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ORIGINAL ARTICLE

Deletion of the α2A/α2C-adrenoceptors accelerates cutaneous wound healing in mice

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SUMMARY

The α2-adrenoceptors regulate the sympathetic nervous system, controlling presynaptic catecholamine release. However, the role of the α2-adrenoceptors in cutaneous wound healing is poorly understood. Mice lacking both the α2A/α2C-adrenoceptors were used to evaluate the participation of the α2-adrenoceptor during cutaneous wound healing. A full-thickness excisional lesion was performed on the dorsal skin of the α2A/α2C-adrenoceptor knockout and wild-type mice. Seven or fourteen days later, the animals were euthanized and the lesions were formalin-fixed and paraffin-embedded or frozen. Murine skin fibroblasts were also isolated from α2A/α2C-adrenoceptor knockout and wild-type mice, and fibroblast activity was evaluated. The *in vivo* study demonstrated that α2A/α2C-adrenoceptor depletion accelerated wound contraction and re-epithelialization. A reduction in the number of neutrophils and macrophages was observed in the α2A/α2C-adrenoceptor knockout mice compared with wild-type mice. In addition, α2A/α2C-adrenoceptor depletion enhanced the levels of nitrite and hydroxyproline, and the protein expression of transforming growth factor-β and vascular endothelial growth factor. Furthermore, α2A/α2C-adrenoceptor depletion accelerated blood vessel formation and myofibroblast differentiation. The *in vitro* study demonstrated that skin fibroblasts isolated from α2A/α2C-adrenoceptor knockout mice exhibited enhanced cell migration, α-smooth muscle actin _protein expression and collagen deposition compared with wild-type skin fibroblasts. In conclusion, α2A/α2C-adrenoceptor deletion accelerates cutaneous wound healing in mice.

Keywords
α2A/α2C-adrenoceptors, collagen fibres, cutaneous wound healing, fibroblasts, knockout mice

Cutaneous wound healing is a highly ordered process that involves inflammation, granulation tissue formation and tissue remodelling (Li *et al.* 2007). The α- and β-adrenoceptors have been identified as candidates for the regulation of wound healing (Pullar *et al.* 2006, 2012; Romana-Souza *et al.* 2009a).

The sympathetic nervous system is composed of three subtypes of each α- and β-adrenoceptor (Bylund *et al.* 1994). Several studies have demonstrated that β-adrenoceptors strongly participate in cutaneous wound healing (Pullar & Isseroff 2006; Romana-Souza *et al.* 2009a,b; Pullar *et al.* 2012). Activation of the β2-adrenoceptors increases fibroblast migration and proliferation (Pullar & Isseroff 2006), whereas β1/β2-adrenoceptor blockade accelerates human keratinocyte migration and re-epithelialization in an *ex vivo* wound healing model (Pullar et al. 2006). Other studies have shown that β1/β2-adrenoceptor blockade delays wound contraction and re-epithelialization in rats, and granulation tissue development in polyurethane (PU) sponge implants (Romana-Souza *et al.* 2009b,c). Nevertheless, β2-adrenoceptor deletion may promote wound angiogenesis and re-epithelialization in mice (Pullar *et al.* 2012). In stressed or burned rodents, β1/β2-adrenoceptor blockade reverses the effects of high epinephrine levels on cutaneous wound healing (Romana-Souza *et al.* 2008, 2010b). In the immune system, β-adrenoceptor activation reduces the antigen-presenting

capacity of Langerhans cells, the activation of natural killer cells, and inhibits tumour necrosis factor-α and interleukin-6 production by dendritic cells (Seiffert *et al.* 2002; Goyarts *et al.* 2008; Tarr *et al.* 2012).

Few studies have described the role of α-adrenoceptors during cutaneous wound healing (Cassuto *et al.* 2005; Eijkelkamp *et al.* 2007; Romana-Souza *et al.* 2009b,c). In rats, α2-adrenoceptor activation reduces plasma extravasation after thermal injury (Cassuto *et al.* 2005). Although α1/α2-adrenoceptor blockade increases polymorphonuclear leucocyte infiltration and cellular proliferation in rat cutaneous lesions, it does not compromise wound closure and granulation tissue development in PU sponge implants (Romana-Souza *et al.* 2009b,c). In mice, α1/α2-adrenoceptor blockade may negate the inhibitory effect of chronic stress on oedema formation and wound contraction (Eijkelkamp *et al.* 2007). Recently, the α2A/α2C-adrenoceptor knockout (KO) mice were developed as a genetic model of heart failure because these animals exhibit cardiac hypertrophy and reduced left ventricle contractility, beginning at 4 months of age, due to chronically increased sympathetic nervous system activity (MacMillan *et al.* 1996; Altman *et al.* 1999; Brum *et al.* 2002). These KO mice may be an alternative model for characterizing the role of the α2-adrenoceptors in cutaneous wound healing.

The aim of this study was to evaluate the effect of α2A/α2C-adrenoceptor deletion on cutaneous wound healing. In addition, this study evaluated the *in vitro* activity of skin fibroblasts isolated from wild-type and α2A/α2C-adrenoceptor KO mice.

Methods

Animals

Ten-week-old wild-type C57BL/6 male mice and α2A/α2C-adrenoceptor KO male mice were obtained from an established breeding colony at the Laboratory of Molecular and Cellular Physiology of Exercise (São Paulo, Brazil). The α2A/α2C-adrenoceptor KO mice were generated by mating the C57BL/6 α2A-adrenoceptor homozygous KO mice to C57BL/6 α2C-adrenoceptor homozygous KO mice, resulting in an F1 generation of compound heterozygous mice (Brum *et al.* 2002). The genotypes were determined by polymerase chain reaction amplification of genomic deoxyribonucleic acid obtained from tail biopsies using primers to detect the intact and disrupted genes. The animals were acclimatized for 2 weeks before the experiments began. The animals had free access to food and water and were maintained in a room with controlled humidity (50%) and temperature (22 °C) on a 12 hour light/dark cycle. All experimental animal work was approved by the Ethical Committee for Animal Use at the State University of Rio de Janeiro (CEA/042/2010).

Wounding models

The α2A/α2C-adrenoceptor KO ($n = 20$) and wild-type ($n = 20$) male mice were intraperitoneally anaesthetized with ketamine (150 mg/kg) and xylazine (15 mg/kg) at 3 months of age (12 weeks after transportation), shaved and cleaned. In the dorsal region, a full-thickness excisional wound (1 cm^2) was created by the excision of the epidermis, dermis and hypodermis, exposing the panniculus carnosus muscle (Romana-Souza *et al.* 2010a). This wound was not sutured or covered, but healed by second intention. The lesions were left uncovered because wound healing is affected by the moist environment maintained by an occlusive dressing and is potentially affected by changing the dressing. In the abdominal region, one circular PU sponge (1.3 cm in diameter, 0.3 cm thick) (Multibrás, São Paulo, Brazil) was implanted in a subcutaneous pocket created on the side of a midline incision. The sponge was hydrated in a 0.9% sodium chloride solution before implantation and tunnelled at least 1 cm away from the incision (Romana-Souza *et al.* 2009b). The incision was sutured with 3-0 nylon (Technew, Rio de Janeiro, Brazil). Polyurethane sponge implantation is an alternative wound model for studying several aspects of granulation tissue formation, such as collagen deposition (Romana-Souza *et al.* 2009b). No signs of infection were observed on the excisional or incisional lesions.

