP3 knockdown (Figure 6c–e). As shown in Figure 6f, UVB irradiation induced cleavage of caspase3 and BNIP3 expression. Intriguingly, knockdown of UVB-induced BNIP3 by RNAi further increased the amount of cleaved caspase3, suggesting that BNIP3 is required for the protection of keratinocytes from UVB-induced apoptosis (Figure 6f).

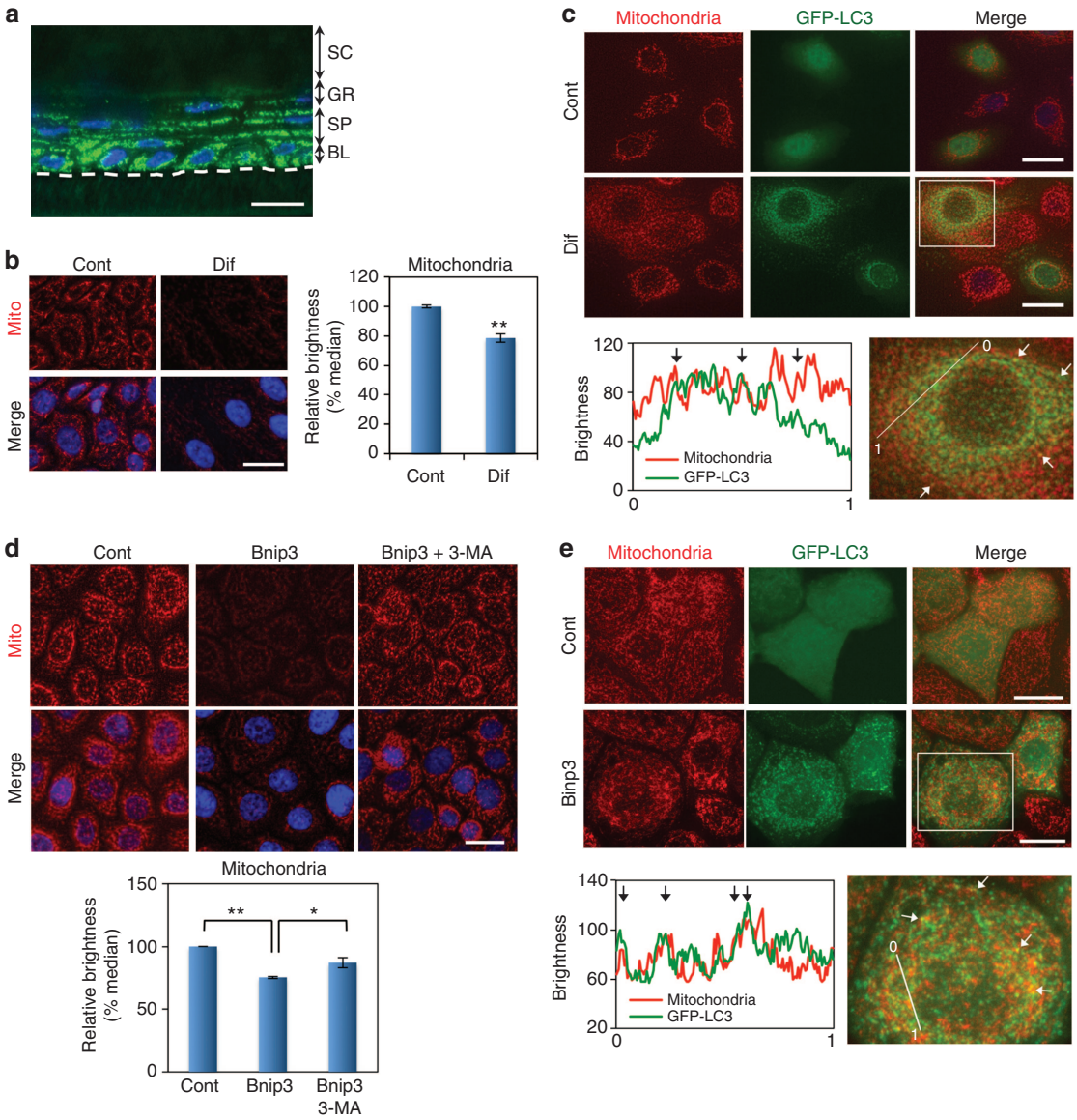

Figure 4. Autophagy stimulates mitochondrial degradation. (**a**) Distribution pattern of mitochondria. The blue signals indicate nuclear staining. The dotted lines indicate the boundary between the epidermis and the membrane. Scale bars = 20 μm. BL, basal layer; GL, granular layer; SC, stratum corneum (cornified layer); SP, spinous layer. (**b**) Nondifferentiated control (Cont) or differentiated human primary epidermal keratinocytes (HPEKs; Dif) were subjected to immunofluorescent staining 2 days after induction of differentiation. Mitochondrial staining is shown in red. The blue signals indicate nuclear staining. Scale bar = 20 μm. The graph indicates the percent of median brightness calculated by BZ Analyzer Software (Keyence) as the mean of three independent experiments ± SD. (**c**) EGFP-LC3-expressing HPEKs were differentiated. Cont or Dif were stained with anti-mitochondria (red) and anti-EGFP (green) 8 hours after induction of differentiation. Graph indicates the linescan analysis of the red and green fluorescent channels. Initial point of linescan is indicated as 0, and terminal point is indicated as 1. The arrows mark the colocalization of the two proteins. (**d**) HPEKs were transduced with enhanced green fluorescent protein (EGFP; Cont) or BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3). As an inhibitor of autophagy, 3-methyladenine 3-MA (5 mM) was added. Cells were then fixed and stained with anti-mitochondria 48 hours after transduction. Scale bar = 20 μm. The graph indicates the percent of median brightness calculated by BZ Analyzer Software (Keyence) as the mean of three independent experiments. $^{**}P<0.01$; $^{*}0.01<P<0.05$. (**e**) EGFP-LC3-expressing HPEKs were transduced with mock (Cont) or BNIP3. Cells were then fixed and stained with anti-mitochondria (red) and anti-EGFP (green) 24 hours after transduction. Graph indicates the linescan analysis of the red and green fluorescent channels. Initial point of linescan is indicated as 0, and terminal point is indicated as 1. The arrows mark the colocalization of the two proteins.

DISCUSSION

In this study, we demonstrated that BNIP3, a potent inducer of autophagy, plays a role in the terminal differentiation and maintenance of epidermal keratinocytes. It has been suggested that autophagy plays a role in the skin epidermis, but few attempts have been made to clarify the involvement of autophagy in skin epidermis.

We found that the HES1 transcriptional repressor directly suppressed BNIP3 expression in mouse epidermis and HPEKs (Figure 1). Moreover, our results revealed that BNIP3 was

