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INTRODUCTION

Skin wound healing is a complex process requiring the coordinated, temporal orchestration of numerous cell types and biological processes to regenerate damaged tissue. Previous work has demonstrated that a functional β-adrenergic receptor autocrine/paracrine network exists in skin, but the role of β2-adrenergic receptor (β2AR) in wound healing is unknown. A range of in vitro (single-cell migration, immunoblotting, ELISA, enzyme immunoassay), ex vivo (rat aortic ring assay), and in vivo (chick chorioallantoic membrane assay, zebrafish, murine wild-type, and β2AR knockout excisional skin wound models) models were used to demonstrate that blockade or loss of β2AR gene deletion promoted wound repair, a finding that is, to our knowledge, previously unreported. Compared with vehicle-only controls, β2AR antagonism increased angiogenesis, dermal fibroblast function, and re-epithelialization, but had no effect on wound inflammation in vivo. Skin wounds in β2AR knockout mice contracted and re-epithelialized faster in the first few days of wound repair in vivo. β2AR antagonism enhanced cell motility through distinct intracellular signalling mechanisms and increased vascular endothelial growth factor secretion from keratinocytes. β2AR antagonism promoted wound repair processes in the early stages of wound repair, revealing a possible new avenue for therapeutic intervention.

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β2AR Antagonists and β2AR Gene Deletion Both Promote Skin Wound Repair Processes

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Skin wound healing is a complex process requiring the coordinated, temporal orchestration of numerous cell types and biological processes to regenerate damaged tissue. Previous work has demonstrated that a functional β-adrenergic receptor autocrine/paracrine network exists in skin, but the role of β2-adrenergic receptor (β2AR) in wound healing is unknown. A range of in vitro (single-cell migration, immunoblotting, ELISA, enzyme immunoassay), ex vivo (rat aortic ring assay), and in vivo (chick chorioallantoic membrane assay, zebrafish, murine wild-type, and β2AR knockout excisional skin wound models) models were used to demonstrate that blockade or loss of β2AR gene deletion promoted wound repair, a finding that is, to our knowledge, previously unreported. Compared with vehicle-only controls, β2AR antagonism increased angiogenesis, dermal fibroblast function, and re-epithelialization, but had no effect on wound inflammation in vivo. Skin wounds in β2AR knockout mice contracted and re-epithelialized faster in the first few days of wound repair in vivo. β2AR antagonism enhanced cell motility through distinct intracellular signalling mechanisms and increased vascular endothelial growth factor secretion from keratinocytes. β2AR antagonism promoted wound repair processes in the early stages of wound repair, revealing a possible new avenue for therapeutic intervention.

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Abbreviations: βAR, β-adrenergic receptor family; β2AR, β2-adrenergic receptor; β2AR+/−, control wild-type mice; β2AR−/−, β2-AR knockout mice; BME, basement membrane extract; CAM, chick chorioallantoic membrane assay; EC, endothelial cell; ERK, extracellular signal-regulated kinase; HDF, human dermal fibroblast; HDMEC, human dermal microvascular endothelial cell; HK, human keratinocyte; MK, murine keratinocyte; MDF, murine dermal fibroblast; NHS, National Health Service; PMN, polymorphonuclear cell; SMA, α-smooth muscle actin; SCM, single-cell migration; TH, tyrosine hydroxylase; VEGF, vascular endothelial growth factor

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RESULTS

β2AR antagonism promotes angiogenesis

Angiogenesis is an essential process in acute wound healing (Tønnesen et al., 2000), and endothelial cell (EC) migration is a critical process in angiogenesis (Eming et al., 2007). To determine whether a β2AR antagonist altered EC migration, single-cell migration (SCM) assays were performed with primary human dermal microvascular ECs (HDMECs) in the presence and absence of β2AR antagonist; however, there was no effect on migration rate (Figure 1a).

