HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2014 September 01.

Published in final edited form as:

J Invest Dermatol. 2014 March ; 134(3): 827–837. doi:10.1038/jid.2013.419.

Bone morphogenetic protein signalling suppresses wound-induced skin repair by inhibiting keratinocyte proliferation and migration

Christopher J. Lewis[1,2], **Andrei N. Mardaryev**[1], **Krzysztof Poterlowicz**[1], **Tatyana Y. Sharova**[3], **Ahmar Aziz**[3], **David T. Sharpe**[2], **Natalia V. Botchkareva**[1,*], and **Andrey A. Sharov**[3,*]

[1]Centre for Skin Sciences, University of Bradford, West Yorkshire, UK

[2]Plastic Surgery & Burns Research Unit, University of Bradford, West Yorkshire, UK

[3]Department of Dermatology, Boston University, Massachusetts, USA

Abstract

Bone morphogenetic protein (BMP) signalling plays a key role in the control of skin development and postnatal remodelling by regulating keratinocyte proliferation, differentiation and apoptosis. To study the role of BMPs in wound-induced epidermal repair, we used transgenic mice overexpressing the BMP downstream component Smad1 under the control of a K14 promoter as an *in vivo* model, as well as *ex vivo* and *in vitro* assays. *K14-caSmad1* mice exhibited retarded wound healing associated with significant inhibition of proliferation and increased apoptosis in healing wound epithelium. Furthermore, microarray and qRT-PCR analyses revealed decreased expression of a number of cytoskeletal/cell motility-associated genes including wound-associated keratins (*Krt16*, *Krt17*) and *Myo5a*, in the epidermis of *K14-caSmad1* mice versus wild-type controls during wound healing. BMP treatment significantly inhibited keratinocyte migration *ex vivo*, and primary keratinocytes of *K14-caSmad1* mice showed retarded migration compared to wild-type controls. Finally, siRNA-mediated silencing of Bmpr-1B in primary mouse keratinocytes accelerated cell migration and was associated with increased expression of Krt16, Krt17 and Myo5a compared to controls. Thus, this study demonstrates that BMPs inhibit keratinocyte proliferation, cytoskeletal organization and migration in regenerating skin epithelium during wound healing, and raises a possibility for using BMP antagonists for the management of chronic wounds.

[*]**Corresponding authors**, Dr. Andrey A. Sharov, Department of Dermatology, Boston University, 609 Albany Street, Boston, MA, USA, Tel: 617 638 5553; FAX: 617 638 5515; drsharov@bu.edu and Dr. Natalia V. Botchkareva, Centre for Skin Sciences, University of Bradford, Richmond Road, Bradford, West Yorkshire BD7 1DP, England, Tel: +44 (0) 1274 233 795; FAX: +44 (0) 1274 309 742; n.botchkareva@bradford.ac.uk.

Conflict of interest

The authors declare no conflict of interest.

Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily, playing key roles in the control of skin development and postnatal remodelling by regulating cell proliferation, differentiation and apoptosis (Botchkarev and Sharov, 2004; Miyazono *et al.*, 2010; Walsh *et al.*, 2010).

BMP signalling is activated by binding of BMP ligands to type I and type II serine-threonine kinase receptors (BMPRs) followed by activation of the BMP-Smad and/or BMP-MAP kinase pathways (Miyazono *et al.*, 2010). BMP signalling activity is controlled by endogenous antagonists, including Noggin (Walsh *et al.*, 2010), which preferentially binds BMP-2 and BMP-4 with very high affinities, preventing their interactions with receptors (Krause *et al.*, 2011; Zimmerman *et al.*, 1996).

During skin and hair follicle (HF) development, BMPs inhibit HF initiation (Botchkarev *et al.*, 1999; Jamora *et al.*, 2003). In adult HFs, BMP signalling is involved in the control of telogenanagen transition by maintaining bulge stem cells in a quiescent state through the binding of BMP-6 to BMPR-1A (Blanpain *et al.*, 2004), and suppressing Wnt/β-catenin and Hedgehog signalling pathways in the stem cell niche (Andl *et al.*, 2004; Botchkarev *et al.*, 2001; Kandyba *et al.*, 2013; Kobielak *et al.*, 2007; Ming Kwan *et al.*, 2004; Plikus *et al.*, 2008; Yuhki *et al.*, 2004; Zhang *et al.*, 2006).

In anagen HFs, BMP signalling inhibits proliferation and promotes differentiation of hair matrix keratinocytes into hair shaft and inner root sheath lineages (Andl *et al.*, 2004; Kobielak *et al.*, 2003; Kulessa *et al.*, 2000; Ming Kwan *et al.*, 2004; Sharov *et al.*, 2006; Yuhki *et al.*, 2004), as well as regulating melanogenesis (Park *et al.*, 2009; Sharov *et al.*, 2005; Singh *et al.*, 2012; Yaar *et al.*, 2006). Additionally, BMPs contribute to the regulation of epidermal differentiation by activating BMPR-1B which is expressed in the suprabasal epidermis (Hwang *et al.*, 2001; Sharov *et al.*, 2003; Yu *et al.*, 2010).

Wound healing is a dynamic process of overlapping phases of inflammation, proliferation, extracellular matrix formation, re-epithelialization and remodelling (Barrientos *et al.*, 2008; Li *et al.*, 2007; Schafer and Werner, 2007; Shaw and Martin, 2009). These stages are characterised by co-ordinated intrinsic cellular responses in keratinocytes, fibroblasts, neutrophils, macrophages, and endothelial cells regulated by an orchestra of mediators including platelet-derived growth factor, tumour necrosis factor-α, insulin-like growth factor-1, epidermal growth factor, TGF-α, vascular endothelial growth factor and platelet factor-IV (Falanga, 1993; Li *et al.*, 2007; Schafer and Werner, 2007).

Skin injury triggers immediate stress responses in epidermal keratinocytes, which begin to proliferate and migrate towards the wound forming a layer of hyper-proliferative epithelium (Falanga, 1993; Li *et al.*, 2007; Schafer and Werner, 2007; Taylor *et al.*, 2000). Stem cells residing in the HF infundibulum and bulge adjacent to the wound become inherently involved in the processes of epithelial regeneration, giving rise to daughter cells that migrate to the sites of injury and assist in skin repair (Cotsarelis, 2006; Hsu *et al.*, 2011; Ito and Cotsarelis, 2008; Ito *et al.*, 2005; Kasper *et al.*, 2011; Langton *et al.*, 2008; Levy *et al.*,

2007; Wong and Reiter, 2011). Similar to the HF, sweat gland stem cells also contribute to wound repair (Lu *et al.*, 2012).

Different members of TGF-beta superfamily also play important but yet distinct roles in the control of wound healing: TGF-beta signalling inhibits skin repair, at least in part by supressing keratinocyte migration, whereas activin signalling stimulates the wound healing response and promotes keratinocyte proliferation (Ashcroft *et al.*, 1999; Hosokawa R, et al., 2005; Wankell M, *et al.*, 2001; Munz *et al.*, 1999). Several indications suggest that BMP signalling can also be involved in the regulation of skin repair. BMP-6 expression has been shown to be induced in response to wounding in both keratinocytes and dermal fibroblasts; additionally, high BMP-6 levels have been found in chronic wounds (Kaiser *et al.*, 1998). Overexpression of BMP-6 in mouse epidermis results in delayed re-epithelialization (Kaiser *et al.*, 1998). It is also known that BMP-2 and BMP-4 exert negative effects on keratinocyte proliferation (Ahmed *et al.*, 2011; Sharov *et al.*, 2006). However, the expression of distinct BMP signalling components and the effects of BMPs and their antagonist Noggin in skin healing after injury remain to be explored.

In this paper, we provide evidence that BMP-2, BMP-4, BMP-7, BMP receptors, and antagonist Noggin exhibit a dynamic expression pattern during wound healing. By using *K14-caSmad1* mice and siRNA-mediated silencing of keratinocyte BMPRs *in vitro*, we demonstrate that BMPs inhibit keratinocyte proliferation, cytoskeletal organization and migration induced by BMPR-1B in regenerating skin epithelium during wound healing, and raises a possibility for using BMP antagonists for the management of chronic wounds.

Results

Bmp pathway components show differential expression patterns during wound healing

To understand the role of the Bmp-Smad pathway in wound repair, the expressions of Bmp ligands (Bmp-2, Bmp-4, Bmp-7), their receptors (Bmpr-1A, Bmpr-1B), intracellular component of the Bmp pathway phosphorylated-Smad-1/5/8 (p-Smad-1/5/8) and Noggin were examined at different stages after application of wounds to mouse skin containing all hair follicles at the telogen (resting) stage of the hair cycle. qRT-PCR analysis revealed significant ($p<0.01$) decreases in the expression of *Bmp-2*, *Bmp-4*, *Bmp-7* and *Noggin* ($p<0.001$) transcripts on days 3 and 5 after skin injury compared to unwounded skin (Figure 1a).

Immunofluorescent analysis showed that Bmpr-1A expression was restricted to the HF bulge in telogen skin (Figure 1b, Supplementary Figure S1a) (Botchkarev *et al.*, 2001; Kobielak *et al.*, 2003). Bmpr-1B expression was found in the basal and suprabasal layers of the epidermis, whilst it was not detected in the HF (Yu *et al.*, 2010) (Figure 1b; data not shown; Supplementary Figure S1a). The expression of pSmad-1/5/8 was seen throughout the epidermis and being more prominent in the suprabasal layers in telogen skin (Figure 1b, Supplementary Figure S1a).

During wound healing, Bmpr-1A expression was progressively decreased followed by its disappearance from the HFs immediately adjacent to the wound bed on day 5 and 7 after

injury, while it remained present in the bulge of HFs distant from the wound (Figure 1b, Supplementary Figure S1a). In contrast, there was prominent expression of Bmpr-1B and p-Smad-1/5/8 in the wound epithelial tongue and in the adjacent unwounded epidermis, HFs and dermal cells (Figure 1b, Supplementary Figure S1a).