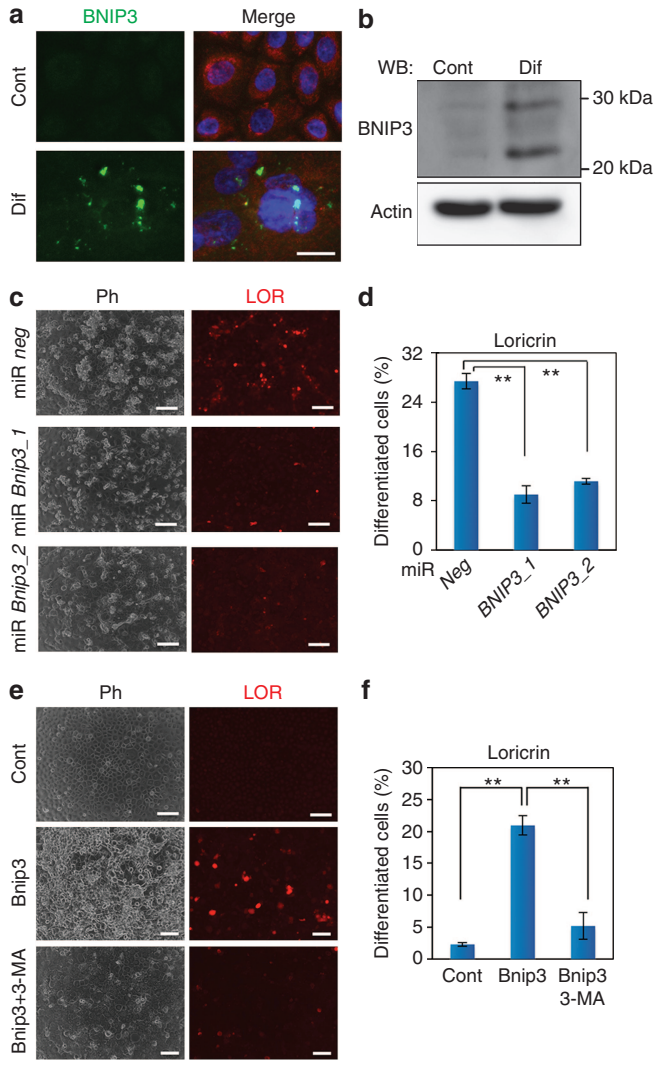

Figure 5. BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) is required for the differentiation of keratinocytes *in vitro*. (**a**, **b**) Human primary epidermal keratinocytes (HPEKs) were differentiated and BNIP3 expression was observed. (**a**) Nondifferentiated control (Cont) or differentiated HPEKs (Dif) were subjected to immunofluorescent staining. BNIP3 staining is shown in green. Mitochondrial staining is shown in red. The blue signals indicate nuclear staining. Scale bar = 20 μm. (**b**) Western blot (WB) analysis. Proteins extracted from Cont or Dif were probed with anti-BNIP3 or anti-actin. (**c**, **d**) HPEKs were infected with adenoviral vectors expressing miR *neg*, miR *BNIP3_1*, or miR *BNIP3_2* followed by induction of differentiation. Cells were then immunostained with a loricrin antibody 9 days after transduction. (**e**, **f**) HPEKs were infected with adenoviral vectors expressing enhanced green fluorescent protein (EGFP; Cont) or BNIP3 and subjected to immunofluorescent staining against loricrin (LOR) 6 days after transduction. As an inhibitor of autophagy, 3-methyladenine 3-MA (5 mM) was added. Phase contrast images (Ph) and LOR staining are shown. Scale bars = 200 μm. (**d**, **f**) Percentages of LOR-positive differentiated cells were calculated by computerized image analysis. The data represent the average of three independent experiments ± SD. $^{**}P<0.01$.

expressed in the granular layers of mouse epidermis, its human skin epidermal equivalent, and its normal human skin epidermis (Figures 1 and 2). These data are consistent with our previous report showing that Hes1 is expressed in the spinous layers, where it represses the regulatory genes for differentiation to maintain the spinous cell fate (Moriyama *et al.*, 2008). Hence, it can be inferred that Bnip3 expression is suppressed in the spinous layers by Hes1, whereas it is upregulated in the granular layers where Hes1 expression is absent. In addition, our finding that BNIP3 is required for keratinocyte differentiation fits our idea that Hes1 represses certain regulatory genes to prevent the premature differentiation of spinous cells. Our *in vitro* data suggest that BNIP3 is involved in keratinocyte differentiation through autophagy (Figures 3–5). The mechanisms underlying the involvement of autophagy in keratinocyte differentiation remain elusive; however, considering that keratinocyte differentiation induced mitochondrial clearance and BNIP3 expression (Figure 4 and 5), BNIP3-induced autophagy may be responsible for the removal of mitochondria that may be required for the terminal differentiation of epidermal keratinocytes. During reticulocyte differentiation, programmed clearance of mitochondria induced by BNIP3L/Nix, a molecule closely related to BNIP3, has been reported to be a critical step (Schweers *et al.*, 2007). Therefore, keratinocytes likely possess the same differentiation mechanism that reticulocytes have, although further investigation will be required for elucidation.