To determine whether β2AR antagonism could alter cell outgrowth from the cut edge of rat aorta (Smith and Staton, 2006), aortic pieces were digested with collagenase and embedded into the basement membrane extract and cultured in the presence or absence of a β2AR antagonist for 5 days. β2AR antagonism increased aortic outgrowth by 66% after 5 days (Figure 1b).

To investigate angiogenesis in a more complex, multicellular environment in vivo, the chick chorioallantoic membrane (CAM) assay, a model of embryonic angiogenesis (Smith and Staton, 2006), was selected. β2AR antagonist treatment increased angiogenesis by 48% in the CAM assay, between days 5 and 9 post fertilization (Figure 1c).

Finally, to determine whether β2AR antagonism could modulate wound angiogenesis in vivo, two full-thickness 6-mm wounds were created in the dorsal skin of wild-type (β2AR+/+) and β2AR knockout (β2AR−/−) mice (Chruscinski et al., 1999). The wounds were treated immediately after wounding and then daily, topically, with hydrogel alone or β2AR antagonist; however, there was no effect on migration rate (Figure 1d).

β2AR antagonism promotes human dermal fibroblast migration in vitro and function in vivo

Within a few days of wounding, fibroblasts proliferate and migrate into the wound bed (Shaw and Martin, 2009). A nonselective βAR agonist increased HDF migration via the src-dependent transactivation of EGFR (Pullar and Isseroff, 2006). To determine the effect of β2AR antagonism on HDF migration, SCM was performed in the presence and absence of a highly selective β2AR antagonist. β2AR antagonism increased the speed of HDF migration by 27% (Figure 2a). In addition, DFs were isolated from the skin of β2AR+/+ and β2AR−/− mice. Although a β2AR antagonist increased the migration speed of β2AR+/+ murine DFs (MDFs) by 49%, the speed of β2AR−/− MDFs was increased by 46% compared with β2AR+/+ MDFs (Figure 2b). The β2AR antagonist had no additional effect on β2AR−/− MDF migration speed, as expected (Figure 2b). Extracellular signal-regulated kinase (ERK) has an important role in cell migration (Klemke et al., 1997). β2AR antagonism increased β2AR+/+ MDF ERK phosphorylation by 90% (Figure 2c).

To explore whether β2AR antagonism could modulate wound DF function, two full-thickness 6-mm wounds were created in the dorsal skin of β2AR+/+ and β2AR−/− mice and treated daily. Wound area was calculated daily until excision to determine the rate of wound contraction (Galiano et al., 2004a), as described in the Materials and Methods section. Wound contraction was accelerated 4-fold in β2AR antagonist–treated β2AR+/+ wounds and 5-fold in control β2AR−/− wounds, 24 hours post wounding (Figure 3a). Wound contraction was still significantly enhanced by 2-fold in β2AR antagonist–treated β2AR+/+ wounds and almost 3-fold in control β2AR−/− wounds and almost 3-fold in control β2AR−/− wounds, 48 hours post wounding. After 3 days, contraction was increased by 19% in β2AR antagonist-treated β2AR+/+ wounds and 40% in control β2AR−/− wounds, but after 4 days, contraction of both β2AR antagonist-treated β2AR+/+ wounds and control β2AR−/− wounds occurred at the same rate as control β2AR+/+ wounds. β2AR antagonist–treated β2AR−/− wounds demonstrated the same rate of wound contraction as control β2AR−/− wounds (Figure 3a).

Expression of α-smooth muscle actin (SMA) is a reliable marker of myofibroblast differentiation, and the rate of wound contraction can directly correlate with SMA expression (Hinz et al., 2001). Sections (7 μm thick) from the wound center, excised after 5 days, were immunostained with an antibody to SMA. Populations of SMA-stained fibroblasts were observed below the wound epithelial margins, 5 days post wounding. The area of SMA staining was increased by 60% in β2AR antagonist–treated wounds in β2AR+/+ mice and was similar in control β2AR−/− and β2AR+/+ wounds (Figure 3b).