Overexpression of Smad1 in the epidermis compromises wound healing

To elucidate a role for Bmp signalling in skin healing, *K14-caSmad1* transgenic (TG) mice overexpressing a constitutively active form of *Smad1* as a key component of the 'canonical' Bmp pathway were employed. *K14-caSmad1* mice were generated using a TG construct containing human K14 promoter, FLAG-tagged human cDNA encoding phospho-mimetic activated Smad1 in which the C-terminal SVS phosphorylation sites (S463 and S465) were mutated into EVE (Fuentealba *et al.*, 2007), and human growth hormone poly-A sequence (Sharov *et al.*, 2009) (Figure 2a). *K14-caSmad1* mice were viable, fertile and showed relatively normal skin and HF development (Supplementary Figure S1b). *K14-caSmad1* mice showed markedly increased *Smad1* expression in both the epidermis and HFs versus corresponding WT mice (Figure 2b, Supplementary Figure S1c). TG genotype was confirmed by Western blot detection of FLAG-tag expression in dorsal skin samples (Figure 2c).

Macroscopically, wound healing in *K14-caSmad1* mice was delayed compared to WT controls, with visibly larger skin wounds at time-matched points (Figure 2d). Histomorphological analysis of skin wounds confirmed that the areas covered by hyper-proliferative epithelium and the epithelial tongue length in *K14-caSmad1* mice were significantly smaller at days 3, 5 and 7 post-wounding than that of controls (Figure 2e, f, g, Supplementary Figure S1d).

K14-caSmad1 mice show altered proliferation/apoptosis and changes in cytoskeletal organization in the wound epithelium

To ascertain whether changes in the dynamics of epithelial regeneration observed in the *K14-caSmad1* mice were associated with altered keratinocyte proliferation and/or apoptosis, a quantitative analysis of Ki-67+ cells and cells positive for active caspase 3 was performed. In telogen skin of *K14-caSmad1* mice, the epidermis showed significantly fewer Ki-67+ keratinocytes (Figure 3a, b, Supplementary Figure S1e) than in the controls. During healing, there was no difference in *K14-caSmad1* wound epithelial proliferation at day 3, but there was a significantly lower proportion of Ki-67+ keratinocytes in *K14-caSmad1* wound epithelium on day 5 and day 7 (Figure 3a, b, Supplementary Figure S1e) after wounding compared to time-matched controls. In contrast, *K14-caSmad1* mice displayed a higher proportion of active caspase-3+ cells in the wound epithelium at days 3, 5 and 7 versus time-matched controls (Figure 3c, d, Supplementary Figure S1f).

Because injury-induced repair is associated with profound changes in cytoskeletal organization, we also examined the expressions of Keratin-16 (Krt16) and Keratin-17 (Krt17), whose expression is induced in response to wounding (Coulombe, 1997; Paladini *et al.*, 1996; Patel *et al.*, 2006). In contrast to Krt14, whose expression was not changed in the wound epithelium of *K14-caSmad1* versus WT mice (Supplementary Figure S1g); Krt16

and Krt17 expressions were dramatically reduced in *K14-caSmad1* mouse wounds compared to WT controls at days 3 and 5 post-wounding (Figure 3e, f, Supplementary Figure S1h, i). Analysis of keratinocyte morphology revealed that the epithelial tongue of WT mice contained more elongated keratinocytes (Figure 3e, f), an important characteristic of actively migrating cells in the wound epithelial tongue (Allard and Mogilner, 2013; Driscoll *et al.*, 2012; Meyer *et al.*, 2012). In contrast, epithelial cells in *K14-caSmad1* mice lost this flattened appearance and showed a more cuboidal shape (Figure 3e, f, Supplementary Figure S1h, i). This suggested that the delayed wound healing in *K14-caSmad1* mice may also be caused by impaired keratinocyte migration.

Global microarray analysis reveals changes in expression of cytoskeletal and cell migration-associated genes in the epidermis of *K14-caSmad1* mice

To define the genetic program regulated by Smad1 in epidermal keratinocytes in the context of the mechanisms underlying alterations in the wound healing in *K14-caSmad1* mice, global microarray analyses of the epidermal keratinocytes isolated from telogen skin of P20 WT and *K14-caSmad1* was performed, as described previously (Fessing et al., 2010; Mardaryev et al., 2011). Microarray data were validated by qRT-PCR analyses of RNA samples isolated from the epidermis of unwounded telogen skin, or from the wound epithelium obtained 3 or 5 days after wound infliction (Mardaryev et al., 2012).

Bioinformatic analyses of the microarray data revealed 2-fold and higher changes in expression of 1600 genes in the epidermis of *K14caSmad1* mice compared to WT controls (Figure 4a; Suppl Tables S1, S2). These genes belonged to different functional categories and encoded distinct adhesion/extracellular matrix molecules, cell cycle/apoptosis regulators, cytoskeletal/cell motility markers, metabolic enzymes, signalling/transcription regulators, etc. (Figure 4a; Suppl Tables S1, S2). Among these functional categories, significant enrichment ($p<0.05$) was found for the genes that encode cytoskeletal/cell motility-associated markers, and qRT-PCR validation showed significant downregulation in expression of the selected epidermal keratins (Krt1, Krt10, Krt16, Krt17) in the wound epithelium of *K14-caSmad1* mice compared to WT controls (Figure 4b).

Furthermore, transcripts for *Myosin VA* (*Myo5a*), an actin dependent protein required for cell motility (Cao *et al.*, 2004; Lan *et al.*, 2010; Sloane and Vartanian, 2007), as well as for other cell motility-associated genes, such as *Ablim2* and *Tubb6* (encoding actin-binding LIM protein family, member 2 and tubulin beta 6, respectively) were significantly more strongly down-regulated in *K14-caSmad1* mice in response to wounding than in controls (Figure 4b). These data suggested that excess of BMP-Smad1 signalling in epidermal keratinocytes inhibits wound healing, at least in part, via alterations in cytoskeletal organization and cell migration.

Bmp inhibitory effects on keratinocyte migration are mediated by Bmpr-1B

To elucidate the effects of Bmp signalling on keratinocyte migration, *ex vivo* skin explants (Mazzalupo *et al.*, 2002) were treated with Bmp-4/7, Noggin or their combination, and cell migratory area from the explants was measured at different time points. Keratinocyte migration was significantly retarded by Bmp-4/7 treatment at both day 5 ($p<0.02$) and day 7

($p<0.03$) compared to control explants (Figure 5a, b). Noggin negated this Bmp-induced inhibition of migration when explants were exposed to both treatments and restored keratinocyte movement back to that seen in controls (Figure 5 a, b). Interestingly, Noggin solo significantly ($p<0.01$) increased keratinocyte migration compared to controls, suggesting that antagonism of Bmp signalling accelerated cell migration in this model (Figure 5 a, b).

Modulation of Bmp activity in the keratinocytes also led to changes in cell morphology, which was determined by Alexa Fluor® 488-phalloidin staining detecting the endogenous actin filament network (Lengsfeld *et al.*, 1974; Meyer *et al.*, 2012; Wulf *et al.*, 1979). In the control group, the majority of migrating keratinocytes displayed an elongated appearance with actin fibres seen across the cell body (Figure 5c), whilst the majority of cells migrating from Bmp-4/7-treated explants had acquired a spherical shape (Figure 5c), and did not show a defined actin fibre network. Notably more polarised cell shapes were observed in Noggin treated samples (Figure 5c), which might reflect their accelerated migration (Figure 5b). Cell morphology of the keratinocytes co-treated with Bmp-4/7 and Noggin was similar to that seen in control cells (Figure 5c).

In addition, inhibitory effects of Bmp on cell migration were studied using transwell assay with primary mouse epidermal keratinocytes (PMEKs) as previously described (Merlo *et al.*, 2009; Yin *et al.*, 2005). Bmp-4/7 significantly slowed ($p<0.001$) PMEK migration compared to control keratinocytes, while it was negated when Bmp was co-administered with Noggin (Figure 5d). Noggin alone significantly ($p<0.04$) accelerated migration compared to controls (Figure 5d). Furthermore, cell migration was significantly ($p<0.0001$) inhibited in the keratinocytes obtained from *K14-caSmad1* versus WT mice (Figure 5e).

To further define the role that individual Bmp receptors play in the regulation of keratinocyte migration, PMEKs were transfected with siRNA to silence Bmpr-1A or Bmpr-1B and processed for transwell assay. Bmpr-1B silencing accelerated ($p<0.01$) PMEK migration compared to controls (Figure 5f); however, no effect was seen with Bmpr-1A silencing. In order to delineate which genes may be responsible for Bmpr-1B-mediated acceleration in migration, transfected PMEKs were processed for qRT-PCR. Following confirmation of significant ($p<0.001$) *Bmpr-1B* silencing by Bmpr-1B siRNA (Figure 5g), we found increased expression of *Myo5a* ($p<0.02$), *Krt16* ($p<0.01$) and *Krt17* ($p<0.001$) in Bmpr-1B-silenced keratinocytes compared to controls. This suggests that Bmp signalling can cause delayed re-epithelialization, at least in part, by inhibition of keratinocyte cytoskeletal organization and migration, the effects which are mediated, at least in part, by Bmpr-1B.

Discussion

In this study, we investigated the effects of BMP signalling on epidermal keratinocytes during skin repair. Using TG mice that overexpress Smad1 in basal epidermal keratinocytes, as well as *ex vivo* and *in vitro* models, we have illustrated that BMP signalling slows wound healing by suppressing keratinocyte proliferation and increasing apoptosis in the wound epithelium, as well as by attenuating keratinocyte migration, an effect at least in part

mediated via BMPR-1B. These data are consistent with previous observations that BMP signalling negates the facets of proliferation and migration seen in wound repair (Ahmed *et al.*, 2011; Kaiser *et al.*, 1998; Sharov *et al.*, 2006).