In contrast to the results from differentiation in two-dimensional culture, we did not observe drastic differentiation defects in the BNIP3 knockdown human epidermal equivalent except for the existence of ''sunburn-like cells'' (Figure 6). This might be because of the incomplete suppression of BNIP3 in the BNIP3 knockdown keratinocytes, and/or might be because of the redundancy between BNIP3 and BNIP3L/Nix, a homolog of BNIP3, as we found in our preliminary study that Bnip3l is also expressed in the epidermis (data not shown). Although the phenotypes of BNIP3-null mice were published in 2007, these researchers found that BNIP3-null mice had no increase in mortality or apparent physical abnormalities (Diwan *et al.*, 2007). Generally, impairment of epidermal differentiation or skin barrier formation results in an obvious defect. Thus, BNIP3-null epidermis seems to exhibit subtle, if any, abnormalities. On the basis of these findings, the involvement of BNIP3 in epidermal differentiation must be investigated in the future. In-depth analysis of the BNIP3-null epidermis phenotype could help elucidate the role of BNIP3 in mouse epidermal differentiation.

Despite the lack of obvious differentiation defects in the human epidermal equivalent, our data showing that BNIP3 knockdown caused the appearance of ''sunburn-like cells'' is regarded as an example of apoptosis (Young, 1987), revealing a new role of BNIP3 in keratinocyte maintenance. Furthermore, requirement of BNIP3 for protection from UV-induced apoptosis was confirmed in two-dimensional keratinocyte cultures (Figure 6e). The underlying mechanism of this prosurvival function of BNIP3 in keratinocytes remains unclear; however, previous reports have demonstrated that hypoxia-induced autophagy through BNIP3 is critical for the prosurvival process (Bellot *et al.*, 2009). Recently, it has been reported that UVA induces autophagy to remove oxidized phospholipids and protein aggregates in epidermal keratino-