Myofibroblasts synthesize collagen III early in the wound repair process, which is later replaced by collagen I in more mature scars (Hinz, 2007). Sections (7 μm thick) from the wound center, excised after 5 days, were immunostained with an antibody to collagen III. β2AR antagonist treatment increased the area of collagen III-stained neo-dermis by 20% in β2AR+/+ wounds (Figure 3c), whereas control β2AR−/− and β2AR+/+ wounds had similar areas of contract.

Vascular endothelial growth factor (VEGF) is a potent proangiogenic factor in acute wounds (Barrientos et al., 2008), and βAR activation can increase VEGF expression in human choroid ECs (Steinle et al., 2008). To determine whether β2AR antagonism increased VEGF secretion from HDMECs, HKs, human dermal fibroblasts (HDFs), neutrophils, and macrophages, ELISAs were performed on control and β2AR antagonist–treated supernatants. VEGF secretion was not detected in HDMECs or HDF supernatants. Although VEGF secretion was detected in the supernatants of neutrophils and macrophages, β2AR antagonist treatment had no effect (results not shown). However, βAR antagonist treatment increased the amount of VEGF secreted from HKs after 24 hours by 23% (Figure 1e).
collagen III staining (Figure 3c). To examine this further, sections were stained with picrosirius red to assess total collagen deposition in the neo-dermis (Junqueira et al., 1979). β2AR antagonist treatment increased the area of collagen-stained neo-dermis, within 1 mm² of the wound edge, by 29% in β2AR⁺/⁺ wounds (Figure 3d), whereas control β2AR⁻/⁻ and β2AR⁺/+ wounds had similar areas of collagen staining (Figure 3d).

β2AR antagonism has no effect on wound inflammation in vivo

Neutrophils and macrophages are recruited to the wound site to quell bacterial infections, where they secrete a cocktail of growth factors and cytokines to activate resident keratinocytes and fibroblasts (Martin, 1997).

To determine whether a β2AR antagonist could alter the guidance of inflammatory cells to a wound, a zebrafish wound model was used. Indeed, zebrafish are known to have
two β2AR genes (Steele et al., 2011), but their role in tissue regeneration is unknown. Zebrafish larvae are transparent and zebrafish neutrophils are identifiable 48 hours post fertilization. Upon transection of the caudal fin, neutrophils are guided to the wound within minutes, but peak neutrophil recruitment occurs after approximately 6 hours (Renshaw et al., 2006). When the wounded zebrafish larvae were immersed in pond water containing a β2AR antagonist, there was no effect on neutrophil guidance to the caudal fin wound (Figure 4a).

To determine whether β2AR antagonist treatment altered wound re-epithelialization in the mouse excisional skin wound model, 7-μm-thick sections from the wound center, excised after 3 and 5 days, were stained with hematoxylin and eosin, and the linear distance covered by keratinocytes (MKs) was measured by enzyme immunoassay in extracts from MKs. MKs similar to HKs (Pullar et al., 2006b), therefore, can also synthesize epinephrine, generating an autocrine and paracrine adrenergic network in the murine epidermis and dermis, respectively.

Finally, to determine the effect of a β2AR antagonist on wound re-epithelialization, 7-μm-thick sections from the wound center, excised after 3 and 5 days, were stained with hematoxylin and eosin, and the linear distance covered by keratinocytes was measured by enzyme immunoassay in extracts from MKs. MKs similar to HKs (Pullar et al., 2006b), therefore, can also synthesize epinephrine, generating an autocrine and paracrine adrenergic network in the murine epidermis and dermis, respectively.

**DISCUSSION**

β2AR antagonist increased keratinocyte migration in vitro and promoted wound re-epithelialization in vivo. Keratinocyte migration from the wound edges is a critical process to restore the barrier function of the epidermis, and they are a valuable source for growth factors in the wound (Shaw and Martin, 2009).