The expression pattern of Bmp receptors we observed in telogen skin was consistent with that previously described, with Bmpr-1A localised to the HF bulge (Blanpain and Fuchs, 2006) and Bmpr-1B in the suprabasal epidermis (Hwang *et al.*, 2001; Sharov *et al.*, 2003; Yu *et al.*, 2010), where they were co-localised with the downstream signalling component pSmad-1/5/8. The prominent expression of Bmpr-1A was consistent with its role in maintaining stem cell quiescence in conjunction with BMP ligands (Blanpain and Fuchs, 2006; Blanpain *et al.*, 2004; Fuchs, 2008; Kobielak *et al.*, 2007; Zhang *et al.*, 2006); indeed, the localised down-regulation of bulge Bmpr-1A expression in those HFs immediately adjacent to the wound, together with reduced levels of Bmp ligands and Smads, suggested a local suppression of the BMP axis in response to wounding (Mathura *et al.*, 2000). Thus, our data suggest that down-regulation of both BMP ligands and Bmpr-1A may facilitate an increase in HF stem cell activity, which accompanies wound healing (Botchkarev *et al.*, 1999; Cotsarelis, 2006; Hwang *et al.*, 2001; Ito and Cotsarelis, 2008; Ito *et al.*, 2005; Kobielak *et al.*, 2003; Plikus *et al.*, 2008; Wong and Reiter, 2011; Zhang *et al.*, 2006).

The extensive expression of Bmpr-1B and pSmad-1/5/8 in the wound epithelium implicates an involvement of BMP signalling in the control of skin repair. Within developing skin, BMPR-1B is involved in the control of cell differentiation of suprabasal epidermal layers (Botchkarev and Sharov, 2004; Fessing *et al.*, 2010; Pardali *et al.*, 2005; Plikus *et al.*, 2008). During wound healing, the expression pattern of Bmpr-1B also show cytoplasmic staining in the epithelial tongue keratinocytes, as opposed to that expected on the cell membrane, which was seen in unwounded epidermis. Intracellular localisation of both BMPRs has been described in human osteoblasts (Singhatanadgit *et al.*, 2008), with BMPR-1B in particular found in the peri-nuclear region, suggesting that in the absence of an appropriate ligand, BMPR-1B undergoes internalisation or represents newly synthesised receptors prior to their transport to the cell surface (Singhatanadgit *et al.*, 2008) to play roles in wound repair.

The *K14-caSmad1* mouse model used in this study provided *in vivo* evidence that constitutive BMP signal activation delays wound healing. These data are consistent with previous studies showing that other components of the TGF-beta signalling pathways, such as Smad2/3 and Smad4 negatively regulate skin repair (Ashcroft *et al.*, 1999; Flanders *et al.*, 2003; Hosokawa *et al.*, 2005; Tomikawa *et al.*, 2012; Yang *et al.*, 2012). Our data provide evidence that Smad1, a crucial downstream regulator of the BMP-Smad pathway (Botchkarev and Sharov, 2004), slows wound healing by attenuating keratinocyte proliferation and migration and augmenting wound epithelial apoptosis. It has previously been shown that activation of BMP signalling slows keratinocyte proliferation (Ahmed *et al.*, 2011; D'Souza *et al.*, 2001; Drozdoff *et al.*, 1994; Kaiser *et al.*, 1998; McDonnell *et al.*, 2001; Park and Morasso, 2002), whereas over-expression of the BMP antagonist Noggin results in epidermal hyperplasia due to hyperproliferation (Sharov *et al.*, 2009) and suppressed apoptosis (Sharov *et al.*, 2003). Thus, our study suggests that Smad1 has an important function in regulating epidermal homeostasis during skin repair.

Our data also assert that BMP signalling, and in particular BMPR-1B, negatively regulates keratinocyte migration during wound repair. Microarray analyses reveal down-regulated expression of a number of genes encoding distinct cytoskeletal proteins (Krt1, Krt10, Krt16, Krt17) and cell motility markers (Myo5a, Ablim2 and Tubb6) in the epidermis of *K14-caSmad1* mice versus WT controls. In *K14-caSmad1* mice, the epithelial tongue was shorter and contained few polarised migratory keratinocytes (Kurosaka and Kashina, 2008), suggesting that Smad1 activation slows migration. Furthermore, BMPR-1B knockdown in keratinocytes caused increased expression of Krt16 and Krt17, which are typically up-regulated following wounding and play roles in the cytoplasmic keratin network reorganisation (Hosokawa *et al.*, 2005; Mazzalupo *et al.*, 2003; McGowan and Coulombe, 1998; Moll *et al.*, 2008; Paladini *et al.*, 1996; Patel *et al.*, 2006; Tomic-Canic *et al.*, 1998; Wawersik and Coulombe, 2000; Wawersik *et al.*, 2001).

Consistently with these data, cultured BMP-treated keratinocytes lacked the defined cytoplasmic actin filament network required for movement, while Noggin-treated keratinocytes displayed an accelerated migration and showed elongated shape with endogenous actin filaments visible (Allard and Mogilner, 2013; Firat-Karalar and Welch, 2011; Fletcher and Mullins, 2010; Kurosaka and Kashina, 2008; Reymann *et al.*, 2012). In addition, Bmp signalling shows a negative effect on expression of Myo5a, an actin-dependent molecular motor involved in cell motility and metastasis (Cao *et al.*, 2004; Eppinga *et al.*, 2008; Kurosaka and Kashina, 2008; Lan *et al.*, 2010; Wang *et al.*, 1996). These data are consistent with previous results showing the inhibitory effects of the TGF-β family on cell migration during skin repair (Hosokawa *et al.*, 2005; Tsuboi *et al.*, 1992), as well as with data demonstrating that Smad2 (Hosokawa *et al.*, 2005), Smad3 (Ashcroft *et al.*, 1999; Flanders *et al.*, 2003) and Smad4 (Yang *et al.*, 2012) inhibit cell movement in other models.

However, additional studies including ChIP-seq and reporter assay analyses are required to define the complete set of the downstream target genes that are regulated by the BMP-Smad pathway in keratinocytes during wound healing. Together with results of this study, demonstrating that BMPs directly inhibit cell proliferation and migration in epidermal keratinocytes during wound healing, this analysis will help in the development of BMP antagonists for the management of chronic wounds.

Materials & Methods

Animals & tissue collection

Animal studies were performed under protocols approved by Boston University (USA) and Home Office Project License (UK). *K14-caSmad1* mice were generated on FVB background using a TG construct containing human K14 promoter, FLAG-tagged human cDNA encoding phospho-mimetic activated Smad1 (EVE) (provided by E. De Robertis) (Fuentealba *et al.*, 2007) and human growth hormone poly-A sequence (Sharov *et al.*, 2009). Skin samples were collected from dorsal skin of newborn mice, as well as from P20 mice with skin wounds (days 0, 3, 5 and 7 after wounding), as previously described (Kaiser *et al.*, 1998; Mardaryev *et al.*, 2011; Wong *et al.*, 2011). In each experiment, at least 4–5 mice of each strain per time point were used for analyses in both experimental and control groups.

Immunohistochemistry and Western Blotting

Formalin-fixed cryosections (10μM) were incubated with primary antibody (Suppl Table 3) overnight followed by application of the corresponding Alexa-546 or Alexa-555-labeled antibodies (Invitrogen, UK) for 45 min at 37°C. To detect endogenous and transgenic expression of Smad1 in *K14-caSmad1* and WT mice, rabbit polyclonal anti-Smad1 antibody (Abcam; Suppl Table S3) was used. Cell nuclei were counterstained with DAPI (Vector Labs, UK). Image analysis was performed using a fluorescent microscope in combination with DS-C1 digital camera and ACT-2U image analysis software (Nikon). Western blot analysis of total tissue proteins obtained from the extracts of full-thickness skin of *K14-caSmad1* and WT mice was performed using mouse monoclonal antibody EPR4759 against DKK1 (FLAG) epitope (Origene, Rockville, MD), as described (Sharov *et al.*, 2006).

Microarray and qRT-PCR analysis

For microarray analysis, total RNA was isolated from primary epidermal keratinocytes of P20 WT and TG mice using RNeasy kit (Qiagen, UK), and processed for one-round RNA amplification using RiboAmp RNA Amplification Kit (Molecular Devices, USA). Gene expression array analysis was performed by Mogene LLC (St. Louis, MO, USA) using 44K Whole Mouse Genome 60-mer oligo-microarray (manufactured by Agilent Technologies). Functional annotation of the overrepresented and underrepresented genes was performed as described before (Fessing et al., 2010; Sharov et al., 2009) using the NIA Array Analysis software (http://lgsun.grc.nia.nih.gov/ANOVA/), and enrichment of the genes in different functional categories was assessed by using the hypergeometric or Fisher's exact tests. Microarray data has been deposited to the Gene Expression Omnibus (GEO). For qRT-PCR, total RNA was isolated from snap-frozen samples of full-thickness wounds using TRIzol (Invitrogen, UK) (Mardaryev *et al.*, 2010) followed by conversion into cDNA using Reverse Transcription System (Promega, UK). PCR primers were designed with Beacon Designer software (Premier Biosoft, Palo Alto; Suppl Table S4). qRT-PCR was performed on MyiQ single-colour real-time PCR detection system (Bio-Rad, UK) using SYBR Green master mix (Applied Biosystems, UK). Differences between samples and controls were calculated using the Genex database software (Bio-Rad, UK) based on the Ct ($^{\Delta\Delta}C_t$) equitation method and normalized to *Gapdh*. Data from triplicates was pooled and statistical analysis was performed using unpaired Student's t test.