Macroscopic analyses

To evaluate wound contraction and re-epithelialization, a transparent plastic sheet was placed over the wound and its margins were traced (Romana-Souza *et al.* 2010a). The lesion was measured soon after wounding and 3, 7, 10 and 14 days after wounding, without scab removal. To evaluate re-epithelialization, the margins of the total wound area and the non-re-epithelialized wound area were traced on a transparent plastic sheet 14 days after wounding. The re-epithelialized wound area was measured only 14 days after wounding because the scab still covered the wound area 10 days after wounding. After digitalization, the wound area was measured using ImageJ software (National Institute of Mental Health, Bethesda, MD, USA). The results were expressed as a percentage of the original wound area or as percentage of the re-epithelialized wound area.

The body weight was measured soon after wounding and weekly thereafter, until the end of the experiment. The results were expressed in grams. The systolic blood pressure and heart rate were non-invasively determined on the day of wounding, 7 and 14 days latter using a tail-cuff system in conscious mice (Letica 5001; Scientific Instrument Inc., West Palm Beach, FL, USA). The mice were acclimatized to the apparatus for 2 weeks before the measurements began. The results were expressed as mmHg and bpm, respectively.

Tissue harvesting and microscopic analyses

Seven or fourteen days after wounding, animals were anaesthetized by intraperitoneal injection (150 mg/kg ketamine and 15 mg/kg xylazine). One hour after the anaesthesia, peripheral blood was collected by cardiac puncture, and the

plasma was frozen at −70 °C. This time lag between anaesthesia and blood collection was chosen to reduce the effects of catecholamine secretion induced by animal manipulation during anaesthesia on the measurement of plasma norepinephrine levels. Subsequently, α2A/α2C-adrenoceptor KO and wild-type mice (10 mice per group) were euthanized by exsanguination. Five lesions and adjacent normal skin per group were formalin-fixed (pH 7.2) and paraffin-embedded. An additional five lesions per group and the PU sponges were frozen at −70 °C. The frozen lesions were macerated in lysis buffer (20 mM Tris–HCl pH 7.5, 138 mM sodium chloride, 10% glycerol, 1% Triton X-100, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 μg/ml leupeptin, 0.025% phenylmethylsulfonyl fluoride; Sigma-Aldrich, St. Louis, MO, USA); after centrifugation, the total protein concentration was determined using the Bradford assay. These lysates were used to perform myeloperoxidase activity, nitrite level and immunoblotting analyses.

The sections were stained with haematoxylin and eosin to quantify the blood vessel number and the migratory tongue length. To quantify the blood vessel number, ten random fields per animal (0.15 mm^2) were analysed in the superficial and deep regions of the granulation tissue using a 40× objective lens (Zeiss Primo Star; Zeiss-Vision, Oberkochen, Germany). The results were expressed as the number of blood vessels per mm^2. To measure the migratory tongue length, images of the wound edge were digitalized using a 5× objective lens and a videomicroscopic system (Axiostar plus microscope and AxioCam Cc1 video-camera; Zeiss-Vision), and the migratory tongue length was measured using the AxioVision LE64 software (Zeiss-Vision) (Tscharntke *et al.* 2007). The length was defined as the distance (in micrometres) from the wound edge to the tip of the tongue. The quantifications were performed blindly and repeated without significant differences. The sections were also stained with Sirius Red and analysed under polarized light to evaluate collagen fibre organization.

Immunohistochemistry and quantification

For the F4/80-positive macrophages quantification, the sections were immunolabelled with a rat anti-F4/80 antibody (1:400; Serotec Inc., Raleigh, NC, USA). For antigen retrieval, the sections were incubated with citrate buffer (pH 6.0) before labelling. Subsequently, endogenous peroxidase activity was inhibited. After washing, the primary antibody was detected using an anti-rat secondary antibody followed by incubation with streptavidin (DAKO, Carpinteria, CA, USA). Diaminobenzidine (DAKO) was used as the chromogen. The sections were counterstained with haematoxylin. No labelling was observed on sections where the primary antibody was omitted. To quantify the number of F4/80-positive macrophages, ten random fields per animal (3636 μm^2) were analysed in the granulation tissue using a videomicroscopic system (an Olympus BH-2 microscope; Olympus Optical Co. Ltd, Tokyo, Japan; a Samsung SDS-415ND video-camera and a Samsung monitor, Samsung Group, Seoul, South Korea). The results were expressed as the number of F4/80-positive macrophages per mm^2.

The quantification of myofibroblasts was performed using sections immunolabelled with a mouse monoclonal antibody in mouse tissue. For this procedure, some modifications to the standard technique were performed as previously described (Romana-Souza *et al.* 2010a). Briefly, the sections were incubated with anti-mouse EnVision™ system reagent (DAKO; 1:60), and endogenous peroxidase activity was inhibited. After washing, the sections were incubated with a mixture of mouse anti-α-smooth muscle actin (α-SMA), antibody (DAKO; 1:100) and anti-mouse EnVision™ system reagent (DAKO; 1:20) overnight. After washing, diaminobenzidine was used as a chromogen, and the sections were counterstained with haematoxylin. No labelling was observed on sections where primary antibody was omitted. Stereological methods were used to estimate the myofibroblast density (Baddeley *et al.* 1986; Gundersen *et al.* 1988). The volume density of myofibroblasts was evaluated using vertical sections and a test system with 16 points. Furthermore, the epidermis was always facing upwards during quantification. For quantification, ten random fields per animal were analysed in the superficial and deep regions of the granulation tissue using a videomicroscopic system (an Olympus BH-2 microscope, Olympus Optical Co. Ltd; a Samsung SDS-415ND video-camera, and a Samsung monitor, Samsung Group). The results were presented as the volume density of myofibroblasts ($Vv_{[myofibroblasts]}$%). All quantifications were performed blindly and repeated without significant differences.

Immunofluorescence staining and microscopy

To determine the expression of the α2A/α2C-adrenoceptors in normal skin and the wound area, sections from wild-type animals were immunolabelled with rabbit anti-α2A-adrenoceptor or anti-α2C-adrenoceptor antibodies (Santa Cruz Biotechnology; 1:200). For antigen retrieval, the sections were incubated with citrate buffer (pH 6.0) before labelling. After washing, the primary antibody was detected using an anti-rabbit secondary antibody conjugated with Alexa Fluor 488 or an anti-goat secondary antibody conjugated with Alexa Fluor 647 (1:200, Invitrogen, Carlsbad, CA, USA). Finally, a mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) was used to mount the coverslips onto glass microscope slides. The slides were viewed on laser scanning confocal microscope (Nikon, Langen, Germany) using a 60× objective lens. No labelling was observed on sections where primary antibody was omitted.

Biochemical analyses

To evaluate whether the α2A/α2C-adrenoceptor KO mice exhibited increase in the plasma catecholamine levels, norepinephrine synthesis was indirectly estimated by measuring the plasma normetanephrine levels. Normetanephrine is a major and stable metabolic end product of norepinephrine

(Pisano 1960). An aliquot of each plasma sample was mixed with 4N ammonium hydroxide (Vetec, Duque de Caxias, Brazil) and 2% sodium metaperiodate (Vetec). The final solution was spectrophotometrically measured at 350 nm. Different concentrations of DL-normetanephrine hydrochloride (Sigma-Aldrich) were used to generate a standard curve. The data were expressed as ng of normetanephrine per μl.