A β2AR antagonist increased keratinocyte migration significantly by 12% (Figure 5a). To confirm that murine keratinocytes (MKS) respond similarly to a β2AR antagonist, MKs were isolated from newborn β2AR+/+ and β2AR−/− mice and the effect of a β2AR antagonist on motility was determined. A β2AR antagonist enhanced MK SCM by 28% (Figure 5b). β2AR−/− MKs migrated 16% faster than β2AR+/+ MKs, whereas a β2AR antagonist had no additional effect on their migration, confirming the absence of the β2AR (Figure 5b). A β2AR antagonist-mediated 2.5-fold increase in ERK phosphorylation, known to have a pivotal role in pro-migratory signaling pathways (Klemke et al., 1997), underpinned its effect on MK motility (Figure 5c).

Tyrosine hydroxylase, the enzyme controlling the rate-limiting step for catecholamine biosynthesis (Nagatsu et al., 1964), was expressed in MKs (Figure 5d), and 100±10 and 75±8 pg mg−1 protein of epinephrine was measured by enzyme immunoassay in extracts from MKs. MKs similar to HKs (Pullar et al., 2006b), therefore, can also synthesize epinephrine, generating an autocrine and paracrine adrenergic network in the murine epidermis and dermis, respectively.
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β2AR Antagonism Promotes Wound Healing

Figure 3. β2-Adrenergic receptor (β2AR) antagonism, or the absence of β2AR, increased dermal fibroblast function in vivo. (a) Two full-thickness 6-mm excisional wounds, created in the dorsal skin of β2AR wild-type (β2AR+/+) and β2-AR knockout (β2AR−/−) mice, were treated and photographed daily, and the area of each wound was calculated; see Materials and Methods. Sections (7 μm), day 5 post wounding, were stained with (b) an anti-smooth muscle actin (SMA) antibody or (c) an anti-collagen III antibody. The mean area of positive staining was calculated; see Materials and Methods. Bar = 1 mm (n = 10–14). Values plotted are means ± SEM. *P<0.05; **P<0.01.

processes, including angiogenesis, DF function, and re-epithelialization, thus accelerating healing.

The mechanism underpinning the β2AR antagonist–mediated increase in angiogenesis could be partly attributed to the β2AR antagonist–mediated increase in VEGF secretion from HKs, known to be an important source of VEGF (Rossiter et al., 2004) and other growth factors in wounds (Werner and Grose, 2003; Barrientos et al., 2008). Indeed, the addition of autologous cultured keratinocytes can aid healing of chronic venous leg ulcers (Liu et al., 2004) and the addition of VEGF promotes healing in chronic diabetic wounds (Galiano et al., 2004b; Brem et al., 2009); therefore, the β2AR antagonist–mediated increase in HK VEGF secretion could be highly beneficial for wound repair. In the complex wound environment, β2AR antagonist treatment could also increase the secretion of other pro-angiogenic factors such as fibroblast growth factor 2 or basic fibroblast growth factor or platelet–derived growth factor (Battegay et al., 1994), from wound cells, which could contribute to the enhanced angiogenesis observed in vivo. In contrast, post-ischemic angiogenesis was impaired in β2AR−/− mice (Ciccarelli et al., 2011).

The increase in wound contraction observed in β2AR−/− wounds (Figure 3a) occurred in the absence of an increase in SMA-expressing cells in the wound bed (Figure 3b), in contrast to the observed increase in both wound contraction and SMA expression in the β2AR antagonist–treated β2AR+/+ wounds. Although the rate of wound contraction is thought to correlate directly with SMA expression (Hinz et al., 2001), there are several reports describing that wound contraction can occur in the absence of SMA-expressing myofibroblasts. Wounds in vanadate-treated rats (Ehrlich et al., 1999) and rat wounds treated with a Smad blocker, SB-505124 (Au and Ehrlich, 2010), contracted normally, but SMA-expressing cells were not detected in the granulation tissue. Finally, a personal communication from J J Tomasek reports that SMA knockout mice heal normally (Hinz, 2007). It appears that wound contraction is not correlated with SMA expression in β2AR−/− mouse wounds and only β2AR antagonist treatment can increase SMA expression (Figure 3b).