Quantitative wound histomorphometry

Wound samples (n=8–10 from each strain) were processed for H&AP staining (Sharov *et al.*, 2006), and analysed using VisiCam (VWR International, UK) software. The epithelial tongue area (μm^2) and length (μm) were measured and compared at time-matched intervals. To assess cell proliferation and apoptosis, the number of Ki-67+, caspase-3+ and DAPI+ cells was counted along the basal layer of the wound epithelial tongue or intact epidermis at time-matched intervals using ImageJ software (National Institutes of Health, Bethesda) as previously described (Mardaryev *et al.*, 2011). Statistical analysis was performed using unpaired Student's *t*-test; differences were deemed significant if $p<0.05$.

Ex vivo skin explant migration assay

Explant migration assay was performed as previously described (Mazzalupo *et al.*, 2002). Explants were treated daily with 1μg/ml recombinant Bmp-4/7 (R&D systems), 600ng/ml Noggin (R&D systems), Bmp-4/7 1μg/ml and Noggin 600ng/ml combined or BSA; all treatments were performed in triplicate. Photomicrographs were taken every 48 hours using a light microscope (Leitz Labovert). Image analysis was performed using the Visicam software (VWR International, UK); the migratory area (mm^2) of PMEKs from the skin explant was measured at each time-point. Following fixation, skin discs were removed and the remaining keratinocytes were stained with Alexa Fluor® 488-phalloidin antibody (Invitrogen, UK).

Cell culture and Transwell migration assay

Primary mouse epidermal keratinocytes (PMEKs) were prepared from newborn mice as described previously (Lichti *et al.*, 2008) and were grown in Eagle's minimal essential medium (EMEM) (Lonza, UK) supplemented with 4% chelated foetal bovine serum (Gibco, UK). Transwell assay was performed as previously described (Merlo *et al.*, 2009; Yin *et al.*, 2005). Following attachment, PMEKs were either transfected with mouse smartpool siRNAs directed against Bmpr-1A or Bmpr-1B and a non-targeting control (Thermo Scientific, UK) using Lipofectamine RNAimax (Invitrogen, UK) according to the manufacturers' protocol, or treated with 1μg/ml recombinant Bmp-4/7 (R&D systems), 600ng/ml Noggin (R&D systems), Bmp-4/7 1μg/ml and Noggin 600ng/ml combined or BSA; all transfection and treatments were performed in duplicate (Ahmed *et al.*, 2011). PMEKs were allowed to migrate over 48 hours through the insert membrane, after which cells adherent to the top surface of the membrane were removed with a cotton swab; cells that had migrated to the bottom surface were formalin-fixed and counterstained with DAPI (Vector Labs, UK). The number of DAPI+ nuclei of migrated PMEKs per microscopic field (10 randomly selected fields/transwell from two transwells per experiment) was counted and compared. Statistical analysis was performed using unpaired Student's *t*-test; differences were deemed significant if $p<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, part of the National Institutes of Health, under Award Number (K01AR056771, AR049778) to AAS. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This study was also supported in part by the Medical Research Council UK via grant MR/K011324/1 to NVB, and by charitable donations to the Bradford Plastic Surgery & Burns Research Unit. We thank Dr. Eddy M. De Robertis (Howard Hughes Medical Institute, University of California, Los Angeles) for plasmid encoding caSmad1 cDNA.

Abbreviations

BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
HF	Hair follicle
K14-caSmad1	transgenic mice over-expressing a constitutively active form of Smad1 under K14 promoter
Krt	keratin
Myo5a	Myosin VA
WT	wild-type

References

Ahmed MI, Mardaryev AN, Lewis CJ, Sharov AA, Botchkareva NV. MicroRNA-21 is an important downstream component of BMP signalling in epidermal keratinocytes. J Cell Sci. 2011; 124:3399–3404. [PubMed: 21984808]

Allard J, Mogilner A. Traveling waves in actin dynamics and cell motility. Current Opinion in Cell Biology. 2013; 25:107–115. [PubMed: 22985541]

Andl T, Ahn K, Kairo A, Chu EY, Wine-Lee L, Reddy ST, et al. Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development. Development. 2004; 131:2257–2268. [PubMed: 15102710]

Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JL, Mizel DE, et al. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol. 1999; 1:260–206. [PubMed: 10559937]

Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen. 2008; 16:585–601. [PubMed: 19128254]

Blanpain C, Fuchs E. Epidermal stem cells of the skin. Annu Rev Cell Dev Biol. 2006; 22:339–373. [PubMed: 16824012]

Blanpain C, Lowry WE, Geoghegan A, Polak L, Fuchs E. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell. 2004; 118:635–648. [PubMed: 15339667]

Botchkarev VA, Botchkareva NV, Nakamura M, Huber O, Funa K, Lauster R, et al. Noggin is required for induction of hair follicle growth phase in postnatal skin. FASEB J. 2001; 15:2205–2214. [PubMed: 11641247]

Botchkarev VA, Botchkareva NV, Roth W, Nakamura M, Chen LH, Herzog W, et al. Noggin is a mesenchymally derived stimulator of hair-follicle induction. Nat Cell Biol. 1999; 1:158–164. [PubMed: 10559902]

Botchkarev VA, Sharov AA. BMP signaling in the control of skin development and hair follicle growth. Differentiation. 2004; 72:512–526. [PubMed: 15617562]

Cao TT, Chang W, Masters SE, Mooseker MS. Myosin-Va binds to and mechanochemically couples microtubules to actin filaments. Molecular biology of the cell. 2004; 15:151–161. [PubMed: 14565972]

Cotsarelis G. Epithelial stem cells: a folliculocentric view. J Invest Dermatol. 2006; 126:1459–1468. [PubMed: 16778814]

Coulombe PA. Towards a molecular definition of keratinocyte activation after acute injury to stratified epithelia. Biochem Biophys Res Commun. 1997; 236:231–238. [PubMed: 9240415]

D'Souza SJ, Pajak A, Balazsi K, Dagnino L. Ca2+ and BMP-6 signaling regulate E2F during epidermal keratinocyte differentiation. The Journal of biological chemistry. 2001; 276:23531–23538. [PubMed: 11319226]

Driscoll MK, McCann C, Kopace R, Homan T, Fourkas JT, Parent C, et al. Cell shape dynamics: from waves to migration. PLoS computational biology. 2012; 8:e1002392. [PubMed: 22438794]

Drozdoff V, Wall NA, Pledger WJ. Expression and growth inhibitory effect of decapentaplegic Vg-related protein 6: evidence for a regulatory role in keratinocyte differentiation. Proc Natl Acad Sci U S A. 1994; 91:5528–5532. [PubMed: 7515504]

Eppinga RD, Peng IF, Lin JL, Wu CF, Lin JJ. Opposite effects of overexpressed myosin Va or heavy meromyosin Va on vesicle distribution, cytoskeleton organization, and cell motility in nonmuscle cells. Cell motility and the cytoskeleton. 2008; 65:197–215. [PubMed: 18044718]

Falanga V. Growth factors and wound healing. The Journal of dermatologic surgery and oncology. 1993; 19:711–714. [PubMed: 8349910]

Fessing MY, Atoyan R, Shander B, Mardaryev AN, Botchkarev VV Jr. Poterlowicz K, et al. BMP signaling induces cell-type-specific changes in gene expression programs of human keratinocytes and fibroblasts. J Invest Dermatol. 2010; 130:398–404. [PubMed: 19710687]

Firat-Karalar EN, Welch MD. New mechanisms and functions of actin nucleation. Current Opinion in Cell Biology. 2011; 23:4–13. [PubMed: 21093244]

Flanders KC, Major CD, Arabshahi A, Aburime EE, Okada MH, Fujii M, et al. Interference with transforming growth factor-beta/ Smad3 signaling results in accelerated healing of wounds in previously irradiated skin. Am J Pathol. 2003; 163:2247–2257. [PubMed: 14633599]

Fletcher DA, Mullins RD. Cell mechanics and the cytoskeleton. Nature. 2010; 463:485–492. [PubMed: 20110992]

Fuchs E. Skin stem cells: rising to the surface. J Cell Biol. 2008; 180:273–284. [PubMed: 18209104]

Fuentealba LC, Eivers E, Ikeda A, Hurtado C, Kuroda H, Pera EM, et al. Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. Cell. 2007; 131:980–993. [PubMed: 18045539]

Hosokawa R, Urata MM, Ito Y, Bringas P Jr. Chai Y. Functional significance of Smad2 in regulating basal keratinocyte migration during wound healing. J Invest Dermatol. 2005; 125:1302–1309. [PubMed: 16354202]

Hsu YC, Pasolli HA, Fuchs E. Dynamics between stem cells, niche, and progeny in the hair follicle. Cell. 2011; 144:92–105. [PubMed: 21215372]

Hwang EA, Lee HB, Tark KC. Comparison of bone morphogenetic protein receptors expression in the fetal and adult skin. Yonsei medical journal. 2001; 42:581–586. [PubMed: 11754140]

Ito M, Cotsarelis G. Is the hair follicle necessary for normal wound healing? J Invest Dermatol. 2008; 128:1059–1061. [PubMed: 18408743]

Ito M, Liu Y, Yang Z, Nguyen J, Liang F, Morris RJ, et al. Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. Nature medicine. 2005; 11:1351–1354.