The wound neutrophil number was estimated by measuring myeloperoxidase activity in the wound lysates (Stark *et al.* 1992). An aliquot of wound lysate was mixed with an 80 mM phosphate buffer containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma-Aldrich). After centrifugation, the supernatant was mixed with 1.9 mg/ml 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma-Aldrich) and 1 mM hydrogen peroxide (Vetec) and then incubated at 37 °C for 15 min. The solution was subsequently mixed with 1.64 M of acetate buffer, and the developed colour was spectrophotometrically measured at 630 nm. The neutrophil number was determined from myeloperoxidase levels corresponding to different concentrations of neutrophils isolated from mice. For this analysis, aliquots with 0, 50, 100, 250, 833 or 1667 cells were lysed by extreme homogenization and subsequently treated as described above. The results were expressed as the number of cells ($\times 10^4$) per μg total protein.

Collagen deposition was quantified by measuring the hydroxyproline levels in frozen PU sponges (Woessner 1961). Dry and defatted tissue samples were hydrolysed in 6 mol/l hydrochloric acid for 18 h at 118 °C. The hydrolysates were then diluted with distilled water, neutralized with 6 mol/l sodium hydroxide and centrifuged at 1031 ***g*** for 15 min. Hydroxyproline levels were measured in these hydrolysates as previously described (Woessner 1961). The results were expressed as ng of hydroxyproline per mg of tissue.

Nitrite levels are used as an index of nitric oxide (NO) synthesis because nitrite is a stable molecule and accounts for more than 90% of total measurable NO metabolites (Schaffer *et al.* 1997). Nitrite levels in the wound lysates were determined by a spectrophotometric method based on the Griess reaction (Schaffer *et al.* 1997). An aliquot of each sample was mixed with 100 μl of Griess reagent and incubated at room temperature for 10 min. Afterwards, the developed colour was spectrophotometrically measured at 557 nm. The results were expressed as μmol of nitrite per μg of total protein.

Immunoblotting

Vascular endothelial growth factor (VEGF) and latent transforming growth factor (TGF)- β 1/2/3 protein expression were analysed by immunoblotting. The protein expression of the α2A/α2C-adrenoceptors was also evaluated to confirm the absence of these receptors in the wound area of the KO mice. Twenty micrograms of protein from each wound lysate was denatured in sample buffer and resolved by 12% or 10% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane. A molecular weight protein standard (Bio-Rad Laboratories Inc, Hercules, CA, USA) was included. The membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich) and probed with a mouse anti-VEGF antibody (45 kDa; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:400) or a rabbit anti-latent TGF-β 1/2/3 antibody (47 kDa; Santa Cruz Biotechnology; 1:400) overnight at 4 °C. Other membranes were probed with a rabbit anti-α2C-adrenoceptor (60 kDa) or a goat anti-α2A-adrenoceptor (70 kDa; Santa Cruz Biotechnology; 1:200). The membranes were then washed and incubated with anti-mouse, anti-rabbit or anti-goat biotinylated secondary antibodies conjugated with horseradish peroxidase (DAKO). The bound antibodies were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK), and densitometry was performed using Adobe Photoshop version 7.01 (Adobe Systems Incorporated; San Jose, CA, USA). Subsequently, the membranes were stripped with a stripping buffer containing β-mercaptoethanol (Sigma-Aldrich) and reprobed with an anti-β-actin antibody (43 kDa; Sigma-Aldrich; 1:1000). Densitometry was performed using Adobe Photoshop version 7.01 (Adobe Systems Incorporated), and the results were expressed as arbitrary units.

Cell culture

Primary fibroblasts were separately isolated from the skin of male wild-type C57BL/6 mice and α2A/α2C-adrenoceptor KO mice (1–2 months of age) by the standard explant technique as previously described (Freshney 2010). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) with 10% (v/v) foetal bovine serum (FBS; Cultilab Ltda, Campinas, Brazil) containing antibiotics (100 UI/ml penicillin, 50 μg/ml kanamycin, 100 μg/ml streptomycin, and 6 μg/ml amphotericin B; Sigma-Aldrich) at 37 °C in a humidified atmosphere with 5% CO_2. For all experiments, the fibroblasts were cultured in DMEM with 10% FBS. Cells between passages 2 and 4 were used in all experiments. The experiments were performed at least three times, in triplicate.

Cell migration was assessed by determining the ability of cells to move into an acellular area in a two-dimensional scratch assay (Romana-Souza *et al.* 2011). Fibroblasts (2×10^5 cells/well) were seeded in a 24-well plate, and the medium was changed to DMEM with 2% FBS after 24 h to inhibit cell proliferation. Shortly thereafter, the cells were scratched in the centre of the well with a 100-μl pipette tip to create a cell-free area. The denuded area was photographed using a microscopic system (Nikon Eclipse TS100 microscope and Nikon Coolpin 4500 camera; Nikon Inc., Melville, NY, USA) soon after scratching, 0.5, 1, 2 and 3 days later. The denuded area was measured using ImageJ software (National Institute of Mental Health), and the data were expressed as the percentage of the initial denuded area.

Fibroblast proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay

(Romana-Souza *et al.* 2011). Fibroblasts (3×10^4 cells/well) were seeded in a 96-well plate and 10 μl of MTT solution (Sigma-Aldrich) at 5 mg/ml were added to each well 1 or 2 days after seeding. After 4 h, the formazan product was solubilized with isopropyl alcohol (Vetec). The developed colour was spectrophotometrically measured at 570 nm. The data were expressed as a percentage of the control (medium only) group. The results obtained by the MTT assay were confirmed by haemocytometer counts.

To evaluate collagen deposition, fibroblasts (7×10^4 cells/well) were seeded on 18-mm glass coverslips, and the medium was changed every third day (Kosla *et al.* 2013). After 15 days, the cells were washed three times in lysis buffer (20 mM Tris pH 6.8, 1% Triton X-100, 0.3% SDS, 5 mM EDTA, 10% glycerol, and 10 μg/ml leupeptin), twice in deionized water, twice in methanol and finally in deionized water. Glass coverslips retaining extracellular matrix material were incubated with Sirius Red for 1 h and washed with 0.5% acetic acid. Bright field images were captured using an Axiostar ZEISS microscope and an Axiocam Cc ZEISS video-camera (Zeiss-Vision) and processed using ImageJ software (National Institute of Mental Health).

To evaluate the α-SMA and latent TGF-β 1/2/3 protein levels, fibroblasts (5×10^5 cells/well) were seeded in a 24-well plate. After 1 or 2 days, the cells were washed with ice-cold PBS and scraped into lysis buffer (20 mM Tris-HCl pH 7.5, 138 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate). The total protein concentration was determined using the bicinchoninic acid protein assay (Thermo Fisher Scientific). The proteins (20 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk powder (Nestlé, São Paulo, Brazil) and probed with a mouse anti-α-SMA (41 kDa; DAKO; 1:200) or a rabbit anti-latent TGF-β 1/2/3 (47 kDa; Santa Cruz Biotechnology; 1:500) overnight at 4 °C. The membranes were then washed and incubated with the appropriate secondary antibodies. Bound antibodies were detected by enhanced chemiluminescence (Santa Cruz Biotechnology), and densitometry analysis was performed using ImageJ software (National Institute of Mental Health). Subsequently, the membranes were stripped using a stripping buffer containing β-mercaptoethanol and reprobed with anti-β-actin (43 kDa; Sigma-Aldrich; 1:1000). The protein expression of the α2A/α2C-adrenoceptors was also detected, as described above, to confirm the absence of these receptors in skin fibroblasts isolated from KO mice.