In addition, blockade of autocrine, endogenously synthesized epinephrine (Figure 5d), inhibitory to keratinocyte migration (Pullar et al., 2003, 2006a), likely contributed to the ability of the β2AR antagonist to promote MK migration in vitro and re-epithelialization in vivo.

Although β2AR antagonist treatment did not alter acute wound inflammation or the secretion of VEGF from inflammatory cells, β2AR blockade has been demonstrated to attenuate the hyper-inflammatory response to traumatic injury in mice (Rough et al., 2009). It is possible, therefore, that β2AR antagonist treatment could reduce persistent or hyper-inflammation, which will be tested in a chronic wound model.

In conclusion, here we report that β2AR antagonism promotes wound angiogenesis, DF function, and re-epithelialization, enhancing wound repair, thus revealing a possible new avenue of therapeutic intervention. To our knowledge, this is previously unreported.
MATERIALS AND METHODS

Animals

The β2AR-deficient FVB/N (β2AR−/−) mice were a kind gift from Dr Brian Kobilka, Stanford University (Stanford, CA) (Chruscinski et al., 1999). The congenic controls, FVB/N (β2AR+/+) mice, were purchased from The Jackson Laboratory (Bar Harbor, ME). The genotypes of the β2AR+/+ and β2AR−/− mice were confirmed by PCR, using published primers (Chruscinski et al., 1999). All animals used in the study were females between 8 and 12 weeks of age. The University of California, Davis Institutional Animal Care and Use Committee (IACUC) approved all animal protocols.

Adult zebrafish were maintained, as described (Westerfield, 1994), in compliance with the Animals (Scientific Procedures; www.legislation.gov.uk/ukpga/1986/14/contents) Act, 1986. Embryos were harvested and raised in egg water (Westerfield, 1994), at 28.5 °C, until the required developmental stage. Staging was performed in accordance with (Kimmel et al., 1995).

Figure 4. β2-Adrenergic receptor (β2AR) antagonism has no effect on wound inflammation. Zebrafish larvae were wounded, untreated, or treated with 500 µM β2AR antagonist and imaged using fluorescein-tyramine signal amplification; see Materials and Methods. (a) The number of neutrophils recruited to each wounded tail was recorded. Bar = 100 µm (n = 15–30). Paraffin-embedded murine wound sections (7 µm), day 3 post wounding, were immunostained with an antibody that recognizes (b) neutrophils (Ly6G-6C) or (c) macrophages (F4-80). The positive cells per field were counted and the data analyzed from 10 images of each wound section; see Materials and Methods (n = 10–14). Values plotted are means ± SEM. FITC, fluorescein isothiocyanate; PMNs, polymorphonuclear cells; TSA, tyramide signal amplification.

Figure 5. β2-Adrenergic receptor (β2AR) antagonism, or the absence of β2AR, increased keratinocyte migration and re-epithelialization. (a) Human keratinocytes (HKs) and (b) murine keratinocytes (MKs), isolated from β2-AR knockout (β2AR−/−) and wild-type (β2AR+/+) neonates, were plated and single-cell migration (SCM) performed; see Materials and Methods (HK: control, n = 224, antagonist, n = 179; MK: β2AR+/+ control, n = 128, β2AR−/− control, n = 102; β2AR−/− control = 105). MKs were treated with media alone (control) or with media containing 10 nM β2AR antagonist, and western blotting for extracellular signal-regulated kinase (ERK) was performed; see Materials and Methods. (c) Blots were scanned and densitometry performed using NIH Image J. (d) MK lysates were prepared and western blotting for tyrosine hydroxylase (TH) was performed. The data shown are representative of three independent experiments. Control and β2AR antagonist-treated β2AR+/+ wounds and control β2AR−/− wounds were excised, fixed, and stained. The percentage of wound re-epithelialization, at days 3 and 5 post wounding, was calculated using Image J; see Materials and Methods (n = 10) (e). Values plotted are means ± SEM. *P<0.05; **P<0.01. P-ERK, phosphorylated-ERK.