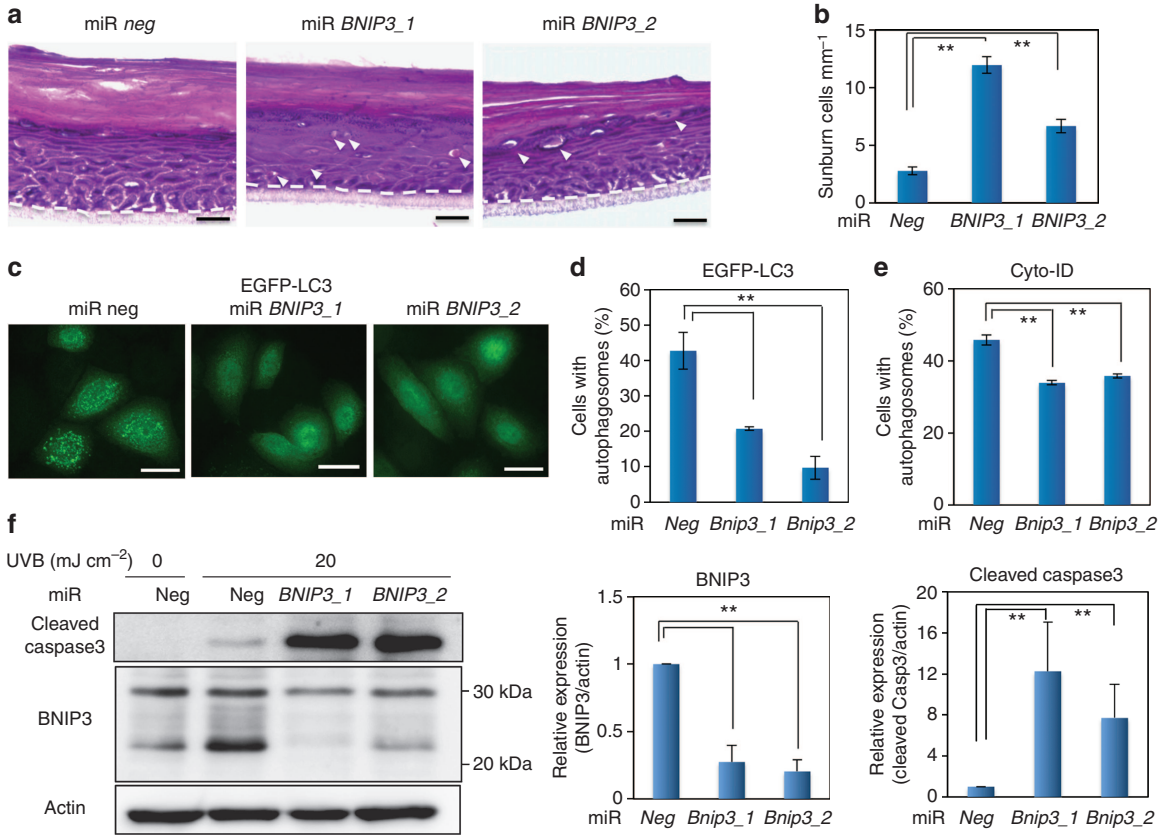

Figure 6. BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) promotes cell survival in the reconstituted epidermis and keratinocytes. (**a**) Morphology of the human skin epidermal equivalents from human primary epidermal keratinocytes (HPEKs) infected with lentivirus expressing miR *neg*, miR *BNIP3_1*, or miR *BNIP3_2*. Arrowheads indicate sunburn-like cells. (**b**) The number of sunburn-like cells per mm was counted and plotted as the means of 10 sections ± SD. (**c**–**e**) HPEKs were infected with adenovirus expressing miR *neg*, miR *BNIP3_1*, or miR *BNIP3_2*, and irradiated with UVB. (**c**) Cells were stained with anti-EGFP at 8 hours after UVB irradiation. (**d**) The percentage of EGFP-LC3-positive cells with more than five puncta were quantified and are presented as the mean of three independent experiments ± SD. (**e**) Autophagy induction was determined by Cyto-ID staining and quantified by flow cytometry. The data represent the average of three independent experiments ± SD. (**f**) Cells were subjected to western blot analysis at 8 hours after irradiation. The blot shown is representative image of three independent experiments. Graphs indicate relative band intensities as determined by ImageJ software and plotted as the means of three independent experiments. Scale bars = 20 μm. $^{**}P<0.01$.

cytes (Zhao *et al.*, 2013). Because our data indicate that UVB-induced autophagy is mediated by BNIP3 (Figure 6c and d), it is possible that autophagy induced by BNIP3 also plays a role in the maintenance of keratinocytes. Further analysis is required to confirm these results.

UV-induced apoptotic cells appear within 12 hours and are predominately located in the suprabasal differentiated keratinocyte compartment of human skin (Gilchrest *et al.*, 1981). Moreover, differentiated keratinocytes appear to be most sensitive to the UV light that induces p53-dependent apoptosis (Tron *et al.*, 1998). Tron *et al.* (1998) demonstrated that differentiated keratinocytes in p53-null mice exhibited only a small increase in apoptosis after UVB irradiation compared with the increase observed in normal control animals (Tron *et al.