Jamora C, DasGupta R, Kocieniewski P, Fuchs E. Links between signal transduction, transcription and adhesion in epithelial bud development. Nature. 2003; 422:317–322. [PubMed: 12646922]

Kaiser S, Schirmacher P, Philipp A, Protschka M, Moll I, Nicol K, et al. Induction of bone morphogenetic protein-6 in skin wounds. Delayed reepitheliazation and scar formation in BMP-6 overexpressing transgenic mice. J Invest Dermatol. 1998; 111:1145–1152. [PubMed: 9856831]

Kandyba E, Leung Y, Chen YB, Widelitz R, Chuong CM, Kobielak K. Competitive balance of intrabulge BMP/Wnt signaling reveals a robust gene network ruling stem cell homeostasis and cyclic activation. Proc Natl Acad Sci U S A. 2013; 110:1351–1356. [PubMed: 23292934]

Kasper M, Jaks V, Are A, Bergstrom A, Schwager A, Svard J, et al. Wounding enhances epidermal tumorigenesis by recruiting hair follicle keratinocytes. Proc Natl Acad Sci U S A. 2011; 108:4099–4104. [PubMed: 21321199]

Kobielak K, Pasolli HA, Alonso L, Polak L, Fuchs E. Defining BMP functions in the hair follicle by conditional ablation of BMP receptor IA. J Cell Biol. 2003; 163:609–623. [PubMed: 14610062]

Kobielak K, Stokes N, de la Cruz J, Polak L, Fuchs E. Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. Proc Natl Acad Sci U S A. 2007; 104:10063–10068. [PubMed: 17553962]

Krause C, Guzman A, Knaus P. Noggin. The international journal of biochemistry & cell biology. 2011; 43:478–481. [PubMed: 21256973]

Kulessa H, Turk G, Hogan BL. Inhibition of Bmp signaling affects growth and differentiation in the anagen hair follicle. EMBO J. 2000; 19:6664–6674. [PubMed: 11118201]

Kurosaka S, Kashina A. Cell biology of embryonic migration. Birth defects research Part C, Embryo today : reviews. 2008; 84:102–122.

Lan L, Han H, Zuo H, Chen Z, Du Y, Zhao W, et al. Upregulation of myosin Va by Snail is involved in cancer cell migration and metastasis. International journal of cancer Journal international du cancer. 2010; 126:53–64. [PubMed: 19521958]

Langton AK, Herrick SE, Headon DJ. An extended epidermal response heals cutaneous wounds in the absence of a hair follicle stem cell contribution. J Invest Dermatol. 2008; 128:1311–1318. [PubMed: 18037901]

Lengsfeld AM, Low I, Wieland T, Dancker P, Hasselbach W. Interaction of phalloidin with actin. Proc Natl Acad Sci U S A. 1974; 71:2803–2807. [PubMed: 4368830]

Levy V, Lindon C, Zheng Y, Harfe BD, Morgan BA. Epidermal stem cells arise from the hair follicle after wounding. FASEB J. 2007; 21:1358–1366. [PubMed: 17255473]

Li J, Chen J, Kirsner R. Pathophysiology of acute wound healing. Clinics in dermatology. 2007; 25:9–18. [PubMed: 17276196]

Lichti U, Anders J, Yuspa SH. Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice. Nature protocols. 2008; 3:799–810. [PubMed: 18451788]

Lu CP, Polak L, Rocha AS, Pasolli HA, Chen SC, Sharma N, et al. Identification of stem cell populations in sweat glands and ducts reveals roles in homeostasis and wound repair. Cell. 2012; 150:136–150. [PubMed: 22770217]

Mardaryev AN, Ahmed MI, Vlahov NV, Fessing MY, Gill JH, Sharov AA, et al. Micro-RNA-31 controls hair cycle-associated changes in gene expression programs of the skin and hair follicle. FASEB J. 2010; 24:3869–3881. [PubMed: 20522784]

Mardaryev AN, Meier N, Poterlowicz K, Sharov AA, Sharova TY, Ahmed MI, et al. Lhx2 differentially regulates Sox9, Tcf4 and Lgr5 in hair follicle stem cells to promote epidermal regeneration after injury. Development. 2011; 138:4843–4852. [PubMed: 22028024]

Mathura JR Jr. Jafari N, Chang JT, Hackett SF, Wahlin KJ, Della NG, et al. Bone morphogenetic proteins-2 and −4: negative growth regulators in adult retinal pigmented epithelium. Investigative Ophthalmology & Visual Science. 2000; 41:592–600. [PubMed: 10670493]

Mazzalupo S, Wawersik MJ, Coulombe PA. An ex vivo assay to assess the potential of skin keratinocytes for wound epithelialization. J Invest Dermatol. 2002; 118:866–870. [PubMed: 11982766]

Mazzalupo S, Wong P, Martin P, Coulombe PA. Role for keratins 6 and 17 during wound closure in embryonic mouse skin. Developmental Dynamics. 2003; 226:356–365. [PubMed: 12557214]

McDonnell MA, Law BK, Serra R, Moses HL. Antagonistic effects of TGFbeta1 and BMP-6 on skin keratinocyte differentiation. Experimental Cell Research. 2001; 263:265–273. [PubMed: 11161725]

McGowan KM, Coulombe PA. Onset of keratin 17 expression coincides with the definition of major epithelial lineages during skin development. J Cell Biol. 1998; 143:469–486. [PubMed: 9786956]

Merlo S, Frasca G, Canonico PL, Sortino MA. Differential involvement of estrogen receptor alpha and estrogen receptor beta in the healing promoting effect of estrogen in human keratinocytes. The Journal of Endocrinology. 2009; 200:189–197. [PubMed: 19008331]

Meyer M, Muller AK, Yang J, Moik D, Ponzio G, Ornitz DM, et al. FGF receptors 1 and 2 are key regulators of keratinocyte migration in vitro and in wounded skin. J Cell Sci. 2012; 125:5690–5701. [PubMed: 22992463]

Ming Kwan K, Li AG, Wang XJ, Wurst W, Behringer RR. Essential roles of BMPR-IA signaling in differentiation and growth of hair follicles and in skin tumorigenesis. Genesis. 2004; 39:10–25. [PubMed: 15124223]

Miyazono K, Kamiya Y, Morikawa M. Bone morphogenetic protein receptors and signal transduction. J Biochem. 2010; 147:35–51. [PubMed: 19762341]

Moll R, Divo M, Langbein L. The human keratins: biology and pathology. Histochemistry and Cell Biology. 2008; 129:705–733. [PubMed: 18461349]

Munz B, Smola H, Engelhardt F, Bleuel K, Brauchle M, Lein I, et al. Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair. EMBO J. 1999; 18:5205–5215. [PubMed: 10508154]

Paladini RD, Takahashi K, Bravo NS, Coulombe PA. Onset of re-epithelialization after skin injury correlates with a reorganization of keratin filaments in wound edge keratinocytes: defining a potential role for keratin 16. J Cell Biol. 1996; 132:381–397. [PubMed: 8636216]

Pardali K, Kowanetz M, Heldin CH, Moustakas A. Smad pathway-specific transcriptional regulation of the cell cycle inhibitor p21(WAF1/Cip1). Journal of Cellular Physiology. 2005; 204:260–272. [PubMed: 15690394]

Park GT, Morasso MI. Bone morphogenetic protein-2 (BMP-2) transactivates Dlx3 through Smad1 and Smad4: alternative mode for Dlx3 induction in mouse keratinocytes. Nucleic Acids Research. 2002; 30:515–522. [PubMed: 11788714]

Park HY, Wu C, Yaar M, Stachur CM, Kosmadaki M, Gilchrest BA. Role of BMP-4 and Its Signaling Pathways in Cultured Human Melanocytes. International Journal of Cell Biology. 2009; 2009:750482. [PubMed: 20130821]

Patel GK, Wilson CH, Harding KG, Finlay AY, Bowden PE. Numerous keratinocyte subtypes involved in wound re-epithelialization. J Invest Dermatol. 2006; 126:497–502. [PubMed: 16374449]

Plikus MV, Mayer JA, de la Cruz D, Baker RE, Maini PK, Maxson R, et al. Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. Nature. 2008; 451:340–344. [PubMed: 18202659]

Reymann AC, Boujemaa-Paterski R, Martiel JL, Guerin C, Cao W, Chin HF, et al. Actin network architecture can determine myosin motor activity. Science. 2012; 336:1310–1314. [PubMed: 22679097]

Schafer M, Werner S. Transcriptional control of wound repair. Annu Rev Cell Dev Biol. 2007; 23:69–92. [PubMed: 17474876]

Sharov AA, Fessing M, Atoyan R, Sharova TY, Haskell-Luevano C, Weiner L, et al. Bone morphogenetic protein (BMP) signaling controls hair pigmentation by means of cross-talk with the melanocortin receptor-1 pathway. Proc Natl Acad Sci U S A. 2005; 102:93–98. [PubMed: 15618398]

Sharov AA, Mardaryev AN, Sharova TY, Grachtchouk M, Atoyan R, Byers HR, et al. Bone morphogenetic protein antagonist noggin promotes skin tumorigenesis via stimulation of the Wnt and Shh signaling pathways. Am J Pathol. 2009; 175:1303–1314. [PubMed: 19700758]

Sharov AA, Sharova TY, Mardaryev AN, Tommasi di Vignano A, Atoyan R, Weiner L, et al. Bone morphogenetic protein signaling regulates the size of hair follicles and modulates the expression of cell cycle-associated genes. Proc Natl Acad Sci U S A. 2006; 103:18166–18171. [PubMed: 17114283]

Sharov AA, Weiner L, Sharova TY, Siebenhaar F, Atoyan R, Reginato AM, et al. Noggin overexpression inhibits eyelid opening by altering epidermal apoptosis and differentiation. EMBO J. 2003; 22:2992–3003. [PubMed: 12805214]

Shaw TJ, Martin P. Wound repair at a glance. J Cell Sci. 2009; 122:3209–3213. [PubMed: 19726630]

Singh SK, Abbas WA, Tobin DJ. Bone morphogenetic proteins differentially regulate pigmentation in human skin cells. J Cell Sci. 2012; 125:4306–4319. [PubMed: 22641693]

Singhatanadgit W, Mordan N, Salih V, Olsen I. Changes in bone morphogenetic protein receptor-IB localisation regulate osteogenic responses of human bone cells to bone morphogenetic protein-2. The International Journal of Biochemistry & Cell Biology. 2008; 40:2854–2864. [PubMed: 18619554]