Culture of primary cells

Primary MKs were isolated from newborn mouse pups, as described previously (Isseroff et al., 1983), and cultured in MK growth media (Epilife and keratinocyte growth supplement), 10 ng ml−1 murine EGF, 0.1 mU cholera toxin (Invitrogen, Paisley, UK), and 0.5% antibiotic solution (25 U ml−1 penicillin and 25 µg ml−1 streptomycin (Sigma Aldrich, Poole, UK), murine keratinocyte complete growth medium). To isolate MDFs, the remaining dermal pieces were minced with sterile scissors and transferred to 100-mm plastic cell culture dishes from Falcon Labware (BD Biosciences, Oxford, UK).
Dermal pieces were allowed to attach for 5 minutes, before the addition of 4 ml of fibroblast growth medium, which consists of DMEM (basal medium), 0.5% antibiotic solution (25 U ml\(^{-1}\) penicillin and 25 μg ml\(^{-1}\) streptomycin; Sigma Aldrich), and 10% fetal calf serum (Invitrogen). The plates were incubated at 37 °C in a humidified atmosphere of 5% CO\(_2\) for 48 hours before adding an additional 6 ml of fibroblast growth medium per dish.

Primary human neonatal DFs were purchased from Invitrogen and cultured as described previously (Pullar and Isseroff, 2006).

Primary HDMECs were purchased from Invitrogen. Cells were grown in EC growth media supplemented with microvascular growth supplement (PromoCell, Heidelberg, Germany) and antibiotics (25 U ml\(^{-1}\) penicillin, 25 μg ml\(^{-1}\) streptomycin; Invitrogen) at 37 °C with 5% CO\(_2\)/95% air in a humidified atmosphere. Cells were grown on attachment factor containing 0.1% gelatin (Invitrogen), and experiments were conducted using cell passages between 3 and 7.

Primary neonatal HKs were purchased from Invitrogen and cultured as described previously (Pullar and Isseroff, 2005). At least three keratinocytes strains, between passages 3 and 6, were used in all experiments.

**SCM assay**

Plastic cell culture dishes (35 mm) were coated with collagen I (60 μg ml\(^{-1}\)) (Invitrogen) in phosphate-buffered saline for 1 hour at 37 °C. MDFs, isolated from both β2AR\(^{-/-}\) and β2AR\(^{+/+}\) mice, and HDFs were plated at a density of 25 cells per mm\(^2\) in fibroblast growth medium, whereas MKs, HKs, and HDMECs were plated at a density of 50 cells per mm\(^2\) in their appropriate complete media (see culture methods), for 2 hours at 37 °C. Cells were incubated with complete media alone (control) or with media containing 10 nM β2AR antagonist (ICI, 118,551; Tocris Biosciences, Bristol, UK) at time 0. The 35-mm dishes were placed in a heating chamber, designed to maintain the medium at 37 °C, and images were taken every 10 minutes for 1 hour with the Improvion software (Perkin Elmer, Cambridge, UK), as described previously (Pullar et al., 2006b).

**Cell treatments for immunoblotting**

MKs or MDFs plated at a density of 1 × 10\(^6\), isolated from both β2AR\(^{-/-}\) and β2AR\(^{+/+}\) mice, were incubated with either murine keratinocyte complete growth medium (MK) or fibroblast growth medium (MDF) alone (control and lysates for catecholamine synthesis enzyme detection), or with media containing 10 nM β2AR antagonist (ICI, 118,551), for 10 minutes. Lysates were prepared and electrophoresed, as previously described for phosphorylated and total ERK (Pullar et al., 2003) and tyrosine hydroxylase (Pullar et al., 2006b).