Statistical analyses

The values for all measurements are expressed as the mean ± the standard error of the mean (SEM). The unpaired and nonparametric Mann–Whitney *U*-test was performed for each day (7 or 14 days after wounding) to compare the wild-type and KO groups. Because different sets of animals were used for each wounding time point, we had one set of animals for the 7-day experiment and another set for the 14-day experiment. The GraphPad Prism software was used to perform the statistical analyses (GraphPad Prism version 5.0, San Diego, CA, USA). The values of $P < 0.05$ were considered statistically significant.

Results

Loss of the α2A/α2C-adrenoceptors increased wound contraction, re-epithelialization, heart rate and catecholamine synthesis

As illustrated in Figure 1a, the wound area appeared to be smaller in the α2A/α2C-adrenoceptor KO mice than in the wild-type mice 7 and 10 days after wounding. Therefore, the wound area was measured to confirm these observations. The wound contraction was enhanced in the α2A/α2C-adrenoceptor KO mice compared with the wild-type mice 7 and 10 days after wounding (Figure 1b). The percentage of the re-epithelialized wound area was increased in the α2A/α2C-adrenoceptor KO mice compared with the wild-type mice 14 days after wounding (Figure 1c). In addition, the length of the migratory tongue was increased in the α2A/α2C-adrenoceptor KO mice compared with the wild-type mice 7 days after wounding (Figure 1d).

No differences in the body weight or the systolic blood pressure were observed between wild-type and α2A/α2C-adrenoceptor KO mice throughout the experiment (Table 1). However, the heart rate was increased in the α2A/α2C-adrenoceptor KO mice compared with the wild-type mice on the day of wounding, 7 and 14 days later (Table 1). In addition, the normetanephrine levels were higher in the α2A/α2C-adrenoceptor KO mice than in the wild-type mice only 7 days after wounding (Table 1).

The normal skin and wound area of wild-type mice contained α2A/α2C-adrenoceptor-positive fibroblasts (Figure 2a–d). Wild-type mice exhibited α2A/α2C-adrenoceptor protein expression in the wound area, while KO mice did not express these receptors in the wound area (Figure 2e).

Deletion of the α2A/α2C-adrenoceptors increased angiogenesis, myofibroblastic differentiation, and collagen deposition, and reduced neutrophil and macrophage mobilization

Representative images of F4/80 immunohistochemical analyses are presented in Figure 3a–d. The macrophage number was decreased in the wounds of α2A/α2C-adrenoceptor KO mice compared with the wild-type mice 7 days after wounding (Figure 4a). The neutrophil number was reduced in the α2A/α2C-adrenoceptor KO mice compared with wild-type mice 7 and 14 days after wounding (Figure 4b). The blood vessel number was higher in the α2A/α2C-adrenoceptor KO mice than in the wild-type mice 7 days after wounding, but it was lower 14 days after wounding (Figure 4c). The nitrite levels were increased in the α2A/α2C-adrenoceptor KO wounds compared with the wild-type wounds 7 days after

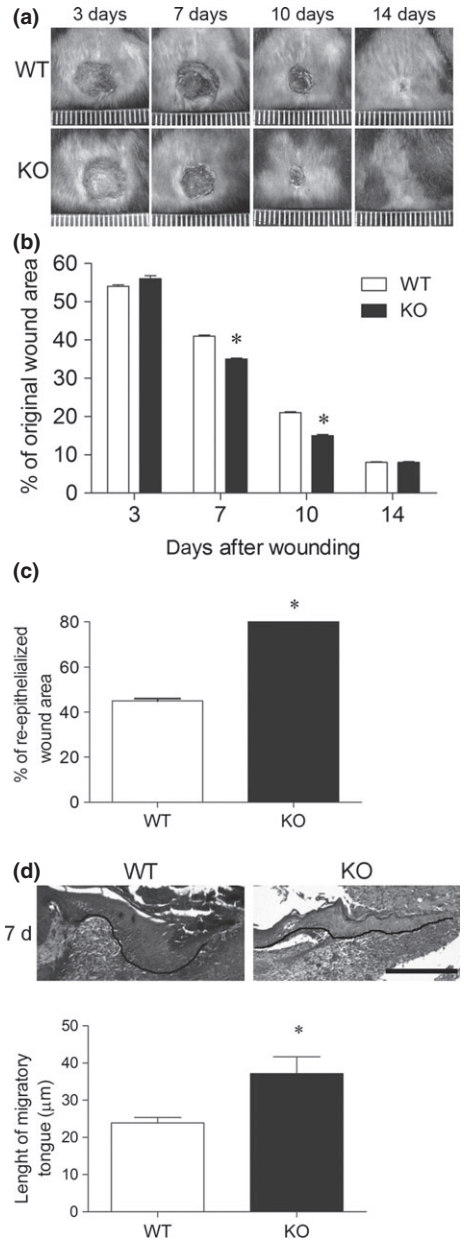

Figure 1 (a) Representative images of wounds from the α2A/α2C-adrenoceptor knockout (KO) mice and wild-type (WT) mice 3, 7, 10 and 14 days after wounding. (b) The percentage (%) of the original wound area is reduced in the α2A/α2C-adrenoceptor knockout mice compared with the wild-type mice 7 and 10 days after wounding. (c) The percentage (%) of the re-epithelialized wound area is increased in the α2A/α2C-adrenoceptor knockout mice compared with the wild-type mice 14 days after wounding. (d) The length of the migratory tongue (μm) is enhanced in the α2A/α2C-adrenoceptor knockout mice compared with the wild-type mice 7 days (7d) after wounding. (d) The black dotted lines show the migratory tongue length. The sections were stained with haematoxylin and eosin. The results (n = 10 per group) are expressed as the mean ± SEM. *P < 0.05 compared with WT. The bars are equal to 50 μm.

Table 1 Biometric parameters and plasma normetanephrine levels in the α2A/α2C-adrenoceptor knockout and wild-type mice on the day of wounding (0 days), 7 and 14 days later

Measurements	Mice (n = 10)	0 days	7 days	14 days
Body weight (g)	WT	23.7 ± 0.8	23.0 ± 0.7	23.7 ± 0.6
	KO	21.3 ± 0.8	23.8 ± 1.0	25.0 ± 0.9
Systolic blood pressure (mmHg)	WT	140 ± 3	148 ± 8	147 ± 4
	KO	138 ± 3	132 ± 6	131 ± 7
Heart rate (bpm)	WT	415 ± 17	436 ± 20	433 ± 17
	KO	514 ± 11*	502 ± 18*	505 ± 17*
Plasma normetanephrine (ng/μl)	WT	–	12.2 ± 2.6	14.9 ± 1.4
	KO	–	24.4 ± 1.6 *	17.7 ± 0.3

All data are shown as the mean ± SEM.
KO, Knockout; WT, wild-type.
*P < 0.05 compared with wild-type mice.