Sloane JA, Vartanian TK. Myosin Va controls oligodendrocyte morphogenesis and myelination. The Journal of Neuroscience. 2007; 27:11366–11375. [PubMed: 17942731]

Song Q, Mehler MF, Kessler JA. Bone morphogenetic proteins induce apoptosis and growth factor dependence of cultured sympathoadrenal progenitor cells. Developmental Biology. 1998; 196:119–127. [PubMed: 9527885]

Taylor G, Lehrer MS, Jensen PJ, Sun TT, Lavker RM. Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell. 2000; 102:451–461. [PubMed: 10966107]

Tomic-Canic M, Komine M, Freedberg IM, Blumenberg M. Epidermal signal transduction and transcription factor activation in activated keratinocytes. Journal of Dermatological Science. 1998; 17:167–181. [PubMed: 9697045]

Tomikawa K, Yamamoto T, Shiomi N, Shimoe M, Hongo S, Yamashiro K, et al. Smad2 decelerates re-epithelialization during gingival wound healing. Journal of Dental Research. 2012; 91:764–770. [PubMed: 22699208]

Tsuboi R, Sato C, Shi CM, Ogawa H. Stimulation of keratinocyte migration by growth factors. The Journal of Dermatology. 1992; 19:652–653. [PubMed: 1293147]

Wach S, Schirmacher P, Protschka M, Blessing M. Overexpression of bone morphogenetic protein-6 (BMP-6) in murine epidermis suppresses skin tumor formation by induction of apoptosis and downregulation of fos/jun family members. Oncogene. 2001; 20:7761–7769. [PubMed: 11753654]

Walsh DW, Godson C, Brazil DP, Martin F. Extracellular BMP-antagonist regulation in development and disease: tied up in knots. Trends Cell Biol. 2010; 20:244–256. [PubMed: 20188563]

Wang FS, Wolenski JS, Cheney RE, Mooseker MS, Jay DG. Function of myosin-V in filopodial extension of neuronal growth cones. Science. 1996; 273:660–663. [PubMed: 8662560]

Wankell M, Munz B, Hübner G, Hans W, Wolf E, Goppelt A, et al. Impaired wound healing in transgenic mice overexpressing the activin antagonist follistatin in the epidermis. EMBO J. 2001; 20:5361–5372. [PubMed: 11574468]

Wawersik M, Coulombe PA. Forced expression of keratin 16 alters the adhesion, differentiation, and migration of mouse skin keratinocytes. Molecular Biology of the Cell. 2000; 11:3315–3327. [PubMed: 11029038]

Wawersik MJ, Mazzalupo S, Nguyen D, Coulombe PA. Increased levels of keratin 16 alter epithelialization potential of mouse skin keratinocytes in vivo and ex vivo. Molecular Biology of the Cell. 2001; 12:3439–3450. [PubMed: 11694579]

Wong SY, Reiter JF. Wounding mobilizes hair follicle stem cells to form tumors. Proc Natl Acad Sci U S A. 2011; 108:4093–4098. [PubMed: 21321207]

Wong VW, Sorkin M, Glotzbach JP, Longaker MT, Gurtner GC. Surgical approaches to create murine models of human wound healing. Journal of Biomedicine & Biotechnology. 2011; 2011:969618. [PubMed: 21151647]

Wulf E, Deboben A, Bautz FA, Faulstich H, Wieland T. Fluorescent phallotoxin, a tool for the visualization of cellular actin. Proc Natl Acad Sci U S A. 1979; 76:4498–4502. [PubMed: 291981]

Yaar M, Wu C, Park HY, Panova I, Schutz G, Gilchrest BA. Bone morphogenetic protein-4, a novel modulator of melanogenesis. The Journal of Biological Chemistry. 2006; 281:25307–25314. [PubMed: 16837459]

Yang L, Li W, Wang S, Wang L, Li Y, Yang X, et al. Smad4 disruption accelerates keratinocyte reepithelialization in murine cutaneous wound repair. Histochemistry and Cell Biology. 2012; 138:573–582. [PubMed: 22644379]

Yin T, Getsios S, Caldelari R, Kowalczyk AP, Muller EJ, Jones JC, et al. Plakoglobin suppresses keratinocyte motility through both cell-cell adhesion-dependent and -independent mechanisms. Proc Natl Acad Sci U S A. 2005; 102:5420–5425. [PubMed: 15805189]

Yokouchi Y, Sakiyama J, Kameda T, Iba H, Suzuki A, Ueno N, et al. BMP-2/-4 mediate programmed cell death in chicken limb buds. Development. 1996; 122:3725–3734. [PubMed: 9012494]

Yu X, Espinoza-Lewis RA, Sun C, Lin L, He F, Xiong W, et al. Overexpression of constitutively active BMP-receptor-IB in mouse skin causes an ichthyosis-vulgaris-like disease. Cell and Tissue Research. 2010; 342:401–410. [PubMed: 21079999]

Yuhki M, Yamada M, Kawano M, Iwasato T, Itohara S, Yoshida H, et al. BMPR1A signaling is necessary for hair follicle cycling and hair shaft differentiation in mice. Development. 2004; 131:1825–1833. [PubMed: 15084466]

Zhang J, He XC, Tong WG, Johnson T, Wiedemann LM, Mishina Y, et al. Bone morphogenetic protein signaling inhibits hair follicle anagen induction by restricting epithelial stem/progenitor cell activation and expansion. Stem Cells. 2006; 24:2826–2839. [PubMed: 16960130]

Zimmerman LB, De Jesus-Escobar JM, Harland RM. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell. 1996; 86:599–606. [PubMed: 8752214]

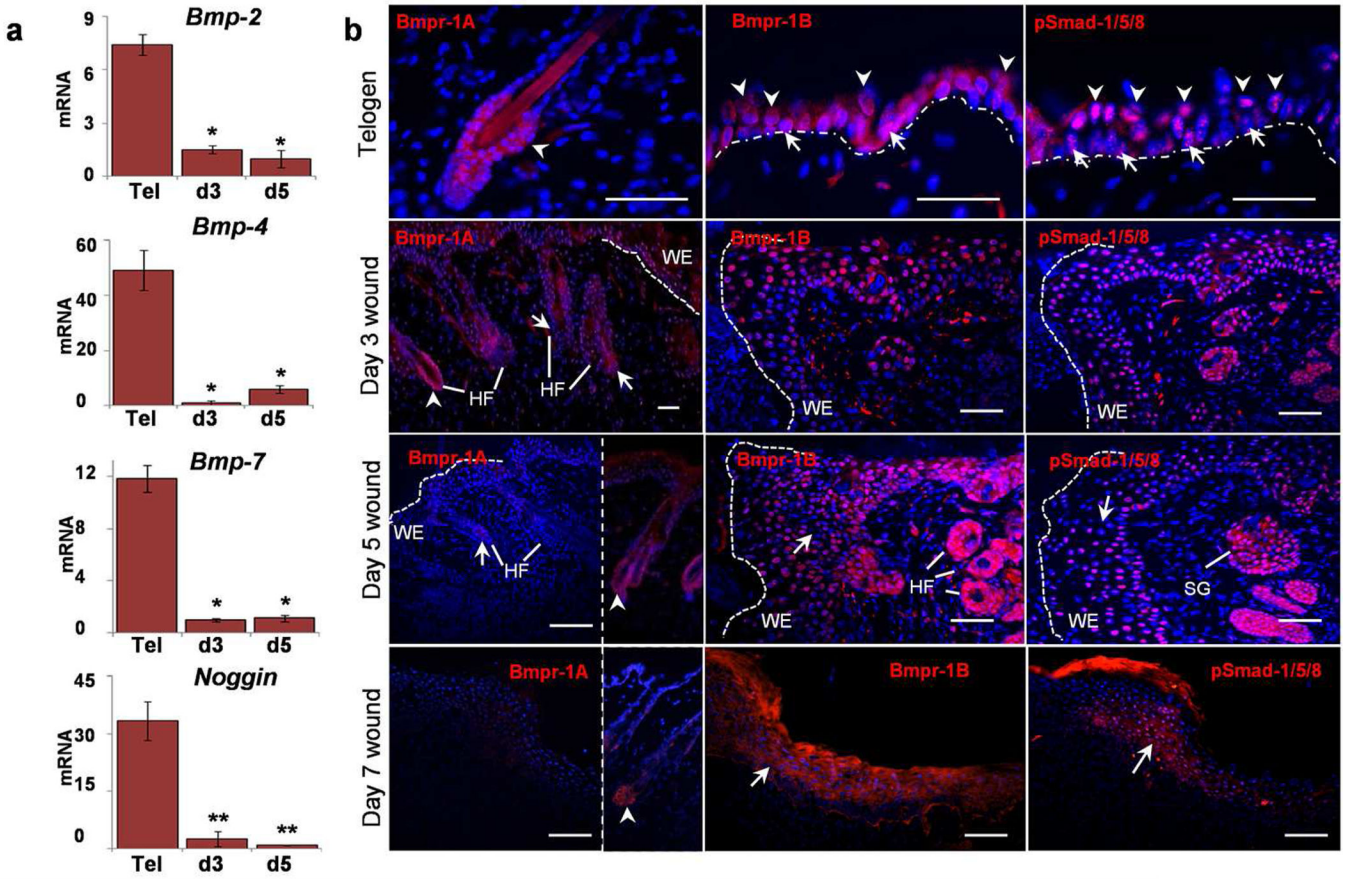

Figure 1. Expression of Bmp pathway components during skin healing

(**a**) qRT-PCR: significant decreases of *Bmp-2*, *Bmp-4*, *Bmp-7* (*p<0.01) and *noggin* (**p<0.001) transcripts on days 3 and 5 post-wounding; (**b**) Immunofluorescence: In telogen skin, Bmpr-1A expression is restricted to the HF bulge (arrowhead); Bmpr-1B is seen in the basal (arrows) and suprabasal epidermal layers (arrowheads); pSmad-1/5/8 is expressed in the basal (arrows) and more prominently in suprabasal epidermal layers (arrowheads). On days 3, 5 and 7 post-wounding, Bmpr-1A expression is low in HF bulges near the wound (arrowed) but remains strongly expressed in those further from the wound (inset, arrowhead); there is strong expression of Bmpr-1B and pSmad-1/5/8 in the wound epithelial tongue and the adjacent unwounded epidermis (arrows); (mean ± SD, *p<0.01, **p<0.0001, Student's t-test). HF – hair follicle, SG – sebaceous gland, WE – wound epithelium, scale bar 100μm.