**Enzyme immunoassay for the quantitative determination of epinephrine in small sample volumes**

MKs (1 × 10\(^5\)), isolated from both β2AR\(^{-/-}\) and β2AR\(^{+/+}\) mice, were extracted in 100 μl 0.1 n HCl and sonicated on ice for 10 minutes. Extracts were tested in triplicate in an epinephrine enzyme immunoassay (Invitrogen) according to the manufacturer’s instructions.

**ELISA**

Cells were plated on collagen I (30 μg ml\(^{-1}\))-coated 6-well plates and incubated for 24 hours in the appropriate complete media to reach 70/80% confluence. Cells were washed with Hank’s balanced salt solution and serum starved for 24 hours in the appropriate basal media. Cells were then incubated with either basal media alone or media containing 10 μM β2AR antagonist for 6, 24, or 48 hours. After each time point, media were collected and stored at −80 °C for analysis with a Duoset VEGF-A ELISA kit (R&D Systems, Abingdon, UK).

**Rat aortic ring assay**

Animals were killed using an approved schedule 1 method. The abdominal and aortic arcs were extracted from adult Wistar rats and placed into sterile phosphate-buffered saline. The fibro-adipose tissue was then removed, and the aortas were washed in warm, sterile phosphate-buffered saline three times. Aortas were cut into uniform, 1-mm sections, placed into 80 μl of basement membrane extract (R&D Systems), and incubated at 37 °C for 30 minutes. Aortas were incubated in EC basal media supplemented with 2% microvascular growth supplement for 3 days, and then with media alone or media containing 10 μM β2AR antagonist (ICI 118,551) for a further 5 days. Aortic endothelial outgrowth was imaged every 24 hours, using a Nikon eclipse microscope and the Improvion Openlab software. The area of outgrowth was analyzed using the Improvion Volocity software.

**Zebrafish caudal fin wound model**

Zebrafish larvae, 72 hours post-fertilization, were immersed in pond water containing 2% tricaine, and complete transection of the tail was performed with a sterile scalpel blade. After wounding, the larvae were incubated in the presence or absence of 500 μM β2AR antagonist (ICI 118,551) for 6 hours, and washed and stained using a fluorescein-tyramide signal amplification method (Perkin Elmer) before fixation in 4% paraformaldehyde. Tails were mounted and photographed on a Nikon TE-2000E inverted microscope at × 20 magnification using a fluoro objective. The number of neutrophils recruited to each wounded tail was recorded.

**CAM assay**

Fertilized eggs were obtained, 1 day post fertilization, from local hatcheries and incubated at 37 °C in a humidified environment for 48 hours. On day 3, approximately 5 ml of albumin was removed from the obtuse poles of the eggs using a 21 G cannula. A square window (2 × 2 cm\(^2\)) was opened into the shell and shell membrane using a Dremel tool (Dremel, Uxbridge, UK). The window was then sealed with Parafilm and incubated horizontally for 48 hours at 37 °C. On day 5, water or 100 μM β2AR antagonist (ICI 118,551) in water was evaporated onto the center of a sterile 13-mm coverslip and then placed face down onto the CAM. CAMs were photographed through the coverslip center, every 24 hours, until day 9 using a stereomicroscope (Prior Scientific, Cambridge, UK). Angiogenesis was analyzed by counting the total number of branch points per field of view through the center of the coverslip.

**Murine wound model**

β2AR\(^{-/-}\) and β2AR\(^{+/+}\) mice were anesthetized by intraperitoneal injection of ketamine (100 mg kg\(^{-1}\)/xylazine (10 mg kg\(^{-1}\)) (Pfizer, Sandwich, UK). Back skin was shaved and two circular, full-thickness 6-mm excisional wounds were created 2 cm apart, in the center of the back, using a sterile 6-mm biopsy punch (SMS, Camberley, UK) to mark the skin for surgical excision. Wounds were treated topically with 100 μl of hydrogel alone (Duoderm, ConvaTec, Uxbridge, UK) or containing 0.1% (1 mM) selective β2AR antagonist (ICI 118,551).
(a concentration previously demonstrated to accelerate mouse epidermal barrier recovery; Denda et al., 2003) immediately after wounding and daily thereafter until harvesting (n = 5–7 mice per group, two wounds per mouse). Each mouse was housed separately after wounding until wound harvest. Wounds were left uncovered and digitally photographed, daily, to determine wound contraction over time. Wounds were harvested 3 and 5 days post wounding by carefully applying an 8-mm punch (SM5) around the original wound and lightly pressing to form an outline on the skin. Scissors were used to excise the wound without damaging the delicate wound bed.