*, 1998). Interestingly, because p53 has been reported to directly suppress BNIP3 expression (Feng *et al.*, 2011), BNIP3 might be abundantly upregulated in suprabasal cells in p53-null animals, resulting in the resistance to UVB-induced apoptosis. Indeed, our preliminary study showed that p53 knockdown enhanced UV-induced BNIP3 expression in HPEKs (data not shown). Therefore, BNIP3 expression in suprabasal cells appears to be important for the protection of differentiated keratinocytes from normal environmental stress such as weak UV exposure *in vivo*.

A recent report on a role for autophagy in epidermal barrier formation and function was identified in *atg7*-deficient mice (Rossiter *et al.*, 2013). The authors showed that autophagy was constitutively active in the suprabasal epidermal layers as we report in this study (Figure 2). However, in contradiction to our results, the authors concluded that autophagy was not essential for the barrier function of the skin. This may be because of the presence of an alternative Atg5/Atg7-independent autophagic pathway (Nishida *et al.*, 2009) in the epidermis. This Atg5/Atg7-independent pathway is also independent of LC3, but forms Rab9-positive double-membrane vesicles. Moreover, protein degradation via this pathway is inhibited by 3-MA and is dependent on Beclin 1. Our data demonstrate that: (1) BNIP3 induced the formation of

EGFP-LC3 puncta (Figure 4) and (2) 3-MA significantly diminished the formation of GFP-LC3 puncta and keratinocyte differentiation induced by BNIP3 (Figure 5). These findings suggest that BNIP3 in the epidermis induced both conventional and Atg5/Atg7-independent autophagy. Intriguingly, GFP cleaved from GFP-LC3 also accumulates in the *Atg7*-deficient epidermis (Rossiter *et al.*, 2013), thereby demonstrating the existence of an alternative autophagic pathway (Juenemann and Reits, 2012) in the epidermis. Further investigation will be required to determine whether Beclin 1 and Rab9 are indispensable for the BNIP3-induced autophagy and subsequent differentiation of keratinocytes.