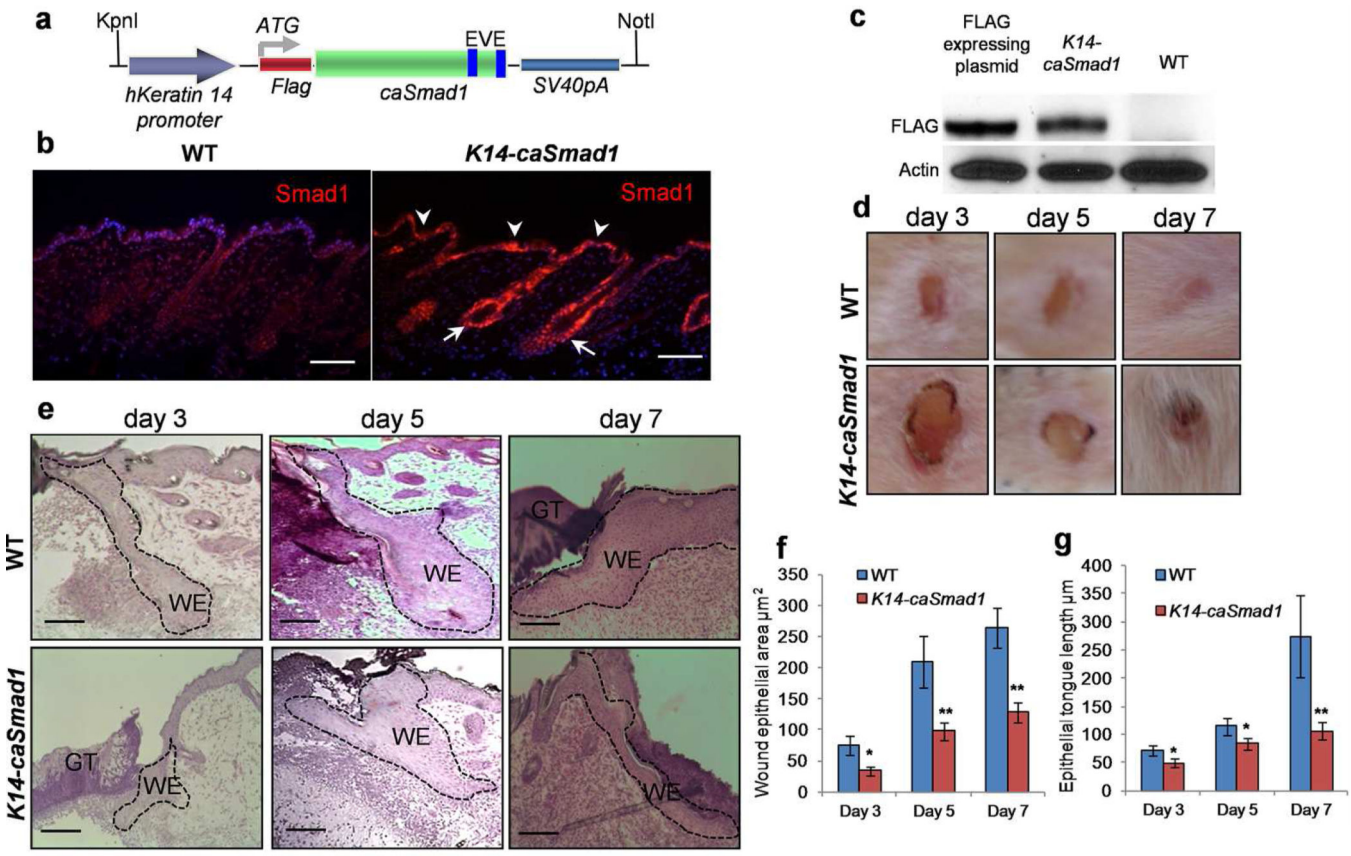

Figure 2. Histomorphological analysis of wound epithelium in *K14-caSmad1* and WT mice

(**a**) Transgenic construct used to generate *K14-caSmad1* mice; (**b**) *K14-caSmad1* mice show markedly increased Smad1 expression in the epidermis and HFs versus control mice as detected by anti-Smad1 antibody; (**c**) Western blot confirmation of FLAG-tag expression in dorsal skin of transgenic *K14-caSmad1* mice versus controls; (**d**) Representative images of macroscopic wound appearance and (**e**) wound histology in *K14-caSmad1* and WT mice 3, 5, and 7 days post-wounding; (**f**) significantly reduced area of wound epithelium in *K14-Smad1* mice on days 3, 5 and 7 after wounding versus WT controls; (**g**) significantly reduced wound epithelial tongue length in *K14-caSmad1* mice on days 3, 5, and 7 post-wounding (mean ± SD, *p<0.01, **p<0.0001, Student's t-test). GT – granulation tissue, WE – wound epithelium, scale bar 100µm.

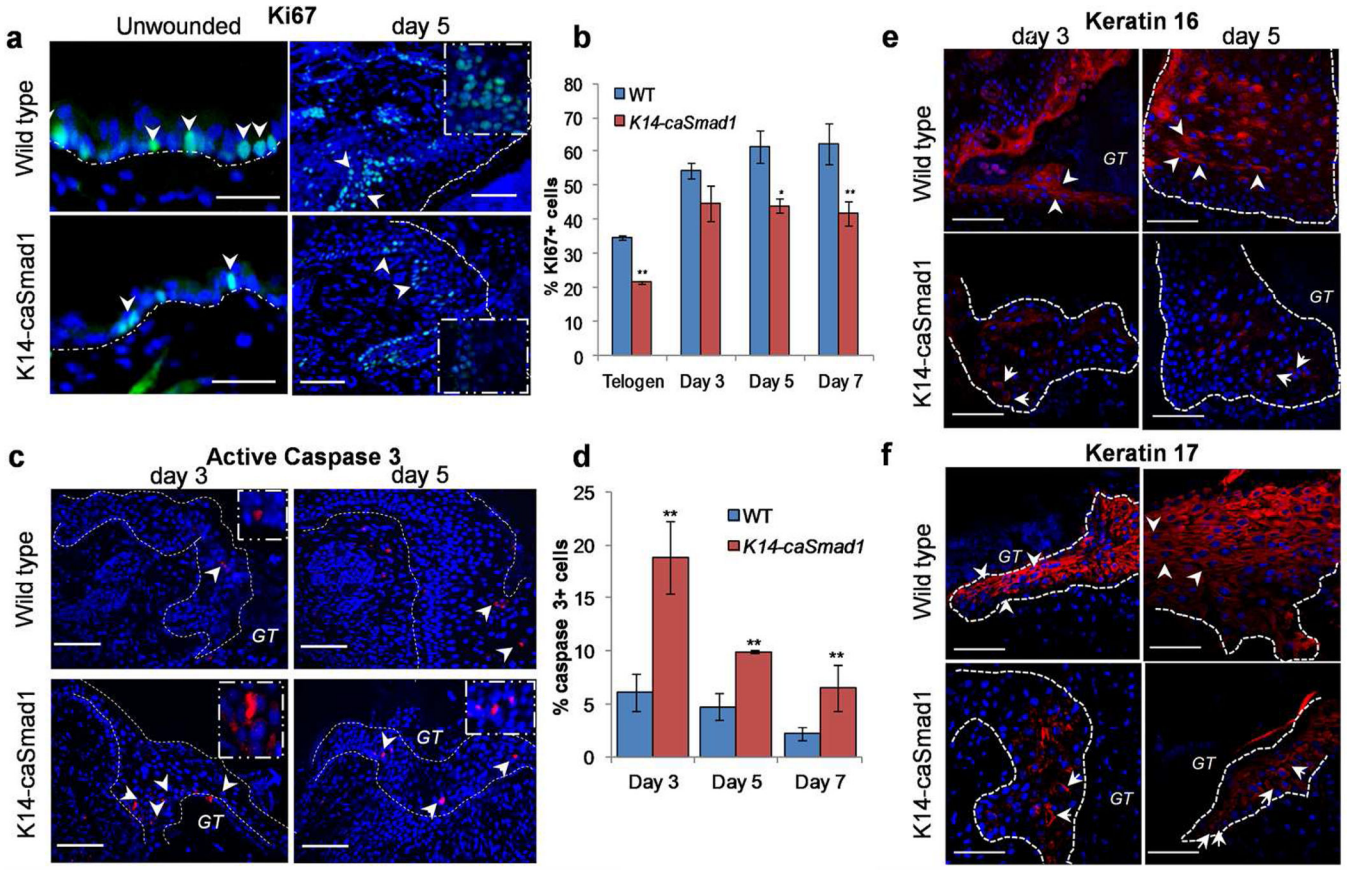

Figure 3. Quantitative analysis of proliferation and apoptosis and assessment of Keratin 16 and Keratin 17 expression in the wound epithelium of *K14-caSmad1* and WT mice

(**a**) Proliferative Ki-67+ cells are seen in the basal layer of telogen skin and in the wound epithelium on day 5 after injury (arrowheads); (**b**) significant reduction in Ki-67+ cells in *K14-caSmad1* telogen skin, on day 5 and 7 after wounding versus WT; (**c**) Apoptotic active caspase 3+ cells are seen in the wound epithelium at days 3 and 5 post-wounding – inset illustrates cell-specific staining; (**d**) significant increase in active caspase 3+ cells in *K14-caSmad1* wound epithelium at days 3, 5 and 7 post-wounding versus WT (mean ± SD, *p<0.01, **p<0.0001, Student's t-test); (**e–f**) reduced expression of keratin-16 and (**e**) keratin-17 expression (**f**) in *K14-caSmad1* wounds and keratinocytes were cuboidal (arrowheads), while those in WT mice were elongated (arrows). GT – granulation tissue, scale bar 100μm.