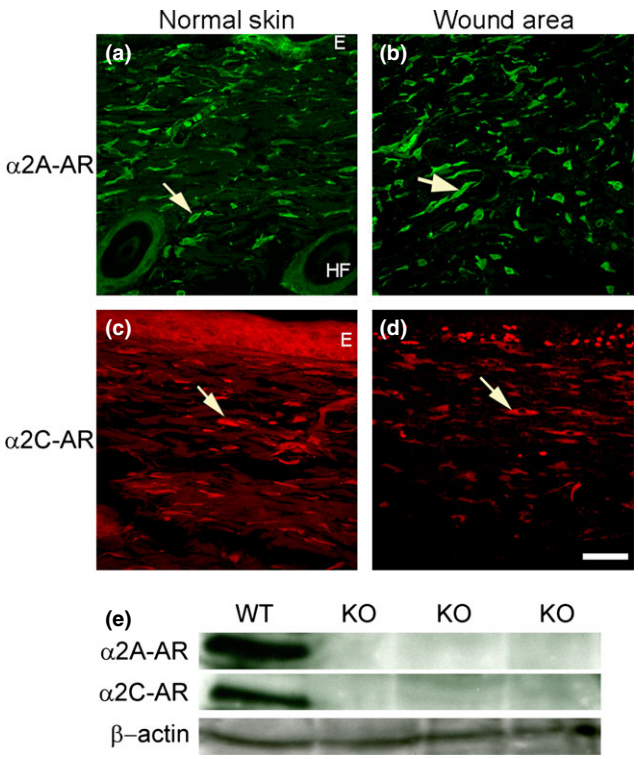

Figure 2 (a–d) The α2A/α2C-adrenoceptor-positive cells in the normal skin and the wound area of wild-type C57BL/6 male mice. Cells positive to α2A-adrenoceptor (α2A-AR) are observed in normal skin (a) and wound area (b), and positive α2C-adrenoceptor (α2C-AR) in normal skin (c) and wound area (d). (e) The expression of the α2A/α2C-adrenoceptor proteins is detected only in the wound area of the wild-type mice. The α2A-adrenoceptor (AR; 70 kDa), α2C-adrenoceptor (AR; 60 kDa) and β-actin (43 kDa) protein expression in the wound area of wild-type (WT) and knockout (KO) mice. E – Epidermis, HF – hair follicle, arrows – fibroblasts. The bars are equal to 50 μm.

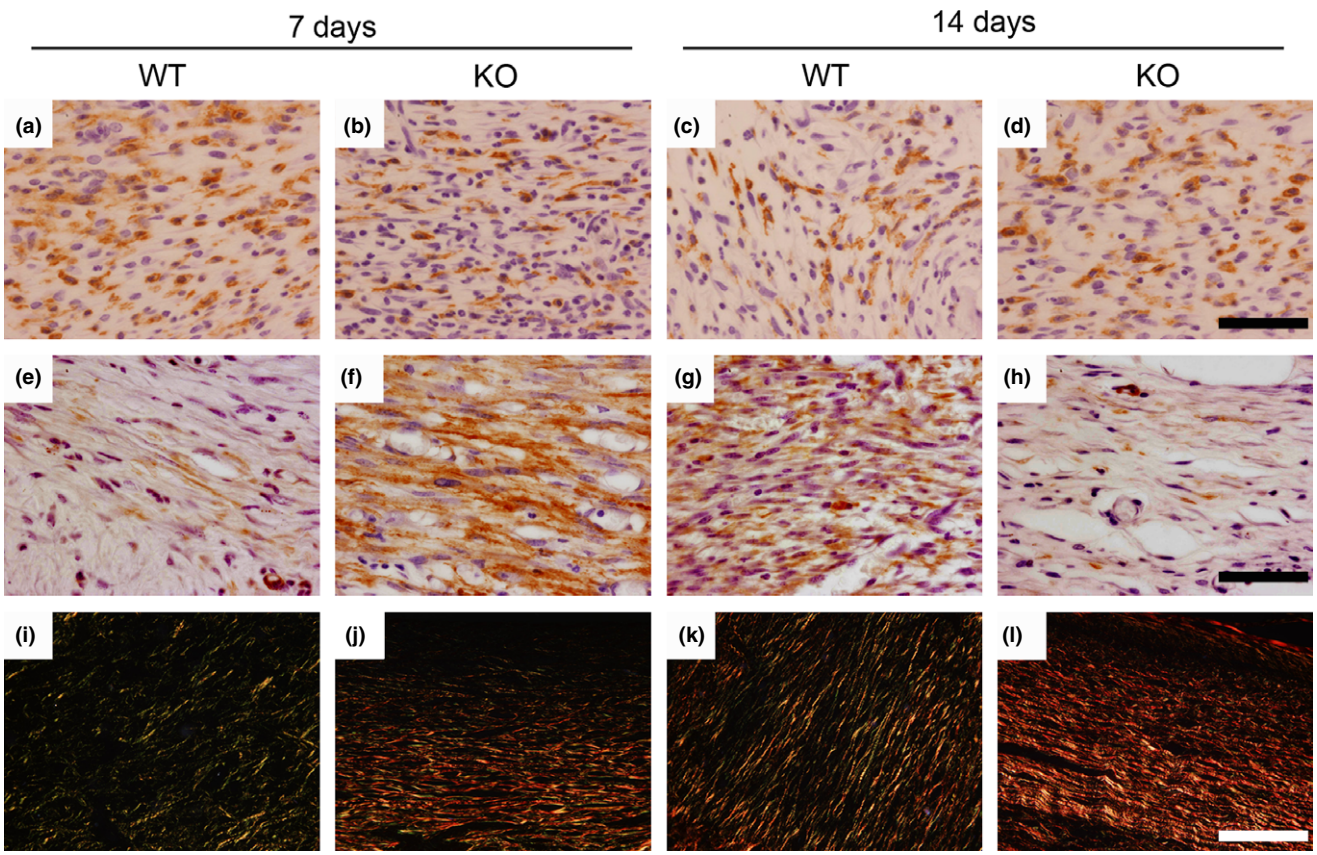

Figure 3 (a, b) F4/80-positive macrophage infiltration is reduced in the α2A/α2C-adrenoceptor knockout (KO) wounds when compared with wild-type (WT) wounds 7 days after wounding. (c, d) There is no difference between the α2A/α2C-adrenoceptor knockout and wild-type wounds 14 days after wounding. (e, f) The number of α-smooth muscle actin-positive myofibroblasts is increased in the α2A/α2C-adrenoceptor knockout wounds 7 days after wounding. (g, h) The number of α-smooth muscle actin-positive myofibroblasts is reduced in the α2A/α2C-adrenoceptor knockout wounds 14 days after wounding. (i, j) The collagen fibres are yellow-greenish in the wild-type wounds 7 days after wounding, but yellow-reddish in the α2A/α2C-adrenoceptor knockout wounds. (k, l) Fourteen days after wounding, collagen fibres are yellow-reddish in the wild-type wounds but yellow-reddish or reddish in the α2A/α2C-adrenoceptor knockout wounds. The bars are equal to 50 μm.