In summary, our data reveal that expression of BNIP3 in granular cells induces autophagy and is involved in the terminal differentiation and maintenance of skin epidermis. Studies on the involvement of autophagy in skin epidermis have attracted considerable attention recently. In addition, increasing evidence suggests the involvement of BNIP3 in the differentiation of several cell types, including oligodendrocytes (Itoh *et al.*, 2003), osteoclasts (Knowles and Athanasou, 2008), and chondrocytes (Zhao *et al.*, 2012); however, the precise role of BNIP3 in this process remains to be investigated. Our study thus provides new insights into the functions of BNIP3 in differentiation and homeostasis.

MATERIALS AND METHODS

Histology and immunofluorescent analysis

Samples and embryos were fixed in 4% paraformaldehyde, embedded in optimal cutting temperature compound, frozen, and sectioned at 10 μm. Sections were then either subjected to hematoxylin and eosin staining or immunohistochemical analysis as previously described (Moriyama *et al.*, 2006). Details are described in Supplementary Materials Online.

Cell culture

HPEKs were purchased from CELLnTEC (Bern, Switzerland) and maintained in CnT-57 (CELLnTEC) culture medium according to the manufacturer's protocol. For induction of differentiation, the medium was changed to CnT-02 (CELLnTEC) at confluent monolayers of HPEKs, followed by adding calcium ions to 1.8 mM. The generation of human skin equivalents was performed using CnT-02-3DP culture medium (CELLnTEC) according to the manufacturer's protocol.

Design of artificial microRNAs and plasmid construction

Oligonucleotides targeting a human BNIP3 sequence compatible for use in cloning into BLOCK-iT Pol II miR RNAi expression vectors (Invitrogen, Carlsbad, CA) were obtained using the online tool BLOCK-iT RNAi Designer. The oligonucleotide sequences used in this study are shown in Supplementary Table S1 online. Cloning procedures were performed following the manufacturer's instructions.

Adenovirus and lentivirus infection

Adenoviruses expressing EGFP, Hes1, BNIP3, and miR *BNIP3* were constructed using the ViraPower adenoviral expression system (Invitrogen) according to the manufacturer's protocol. Lentivirus expressing EGFP-LC3 (from Addgene plasmid 21073, Cambridge, MA) and miR *BNIP3* plasmid was constructed and used to infect keratinocytes as previously described (Moriyama *et al.*, 2012; Moriyama *et al.*, 2013).

RNA extraction, complementary DNA generation, and Q-PCR

Total RNA extraction, complementary DNA generation, and Q-PCR analyses were carried out as previously described (Moriyama *et al.*, 2012). Details of the primers used in these experiments are shown in Supplementary Table S2 online.

Western blot analysis

Western blot analysis was performed as previously described (Moriyama *et al.*, 2012; Moriyama *et al.*, 2013). Details are described in Supplementary Materials Online.

ChIP assay

The ChIP assay was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions. Hemagglutinin-tagged Hes1 was immunoprecipitated with rabbit polyclonal antibody against hemagglutinin tag (ab9110, Abcam, Cambridge, MA). Immunoprecipitated DNA was analyzed by Q-PCR. Relative quantification using a standard curve method was performed, and the occupancy level for a specific fragment was defined as the ratio of immunoprecipitated DNA over input DNA. Details of the primers used in these experiments are shown in Supplementary Table S2 online.

Flow cytometry analysis

For autophagy detection, Cyto-ID Autophagy detection kit (Enzo Life Sciences, Plymouth Meeting, PA) was used according to the manufacturer's instructions. Details are described in Supplementary Materials Online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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

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