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

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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.
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; Kobiela 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; Kobiela 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.,
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 suppressing 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 HF’s 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 5a, 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 5a, 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; Kobiela 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; Kobiela 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 Morasco, 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 (ΔΔCt) 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 (µm²) 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.

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Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| 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 |

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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.