wounding (Figure 4d). Representative images of α-SMA immunohistochemical analyses are presented in Figure 3e–h. The myofibroblast density was increased in the α2A/α2C-adrenoceptor KO mice when compared with wild-type mice 7 days after wounding, but it was reduced 14 days after wounding (Figure 4e). Seven days after wounding, the collagen fibres were mainly yellow-greenish, thin and arranged parallel to the surface in wild-type mice, whereas these fibres were yellow-reddish, thin and arranged parallel to the surface in α2A/α2C-adrenoceptor KO mice (Figure 3i,j). Fourteen days after wounding, the collagen fibres were yellow-reddish, thin and arranged parallel to the surface in wild-type mice, whereas these fibres were mainly yellow-reddish or reddish, thick and arranged parallel to the surface in α2A/α2C-adrenoceptor KO mice (Figure 3k,l). These results indicate that α2A/α2C-adrenoceptor deletion increased collagen deposition in the wound area of mice. To confirm this observation, hydroxyproline levels were measured in subcutaneously implanted PU sponges 7 and 14 days after implantation. The hydroxyproline levels were higher in the α2A/α2C-adrenoceptor KO mice than in the wild-type mice 7 and 14 days after implantation (Figure 4f). VEGF protein expression was increased in α2A/α2C-adrenoceptor KO mice when compared with wild-type mice 7 days after wounding (Figure 4g). Latent TGF-β 1/2/3 protein levels were higher in the α2A/α2C-adrenoceptor KO mice than in the wild-type mice 7 days after wounding (Figure 4h). Latent TGF-β 1/2/3 was not detected in the wound area of either α2A/α2C-adrenoceptor KO or wild-type mice 14 days after wounding (data not shown).

Deletion of the α2A/α2C-adrenoceptors improved α-SMA and TGF-β expression, cell migration and collagen deposition in murine skin fibroblast cultures

The cell proliferation rates were similar in skin fibroblasts isolated from α2A/α2C-adrenoceptor KO and wild-type mice 1 and 2 days after seeding (Figure 5a). Accelerated

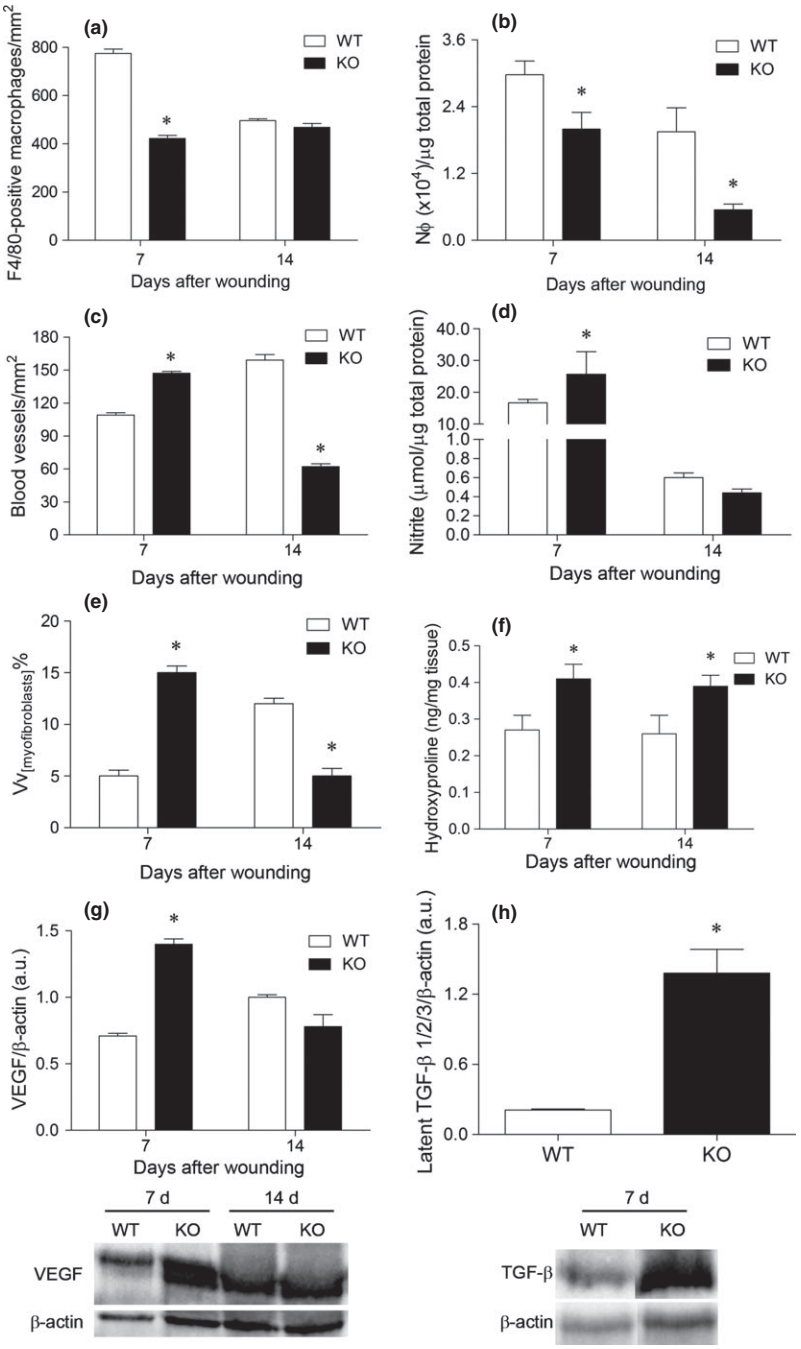

Figure 4 (a) The number of F4/80-positive macrophages is decreased in the α2A/α2C-adrenoceptor knockout (KO) mice when compared with wild-type (WT) mice 7 days after wounding. (b) The number of neutrophils (Nϕ, $\times 10^4$) per μg total protein is reduced in the α2A/α2C-adrenoceptor knockout wounds when compared with wild-type wounds 7 and 14 days after wounding. (c) The blood vessel number is increased in the α2A/α2C-adrenoceptor knockout wounds when compared with wild-type wounds 7 days after wounding, but reduced in the α2A/α2C-adrenoceptor knockout wounds 14 days after wounding. (d) The nitrite levels are higher in the α2A/α2C-adrenoceptor knockout mice than in the wild-type mice 7 days after wounding. (e) The myofibroblast density is increased in the α2A/α2C-adrenoceptor knockout wounds compared with the wild-type wounds 7 days after wounding, but decreased 14 days after wounding. (f) The hydroxyproline levels in the polyurethane sponges are higher in the α2A/α2C-adrenoceptor knockout mice than in the wild-type mice 7 and 14 days after implantation. (g) Vascular endothelial growth factor (VEGF) protein expression is enhanced in the α2A/α2C-adrenoceptor knockout wounds compared with the wild-type mice 7 days (7d) after wounding, but there is no difference at 14 days (14d). (h) Latent TGF-β 1/2/3 protein expression is increased in the α2A/α2C-adrenoceptor knockout wounds compared with the wild-type wounds 7 days after wounding. The β-Actin (43 kDa) protein was used as a loading control. The results ($n = 5$ per group) are expressed as the mean ± SEM. *$P < 0.05$ compared with WT.