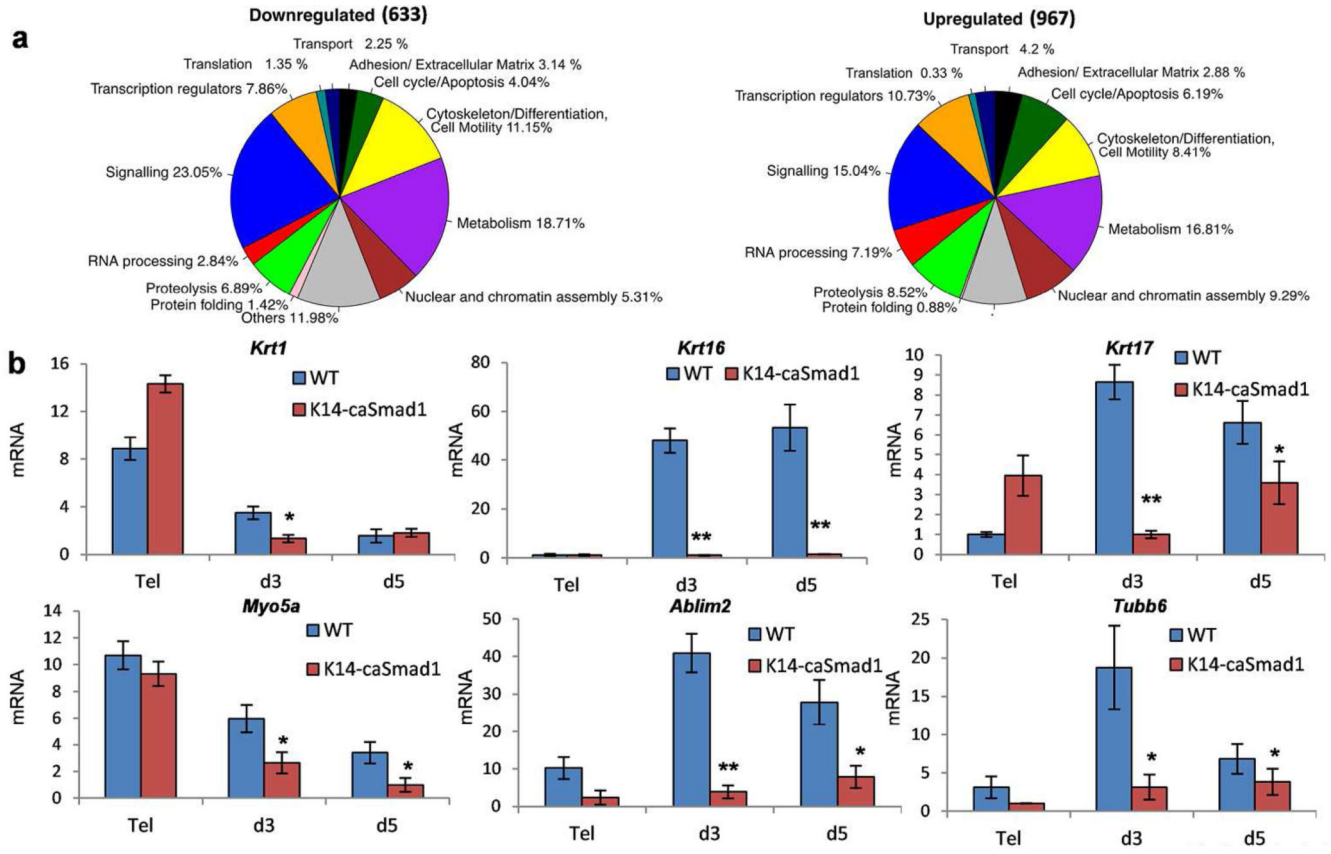

Figure 4. Global gene expression profiling of analyses of the epidermal keratinocytes isolated from telogen skin of WT and *K14-caSmad1*

(**a**) Microarray analysis of the global gene expression in the keratinocytes *K14-caSmad1* versus WT: functional assignments of the genes with altered expression; (**b**) *K14-caSmad1* mice displayed a significant decrease in *Keratin - 1, 16* and *-17* expression (upper panel) and *Myo5a, Ablim2, Tubb6* (lower panel) in response to wounding versus WT (mean ± SD, *p<0.01, **p<0.0001, Student's t-test).

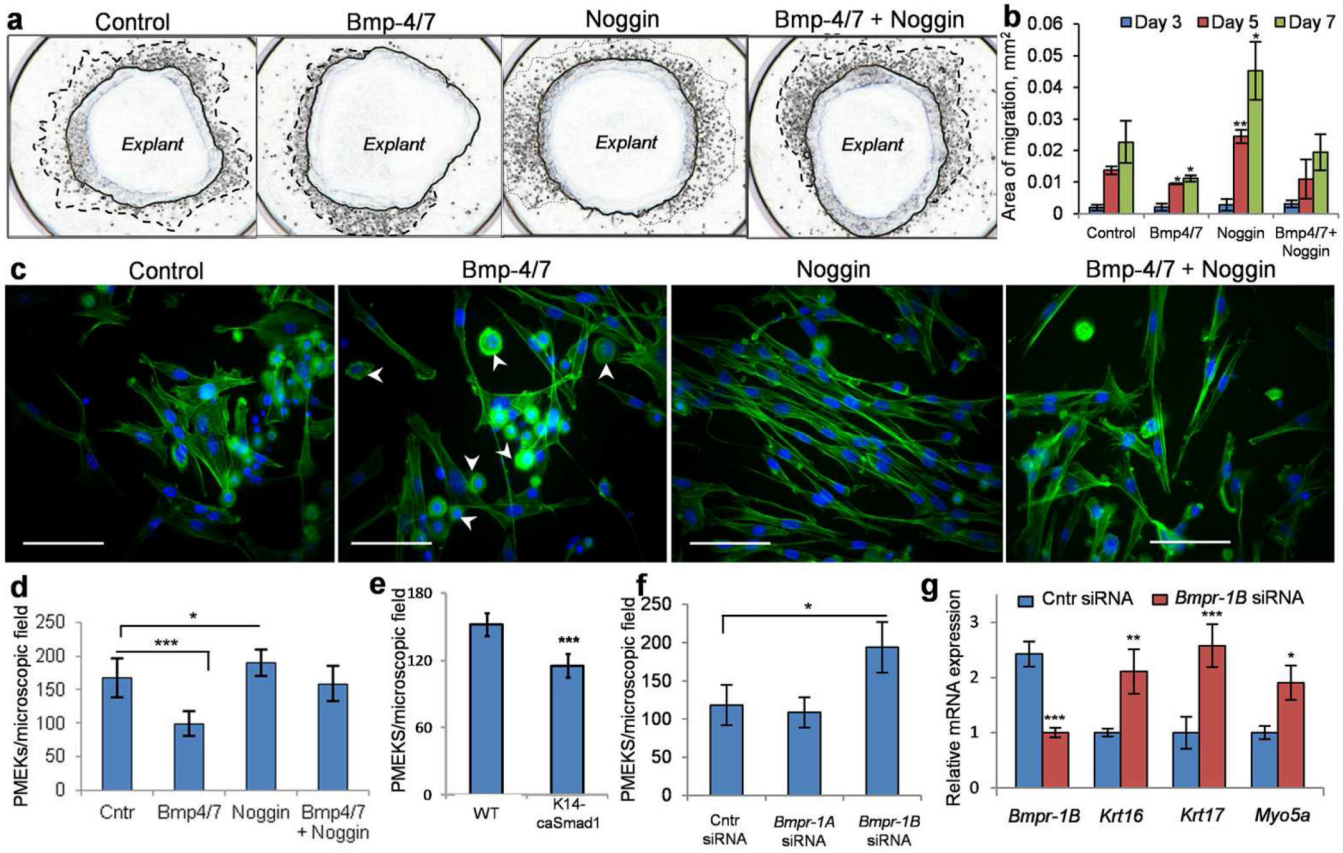

Figure 5. BMP pathway modulation alters keratinocyte migration and morphology

(**a–b**) Skin explant model; (**b**) Bmp-4/7 inhibited migration at days 5 (*p<0.05) and 7 (*p<0.05); Noggin alone increased migration at days 5 (**p<0.01) and 7 (*p<0.05); (**c**) Phalloidin staining of actin filament networks; control keratinocytes were elongated with actin fibres across the cell body; Bmp-4/7-treated keratinocytes were spherical and lacked defined actin fibres (arrowheads); Noggin increased cell polarity and actin formation; (**d–f**) transwell assay: Bmp-4/7 inhibited migration (**p<0.001); Noggin (*p<0.05) increased migration; (**d**); significant delay in PMEK migration obtained from *K14-caSmad1* mice (***p<0.0001); Bmpr-1B knockdown accelerated (*p<0.01) PMEK movement (**f**); (**g**) qRT-PCR confirmation of *Bmpr-1B* silencing (***p<0.001); Bmpr-1B siRNA up-regulated *Myo5a* (*p<0.02), *Krt16* (**p<0.01) and *Krt17* (***p<0.001) transcripts. PMEK – primary mouse epidermal keratinocyte, scale bar 100μm, mean±SD, Student's t-test.