For histological analysis, the wounds were fixed in an IHC zinc fixative (BD Biosciences). The zinc-fixed biopsies were bisected, to ensure that sections were taken from the center of the wound, dehydrated through an ethanol-xylene series, and embedded in paraffin. Cross-sections, 7 μm thick, were stained with the hematoxylin-eosin technique or with picrosiris red, to visualize collagen in the neo-dermis (Junqueira et al., 1979), as described by Dapson et al. (2011). Sections were immunostained with an antibody against SMA (Dako, Ely, UK), collagen III (Roche, Lorne Laboratories, Reading, UK), Ly6G-6C (a neutrophil marker; BD Pharmingen, Oxford, UK), F4-80 (a macrophage marker; Serotec, Kidlington, UK), or CD31 (an EC marker; BD Pharmingen), followed by DAB detection (BD Pharmingen) according to the manufacturer’s protocols.

Specimens that were damaged in the histological process or otherwise non-interpretable were excluded from the study. Re-epithelialization was determined by examining all hematoxylin- and eosin-stained sections by light microscopy, using a Q-imaging Retiga-EX camera attached to a Nikon T-100 inverted microscope, controlled by the Improvision software. Image J was used to measure the linear distance covered by new epithelium and the linear distance between the original wound edges, to determine the percentage of re-epithelialization. Wound closure was quantified by measuring the wound area of each wound from the digital pictures taken every day with Image J. Each picture was calibrated individually. Images of picrosirus red-stained sections were captured on an Olympus BX51 upright microscope equipped for dark field. The area of the collagen-stained neo-dermis was analyzed by measuring the red-stained area within 1 mm² of each wound edge on the dark-field pictures with the Improvision software. Image J was used to determine the area of SMA and collagen III staining in each wound to calculate the mean area of SMA and collagen III staining in each group. Images of the CD31- (× 40 magnification), Ly-6C-G6C, and F4-80-stained (× 60 magnification) wounds were also captured on the Nikon Eclipse microscope. The number of stained cells/vessels in each image was counted in a double-blind manner, and the average cell/vessel number was calculated for each group. For all image analysis, 10 fields were selected from the dermis below the wound margins and across the wound bed using a template to ensure that images were captured from similar areas in each wound.

**Statistical analysis**

Unless mentioned in a specific method, categorical variables were compared with a two-tailed Fisher’s exact test or a one-way analysis of variance, followed by the Dunnett’s test, whereas each continuous variable test group was compared with the control using the two-tailed Student’s t-test for unpaired data with unequal variance. P<0.05 (*) was significant and P<0.01 (**) highly significant.

**CONFLICT OF INTEREST**

CEP/RRI are coinventors on a patent held at University of California, Davis, entitled “β2AR modulation of wound healing”. The authors declare that there are no other competing interests.

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**Author contributions**

CEP and RRI initiated the concept for the murine wound study. RRI contributed to the initial analysis of the murine wound study and the proof-reading of the final article. CEP initiated the concept for all mechanistic studies, designed, acquired, analyzed, and interpreted the majority of the data, and drafted and revised the article. GSLP, APO, SE, and BB substantially contributed to the design of some experiments, the acquisition, analysis, and interpretation of the majority of the associated data, and either the drafting or the proofreading of the article.

**SUPPLEMENTARY MATERIAL**

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

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