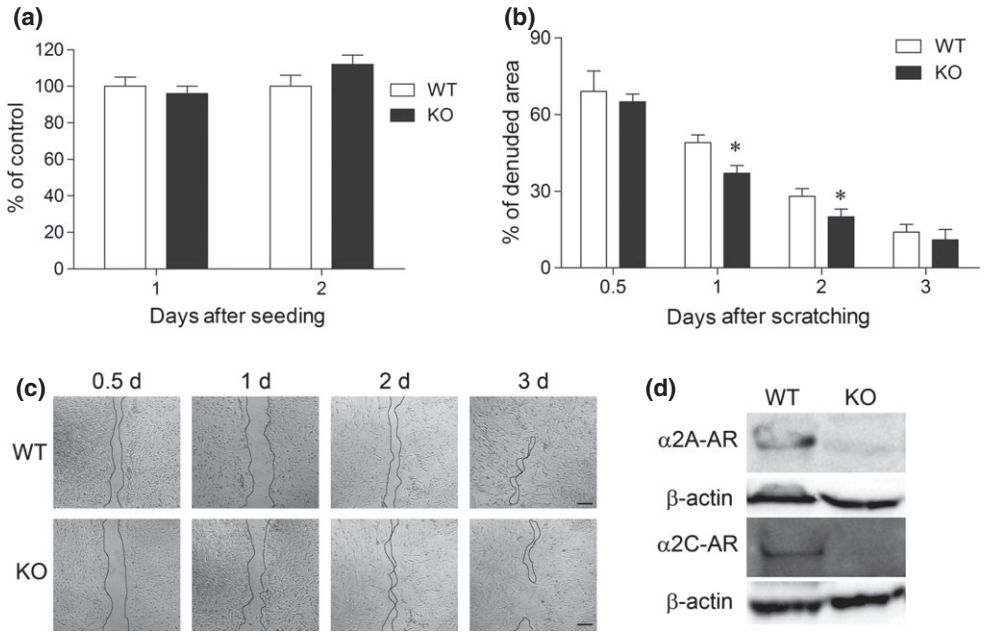

Figure 5 (a) The cell proliferation rates are similar in skin fibroblasts isolated from α2A/α2C-adrenoceptor knockout (KO) mice and wild-type (WT) mice 1 and 2 days after seeding. (b) Cell migration is accelerated in the skin fibroblasts isolated from α2A/α2C-adrenoceptor knockout mice compared with the skin fibroblasts isolated from wild-type mice 1 and 2 days after scratching. (c) Representative images of the denuded area (fibroblast-free area) in skin fibroblast cultures isolated from α2A/α2C-adrenoceptor knockout mice and wild-type mice 0.5 (0.5d), 1 (1d), 2 (2d) and 3 (3d) days after scratching. (d) The α2A-adrenoceptor (AR; 70 kDa) and α2C-adrenoceptor (AR; 60 kDa) protein expression is only detected in skin fibroblast cultures isolated from wild-type mice. The β-actin (43 kDa) protein was used as a loading control. The data (n = 9) are expressed as the mean ± SEM. *P < 0.05 compared with WT. Bar = 200 μm.

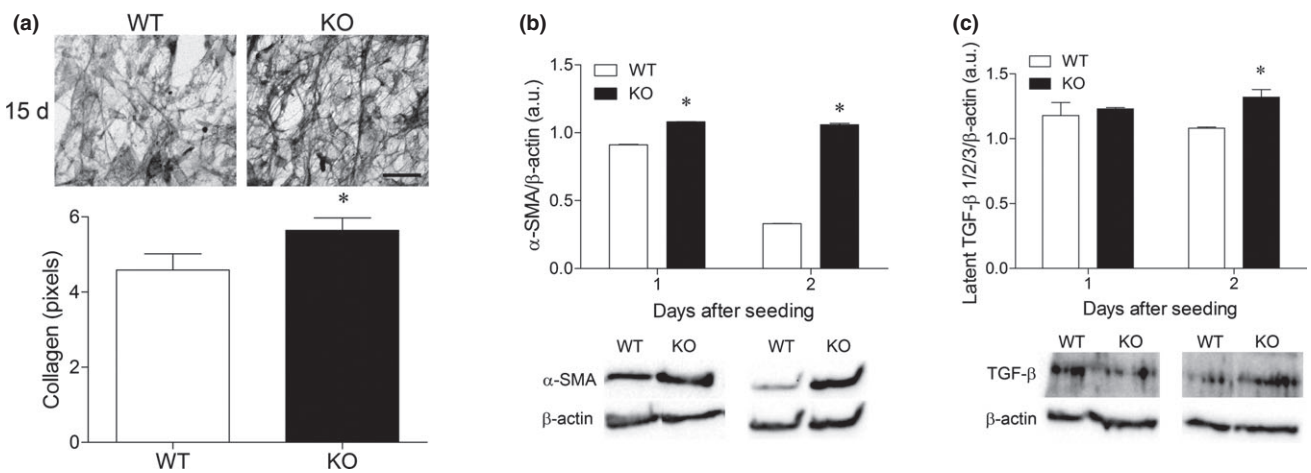

Figure 6 (a) Collagen deposition (pixels) is enhanced in skin fibroblasts isolated from α2A/α2C-adrenoceptor knockout (KO) mice compared with skin fibroblasts isolated from wild-type (WT) mice 15 days after seeding. (b) The levels of α-smooth muscle actin (α-SMA; 41 kDa) protein expressed as arbitrary units (a.u.) are increased in skin fibroblast isolated from α2A/α2C-adrenoceptor knockout mice compared with skin fibroblasts isolated from wild-type mice 1 and 2 days after seeding. (c) Latent transforming growth factor-β (TGF-β) 1/2/3 (47 kDa) protein levels expressed as arbitrary units (a.u.) are increased in skin fibroblasts isolated from α2A/α2C-adrenoceptor knockout mice compared with skin fibroblasts isolated from wild-type mice 2 days after seeding. The β-actin (43 kDa) protein was used as a loading control. The data (n = 9) are expressed as the mean ± SEM. *P < 0.05 compared with WT. Bar = 50 μm.

cell migration was observed in skin fibroblasts isolated from the α2A/α2C-adrenoceptor KO mice compared with wild-type mice 1 and 2 days after scratching (Figure 5b,c). Skin fibroblasts isolated from wild-type mice expressed the α2A/α2C-adrenoceptor proteins, whereas skin fibroblasts isolated from KO mice did not express these receptors (Figure 5d). Collagen deposition was increased in the skin fibroblasts isolated from α2A/α2C-adrenoceptor KO mice compared with the skin fibroblasts isolated from wild-type mice 15 days after seeding (Figure 6a). The expression of α-SMA protein was higher in the skin fibroblasts isolated from α2A/α2C-adrenoceptor KO mice compared with the skin fibroblasts isolated from wild-type mice 1 and 2 days after seeding (Figure 6b). Latent TGF-β 1/2/3 protein expression was increased in the skin fibroblasts isolated from α2A/α2C-adrenoceptor KO mice compared with the skin fibroblasts isolated from wild-type mice 2 days after seeding (Figure 6c).

Discussion

The α2A/α2C-adrenoceptors inhibit the presynaptic release of catecholamines from sympathetic fibres and noradrenergic neurons. The α2A-receptors modulate neurotransmission at higher levels of nerve activity, whereas the α2C-receptors modulate neurotransmission at lower levels (MacMillan *et al.* 1996; Hein *et al.* 1999). The α2A/α2C-adrenoceptor KO mice exhibit a chronically increased sympathetic activity that leads to cardiac hypertrophy with decreased left ventricular contractility by 4 months of age (Altman *et al.* 1999; Hein *et al.* 1999; Brum *et al.* 2002). Therefore, these mice are used as a genetic model of heart failure induced by sympathetic hyperactivity (Brum *et al.* 2002). We propose that the α2A/α2C-adrenoceptor KO mice provide a new approach for studying the role of the α2A/α2C-adrenoceptors on cutaneous wound healing. We hypothesize that deletion of the α2A/α2C-adrenoceptors alters cutaneous wound healing. We used α2A/α2C-adrenoceptor KO mice at 3 months of age, when they do not exhibit significant cardiovascular alterations (only increased heart rate and catecholamine levels), thus excluding the influence of cardiomyopathy (Brum *et al.* 2002). Previous studies have not demonstrated the distribution of the α2A/α2C-adrenoceptors in normal mouse skin or cutaneous lesions. We observed α2A/α2C-adrenoceptor-positive fibroblasts in the normal skin and the wound area of wild-type mice using immunofluorescence staining. We believe that this is the first demonstration of α2A/α2C-adrenoceptor expression in the fibroblasts of normal mouse skin or cutaneous lesions. Thus, α2A/α2C-adrenoceptor expression is not altered by wound formation.

In rat, the activation of the α2-adrenoceptors reduces plasma extravasation in full-thickness burn injuries through vasoconstriction (Cassuto *et al.* 2005). However, α1/α2-adrenoceptor blockade slightly increased the number of polymorphonuclear leucocytes recruited to the wound area, but did not compromise the wound closure of cutaneous rat lesions (Romana-Souza *et al.* 2009c). Deletion of the α2A/α2C-adrenoceptors reduced the neutrophil and macrophage numbers in the wound area. These results seem to be controversial; however, non-selective pharmacological blockade of the α-adrenoceptors may inhibit an inflammatory signalling pathway that is modulated by the α2-adrenoceptor. In addition, we should keep in mind that the pharmacological blockade is not 100% effective (Zebrowska-Lupina & Szurska 1986). Nevertheless, deletion of the α2A/α2C-adrenoceptors allowed an observation of the specific role of these receptors on neutrophil infiltration. The α2A/α2C-adrenoceptor KO mice exhibited a decrease in neutrophil infiltration that may have accelerated inflammation, angiogenesis, dermal reconstruction and wound contraction. This hypothesis may be confirmed by other studies demonstrating that a reduction in inflammation accelerated the development of subsequent wound healing phases (Romana-Souza *et al.* 2008, 2009a, 2010b; Georgii *et al.* 2011; dos Santos & Monte-Alto-Costa 2013). Radioligand binding studies have indicated that the bone marrow cells and thymocytes express the α-adrenoceptors. Alpha1-adrenoceptor blockade can enhance myelopoiesis and platelet formation in normal mice and increase the thymic weight, cortical size and cellularity in young adult rats (Maestroni & Conti 1994; Plecas-Solarovic *et al.* 2005). In addition, chronic exposure of rats to noradrenaline may enhance thymocyte apoptosis via the α-adrenoceptors (Stevenson *et al.* 2001). However, previous studies have not demonstrated whether the deletion of the α2A/α2C-adrenoceptors alters haematopoiesis or the thymus.

Re-epithelialization is the process of restoring the epidermis. Fourteen days after wounding, re-epithelialization was increased in the α2A/α2C-adrenoceptor KO mice when compared with wild-type mice. In addition, the α2A/α2C-adrenoceptor KO mice displayed an increased length of the migratory tongue when compared with wild-type mice 7 days after wounding, indicating that keratinocyte migration was increased by α2A/α2C-adrenoceptor ablation. These results suggest that deletion of the α2A/α2C-adrenoceptors may improve re-epithelialization through an increased keratinocyte migration that contributes to accelerated wound closure.

Vascular endothelial growth factor is the most important positive regulator of angiogenesis because it stimulates endothelial cell migration and proliferation (Barrientos *et al.* 2008). In rats, α1/α2-adrenoceptor blockade does not alter blood vessel density in excisional lesions or PU sponge implants (Romana-Souza *et al.* 2009b,c). Nevertheless, the α2A/α2C-adrenoceptor KO mice exhibited accelerated new blood vessel formation and increased VEGF protein levels. The deletion of the α2A/α2C-adrenoceptors may improve angiogenesis through increased VEGF synthesis that permits enhanced granulation tissue development.

In rodents, myofibroblast-mediated contraction is the major mechanism of wound closure in the skin (Davidson 1998). Myofibroblastic differentiation, collagen deposition and wound contraction in excisional lesions or PU sponge

implants were not compromised by α1/α2-adrenoceptor blockade (Romana-Souza *et al.* 2009b,c). The deletion of the α2A/α2C-adrenoceptors accelerated myofibroblastic differentiation and increased collagen deposition. These results may be associated with high levels of latent TGF-β 1/2/3 induced by α2A/α2C-adrenoceptor ablation because TGF-β1 is one of the main factors that stimulates the fibroblast to myofibroblast transition and type I collagen production by fibroblasts and myofibroblasts (Greenwel *et al.* 1997; Gabbiani 2003). Thus, the α2A/α2C-adrenoceptor deletion-induced increase in TGF-β 1/2/3 synthesis may have promoted collagen deposition and myofibroblastic differentiation that resulted in faster wound contraction. This hypothesis was confirmed by the *in vitro* studies demonstrating that skin fibroblasts isolated from α2A/α2C-adrenoceptor KO mice expressed higher levels of α-SMA and TGF-β, deposited more collagen and migrated faster than skin fibroblasts isolated from wild-type mice. Furthermore, α2A/α2C-adrenoceptor deletion-stimulated NO synthesis may have contributed to increased collagen deposition because NO stimulates collagen deposition in skin fibroblast cultures (Schaffer *et al.* 1997).

An increase in plasma catecholamine and heart rate is observed in the α2A/α2C-adrenoceptor KO mice beginning at 3 months of age (Hein *et al.* 1999). In this study, the α2A/α2C-adrenoceptor KO mice exhibited plasma catecholamine levels that were twofold higher than WT mice at 3 months of age. Nevertheless, high levels of catecholamines (an increase of three to tenfold) were observed in severely burned children, stressed patients and animals suffering from a prolong inflammatory response, leading to impaired wound closure (Padgett *et al.* 1998; Sedowofia *et al.* 1998; Romana-Souza *et al.* 2008, 2010a; Sivamani *et al.* 2009). Stress-stimulated high glucocorticoid and norepinephrine levels reduce inflammatory cell activation and pro-inflammatory cytokine synthesis due to immunosuppressive effects (Bamberger *et al.* 1996; Glaser *et al.* 1999; Stark *et al.* 2001; Mercado *et al.* 2002; Avitsur *et al.* 2005; Gosain *et al.* 2007). Nevertheless, long-term exposure to norepinephrine and glucocorticoids leads to inflammatory cell (such as neutrophils and macrophages) resistance, inducing an increase in the inflammatory response during the final phases of wound healing (Romana-Souza *et al.* 2010a). The α2A/α2C-adrenoceptor KO mice exhibited increased catecholamine levels; however, these levels were lower than the catecholamine levels of chronically stressed mice. Therefore, the increased catecholamine levels may not correlate with the reduced inflammatory response observed in the α2A/α2C-adrenoceptor KO mice.

In summary, α2A/α2C-adrenoceptor deletion strongly accelerates cutaneous wound healing by reducing the local inflammatory response and increasing TGF-β 1/2/3 expression, keratinocyte migration, collagen deposition, angiogenesis and myofibroblastic differentiation, thereby leading to accelerated wound closure. In addition, α2A/α2C-adrenoceptor ablation promotes cell migration and collagen deposition and increases α-SMA and TGF-β protein expression in murine skin fibroblast cultures.

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Conflicts of interest

The authors declare no conflict of interest.

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